Senescence or Ageing in Wallflower Petals?

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by

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Erysimum linifolium 'Bowles Mauve'

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Abstract

Senescence or Ageing in Wallflower Petals?

Senescence is the final event in the life of many plant tissues. It is concerned with remobilisation of metabolites from the senescing organ and is distinct from other forms of cell death. It is a highly regulated process involving structural, biochemical and molecular changes. Gene expression is tightly regulated, with many genes down regulated and others induced. Conversely, ageing is a passive degradation. Petals and leaves are good model organs for developmental studies, as petal development is irreversible and tightly controlled, and leaves are an extensively studied senescent system. Similarities between these organs allow direct comparison of late developmental events to establish whether petals senesce, as leaves do, or age; however, important differences in function lead to the hypothesis that petal deterioration and leaf senescence are distinct. Wallflowers were chosen as they are closely related to *Arabidopsis* and *Brassica* spp., have large petals, and are commercially relevant ornamental plants. Changes in wallflower morphology and physiology were examined over development. A high degree of gene homology was shown between wallflowers and *Arabidopsis* by northern blotting. 1632 genes upregulated in old wallflower tissues were cloned using subtractive selective hybridisation and a microarray of these genes, along with 91 *Arabidopsis* senescence associated genes, was constructed. Probing the microarray with RNA from different leaf and petal developmental stages identified 298 common genes significantly upregulated during both leaf senescence and late petal development, and expression patterns were compared between the two tissues. The expression patterns of selected genes were confirmed by RT-PCR. Late development in petals was shown to be an active process, and was tentatively concluded to be senescence due to upregulation of genes involved with nutrient remobilisation and the strong upregulation of the *SAG12* gene - a molecular marker for leaf senescence that is not upregulated during other forms of death in leaves.

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Abbreviations

A,C,G,and T	four bases of DNA (A=adenine; C=cytosine; G=guanine; T=thymine)
ABA	abscisic acid
ABI	Applied Biosystems
ACC	1-amino-cyclopropane-1-carboxylic acid
ADH	alcohol dehydrogenase
amp100	100μg/ml ampicillin
APX	ascorbate peroxidase
aRNA	amplified RNA
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
AU	absorbence units
BCA	bicinchloronic acid
BCB	blue copper binding protein
BFN	bifunctional nuclease
BH FDR	Benjamini and Hochberg false discovery rate
bp	base pair
BSA	bovine serum albumin
CAT	catalase
CCH	copper chaperone
cDNA	deoxyribonucleic acid copy of messenger ribonucleic acid
Ch	chlorophyll
ctDNA	chloroplast DNA
CTP	cytosine triphosphate
DAD	defender against death
DAG	diacylglycerol
dATP	2'-deoxyadenosine 5'triphosphate
dCTP	2'-deoxycytosine 5'triphosphate
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
dGTP	2'-deoxyguanidine 5'triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	2'-deoxynucleoside 5'triphosphate
dsDNA	double-stranded deoxyribonucleic acid
DTT	dithiothreitol
dTTP	2'-deoxythymanine 5'triphosphate
dUTP	2'-deoxyuracil 5'triphosphate
DW	dry weight
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
ERD	early response to dehydration
EST	expressed sequence tag
et al.	*et alia* (and others)
EtBr	ethidium bromide
FW	fresh weight
G	gravitational force
GDP	guanidine diphosphate
GPCR	G-protein coupled receptors

GS	glutamine synthase
GST	glutathione S transferase
GTP	guanidine triphosphate
GUS	β-glucuronidase
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
HR	hypersensitive response
HRI	Horticulture Research International
HSP	heat shock protein
iAFLP	introduced Amplified Fragment Length Polymorphisms
i.e.	*id est* (that is)
IGF	insulin growth factor
IP_2	inositol 1, 4-diphosphate
IP_3	inositol 1,4,5 triphosphate
IPP	inositol polyphosphate 5- phosphatase
IPTG	isopropyl-1-thio-β-D-galactopyranoside
JA	jasmonic acid
JA-Me	methyl jasmonate
Kb	kilo base
LB	Luria-Bertani
LD PCR	long distance polymerase chain reaction
LEA	late embryogenesis abundant
LHCP	light harvesting complex protein
LMM	lesion mimic mutants
LOX	lipoxygenase
LSC	leaf senescence clone
LTP	lipid transfer protein
MAGE-ML	microarray gene expression mark-up language
MAP kinase	mitogen-activated protein kinase
MGED	microarray gene expression data society
MIAME	minimum information about a microarray experiment
mins	minutes
MOPS	3-(N-morpholino)propane sulphonic acid
mRNA	messenger ribonucleic acid
MTC	multiple testing correction
NAD	nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
NHS	N-hydroxyl succinimidyl
nt	nucleotide
OD	optical density
OL	old leaf
OP	old petal
OPDR	oxophytodienoate reductase
ORF	open reading frame
PARP	poly-(ADP-ribose) polymerase
PCD	programmed cell death
PCR	polymerase chain reaction
PGR	plant growth regulators
pH	potential of Hydrogen. Negative 10-base log (power) of the positive hydrogen ion concentration (measure of acidity)
Pi	inorganic phosphorus

PIP_2	phosphatidylinosotol 4,5-bisphosphate
PLA	phospholipase *a*
PLC	phospholipase c
POP dikinase	pyruvate orthophosphate dikinase
PVP	polyvinylpyrolidone
QC	quality control
RBCS	ribulose bisphosphate carboxylase small subunit
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
Rubisco	ribulose bisphosphate carboxylase/oxygenase
SA	salicylic acid
SAG	senescence associated gene
SAGE	serial analysis of gene expression
SAM	S-adenosyl methionine
SD	standard deviation
SDS	sodium dodecyl sulphonate
sdw	sterile distilled water
SE	standard error
SEVAG	24:1 chloroform:isoamyl alcohol
SOD	superoxide dismutase
SRP	signal recognition particle
ssDNA	single-stranded deoxyribonucleic acid
SSH	suppression subtractive hybridisation
STS	silver thiosulphate
T-DNA	transferred deoxyribonucleic acid
TAE	tris-acetate-EDTA
Taq	*Thermus aquaticus* (DNA polymerase from this source)
TCA	trichloroacetic acid
TE	Tris-EDTA
Tm	melting temperature
TNE	tris-NaCl-EDTA
Tris	2-amino-2-hydroxymethyl-1-3-propanediol
tRNA	transfer RNA
TUNEL	terminal dUTP nick end labeling
U	unit
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
v/v	volume:volume ratio
w/v	weight:volume ratio
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XML	extensible markup language
YL	young leaf
YP	young petal

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1 Introduction

So dramatic are the autumn displays of the red and gold foliage of deciduous leaves and yellowing wheat, that they change the earth's appearance from space, as recorded by orbiting satellites (Hendry, 1988). The economic implications are also considerable: cereals are very important economic crops and the possibility of increased yield and productivity by manipulating senescence to increase the photosynthetically active period could make them even more valuable (Nelson, 1988), and the spectacular changes of autumn foliage is a major tourist attraction, particularly in the North-Eastern United States (Hendry, 1988). The commercial value of ornamental flowers is also considerable, at around £674 million annually (NFU, 2002) and cut flowers worth $7 billion a year globally (Gollnow, 2002) and over £1.45 billion annually in the UK, representing an average annual spend of £26 per person (Flowers and Plants Association, 2004), so the ability to influence flower longevity to increase vase life has great economic implications, whilst the length of the effective pollination period of a flower will influence fruit and seed set (Stead and van Doorn, 1994).

Even under ideal growth conditions, the leaves of annual and perennial plants have limited lifespans and are programmed to senesce (Bleecker and Patterson, 1997). Some leaves have extremely short lives, such as the cotyledons of certain species that begin to senesce almost as soon as they have reached maturity, whereas other leaves, like those of the bristle-cone pine tree, remain photosynthetic for up to 30 years (Woolhouse, 1982). Similarly, the flowers of some species of ephemeral flowers live for only a few hours, whereas some species of orchid can live for several months (Stead and van Doorn, 1994). However, senescence is not simply concerned with death, but is a complex and highly regulated developmental phase.

In this thesis, the work undertaken compares the late developmental events in leaves (leaf senescence) with those in petals, to address the question: do petals senesce in the same way as leaves do, or is the process of petal ageing distinct from that of leaf senescence? Thus, in the introduction, the terms 'senescence' and 'ageing' are defined and the process of senescence described in relation to cells, organs and whole organisms. In order not to prejudge the issue, the term 'late development' will be

used to refer to late petal stages. The reasons for choosing leaves and petals over other organs as model systems for studying senescence are evaluated and the validity of comparing events between the two organs is assessed. Senescence is considered in relation to programmed cell death (PCD) and compared to other processes resulting in cell or organ death in both plants and animals. What is known about the regulation of senescence and the progression of the process is described, noting key physiological, biochemical and molecular features and their timing in relation to each other. Some of the different methods used in the past for studying senescence are described and their merits and shortcomings are evaluated. Finally, the use of wallflowers as a model species for senescence studies is explained, giving reasons for its choice, before formulating the hypotheses on which this study is based.

1.1 'Senescence' and 'ageing'

No consensus has yet been reached regarding where the boundaries lie between the terms 'senescence' and 'ageing', and where there are overlaps between these distinct events, along with a host of other terms relating to late and terminal events in plant development. Different authors use these terms in different ways, with some appreciating the distinction between them (eg. Thomas *et al.*, 2003) and others using the terms almost interchangeably (eg. Jing *et al.*, 2003). In this thesis, senescence is defined as an active, highly controlled process of differentiation and deterioration, concerned with the recycling of nutrients from the dying organ, whereas ageing is a passive degeneration over time, or 'wearing out' of the organ, with little, if any, control and no salvage of nutrients.

1.1.1 Senescence

Senescence is the final stage of development in the life of many plant organs. During leaf senescence, photosynthesis ceases and the organelles undergo biochemical changes. Chlorophyll, lipids, nucleic acids and proteins are catabolised and the breakdown products removed and reused (Quirino *et al.*, 2000; Buchanan-Wollaston, 1997). The process is endogenously controlled (Quirino *et al.*, 2000; Matile, 2000; Noodén *et al.*, 1997; Smart, 1994) and is a highly regulated and ordered sequence of genetically programmed structural, physiological, biochemical and molecular changes. Tight metabolic regulation is shown throughout the senescence process

(Thomas, 1992; Matile, 1992; Stoddart and Thomas, 1982; Thomas and Stoddart, 1980), and *de novo* gene transcription and protein synthesis are both required (reviewed by Noodén, 1988*a*). Membrane integrity and cellular compartmentalisation are maintained until late in the senescence programme, with little or no leakage of cellular contents, with the leaves remaining turgescent throughout (Matile, 2000; Noodén, 1988*a*).

Despite being an active process, senescence is deteriorative; ultimately leading to the death of the organ, which in many cases bears the hallmarks of programmed cell death (PCD) (Buchanan-Wollaston and Morris, 2000; Rubinstein, 2000; Noh and Amasino, 1999*a,b*; Pennell and Lamb, 1997; Makrides and Goldthwaite, 1981). Although PCD is the final stage of senescence, the two processes are distinct in that the cells must remain alive and viable for senescence to be initiated and to proceed. This explains how herbarium samples and frozen vegetables remain pre-senescent, as they are made non-viable, and so senescence can not occur. Thus, the PCD phase of the process only begins once senescence has ended, having accomplished its purpose, and so PCD is actively delayed until maximum mobilisation has occurred. However, senescence is not a prerequisite for PCD, which can occur in the absence of senescence and so as well as being distinct; the two processes are also mechanistically independent.

The function of senescence, which is dependent on the viability of the cells, is to maximise the salvage of nutrients, including nitrogen, phosphorous and minerals in particular, but also carbon, which are recycled to the growing parts of the plant or stored until the next growing season (Noh and Amasino, 1999*a,b*; Buchanan-Wollaston, 1997; Bleecker and Patterson, 1997; Noodén, 1988*a,b*). This saves energy (Leopold, 1961) and is particularly valuable in environments where certain nutrients or minerals are limiting, with phosphate being a prime example of such a nutrient. It has been suggested, therefore, that death is merely a pleiotropic consequence of a programme developed primarily for nutrient salvage (Bleecker, 1998; Noodén, 1988*a*). Paradoxically, senescence and death are thus of great value for the survival of the plant - as once an organ is of no further use to the plant, or is under stress, or attack from pathogens, it senesces so that the nutrients can be used by other parts of the plant. This improves the efficiency of the whole plant and ensures that the

stressed or infected organ does not adversely affect the whole plant, since it is shed (Leopold, 1961). Senescence is also important for escape from seasonal adversity, such as low water availability either due to low rainfall or freezing, and high winds; leaving only the most resistant structures to survive stressful conditions (Larcher, 1973). Another application of senescence is in evolution- as nutrients from the plant are eventually translocated to the seed, which may produce a plant that is better suited to the environment than the parent plant (Leopold, 1961). Nutrients are recycled and translocated so efficiently that a single *Lolium temulentum* seed grown on a hydroponic medium lacking phosphorus produced a mature (if underdeveloped) plant bearing a solitary fertile seed, by recycling the phosphorus present in the original seed through the consecutive leaves and into the new seed (Thomas *et al.*, 2003).

Senescence is reversible in the leaves of many, if not all, plant species (Zavaleta-Mancera *et al.*, 1999*a, b*), showing that senescence can occur without PCD, further proving them to be distinct processes. Leaves do eventually reach a point when senescence can no longer be reversed late in the process (Brady, 1988; Noodén and Leopold, 1978), which may mark the transition between senescence and death. Thus, senescence and its reversal, regreening, are both processes of transdifferentiation ('change of a cell or tissue from one differentiated state to another'; *Dictionary of Cell and Molecular Biology Online*) or metaplasia ('a reversible change in the character of a tissue from one mature cell type to another'; Underwood, 2000).

1.1.1.1 Senescence in cells, organs and organisms

PCD is exactly that, a way that particular cells can die in a planned manner- and can be described as cellular suicide, as the cell actively participates in its own demise. Senescence refers to a whole organ, thus making it unique to plants, and is a prelude to death- a way in which the plant can salvage as much as possible from the organ before it dies. However, organs such as leaves and petals are made up of several tissue types which are neither homogenous nor synchronous in their development, and thus organs neither senesce nor die uniformly. Xylem tissue dies early in leaf development and the phloem elements also autolyse their contents as they mature (Woolhouse, 1984), whereas stomatal cells retain fully green chloroplasts almost indefinitely (Heaton *et al.*, 1987). Furthermore, even within a given tissue,

senescence may be initiated at different times and progress at different rates in different cells, because for the nutrients recycled from the senescing leaf to be exported efficiently, a connection must be maintained between the cells and the vascular tissue, thus the areas surrounding the vascular bundles senesce later than the marginal tissue. Similarly, the marginal cells of flower petals degrade early, and in fact have often completely degraded by the time the flower has opened, as observed in *Alstroemeria* (Wagstaff *et al.*, 2003). Even the process of senescence itself may differ, depending on the contents and purpose of the senescing cell. In terms of cellular contents, a mesophyll cell can contain more than a hundred chloroplasts (Matile, 2000), yet in a mature leaf, only about a half of the cells contain any chloroplasts at all (Pyke, 1994). For a plant growing in a phosphorus-limited environment, an organ may senesce so that the phosphorus can be recycled, and the remobilisation of protein and other nutrients may not be a priority (Thomas and Sadras, 2001). However, for another plant, phosphate may be freely available and nitrogen be limiting and thus it is more important to recycle the nitrogen efficiently and fully. Degreening of the single celled algae *Chlorella* appears to be similar in many ways to senescence in plants, including similar methods of chlorophyll breakdown, protein degradation and mobilisation, some similar gene expression patterns (Hörtensteiner *et al.*, 2000) and the reversibility of the process (Aoki *et al.*, 1965). Thus, although senescence is a whole organ phenomenon, this suggests that the individual cells within the organ may have the capacity for autonomous senescence, just as PCD is a cell autonomous process. Thus, senescence could be 'scaled down' to a single cellular level, and furthermore that the process following on from senescence, PCD, can be 'scaled up' to occur on a whole organ level.

Plants can be seen as being modular, comprising a number of structural modules, or organs, making up an integrated whole. These modules interact with each other within the individual in a similar way to which individuals interact with each other within a population (Thomas, 2002)- competing for resources, with the most successful surviving, and others dying and recycling the nutrients contained within them to a common pool for reallocation. This happens through sequential senescence, where leaves, or modules, senesce sequentially, oldest first; and the nutrients are recycled through successive generations of modules. Annual plants take senescence

as an escape from seasonal adversity to the extreme, and undergo complete senescence of the whole organism or monocarpic senescence. This follows the reproductive phase of the plant's lifecycle, and the nutrients from every vegetative part of the plant are eventually recycled to the reproductive structures, such as the fruits and seeds, leaving only these to survive the more stressful conditions. As the vast majority of nitrogen (Feller and Keist, 1986) and phosphorus (Thomas *et al.*, 2003) for grain filling is recycled from the vegetative parts of the plant, senescence is central to the nutrient budget of seed crops. This leads to a conflict between the nitrogen needs of the seed, which requires degradation of leaf protein, and its requirement for carbon, for which the nitrogen-rich photosynthetic machinery must be conserved (eg. Sadras *et al.*, 1993). Again, this is not a uniform process, and the senescence of each organ and the organs' constituent tissues differ in the timing of their initiation and in the rate of their progression. Predictably, this is a complex series of events, due to its large scale, but leaf senescence appears to be central to monocarpic senescence (Noodén *et al.*, 1997).

1.1.2 Ageing

Ageing has been defined as the changes that occur over time that do not necessarily result in death (Medwar, 1957) and, as opposed to senescence, is a passive degeneration (Leopold, 1975). An example of the passive accumulation of lesions with age is the steady loss of viability of seeds during storage (Roberts, 1988; Priestley, 1985; Noodén and Leopold, 1978). It has been suggested that as reactive oxygen species (ROS) increase with increasing age and levels of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) drop, that the inbuilt processes that protect against free-radical damage break down (e.g. Munne-Bosch and Alegre, 2002; Bialski, 2002; Orendi, 2001; Ye *et al.*, 2000*b*; Jimenez *et al.*, 1998). Another possibility is that a deficiency in some critical component(s) is induced in the tissue, either due to changes in metabolism resulting in reduced biosynthesis, or as essential materials cease to be translocated, or due a combination of these factors; leading to the tissue ageing and 'wearing out' (Thomas *et al.*, 2003). Thomas (2002) and Dangl *et al* (2000) note that ageing may be a starvation or neglect procedure, and ask what this means for an autotrophic organism, where energy and raw materials are generally not limiting. In the case of petals, which act as sink organs, the plant may cease to allocate nutrients to them once their

useful or predetermined life is over, allowing them to decline passively with increasing age.

1.1.3 Leaves senesce, petals...?

The chloroplasts, which are an important organelle in most healthy, mature leaf cells, transdifferentiate into gerontoplasts (a term coined by Sitte in 1980) in senescing tissues (Parthier, 1988), losing their green colour as the chlorophyll is degraded and the yellow carotenoids are unmasked (Matile, 1992); then return to being chloroplasts should senescence be reversed and the tissue regreen. Similarly, the chloroplasts of immature petals transdifferentiate into chromoplasts, containing some of the coloured pigments responsible for flower colour rather than chlorophyll. Regreening of petals has not been induced experimentally, however in certain orchids the chromoplasts of the perianth can revert to chloroplasts following pollination, and the organ regains photosynthetic capability (Curtis, 1943). Likewise, the petals of some species of Liliaceae and Orchidaceae species become green rather than wilting or abscising (Pfeiffer, 1928; Fitting, 1921). Senescing lower leaves regreen once the upper shoots are removed and similarly, flower longevity can be increased by removing the young buds (eg. Chanasut *et al.*, 2003). Thus, there is an argument for the similarity of the late developmental processes of petals to leaf senescence, and it is possible that petals may senesce in a similar way to leaves, recycling the nutrients actively. Ashman and Schoen (1994) assume the plant has a fixed pool of resources from which to draw for flowering, but do not consider the possibility that the flower may pay back into this pool before it dies. However, petals may not need to recycle their nutrients, which is the defining feature of senescence, in order to benefit the plant, since their removal may be worthwhile in itself as they cease being a nutrient sink and a source of transpiration; and thus, they may age passively. Daylily flowers senesce, exporting sugars, chiefly sucrose, and amino acids, mainly hydroxyproline and glutamine, via the phloem for many hours after wilting, translocating these compounds to other parts of the plant, particularly developing flower buds. This recycling is only seen in flowers still attached to the plant, with no export of nutrients seen in detached flowers (Bieleski, 1995). Similarly, in *Gladiolus*, material must be recycled from senescing to developing florets, as removal of older flowers reduces the size of younger flowers when they open (Waithaka *et al.*, 2001).

From these definitions of senescence and ageing, it could be argued that senescence anticipates ageing, concluding in death on the plant's own terms (Thomas, 1994).

1.2 Leaves and petals as model systems

In this section, the value and accessibility of using different plant parts for senescence studies is assessed, with reasons given for the eventual choice of leaves and petals. The validity of comparing the two organs is discussed, with regard to the similarities and differences between them and to what can be learnt from the comparison. The late developmental events of leaves and petals are considered, along with the merits of their study and of comparing these events between the two organs with regard to the purpose and function of senescence.

Plants comprise three distinct parts : roots, stems, and leaves and flowers (as all of the floral organs are, in fact, modified leaves; von Goethe, 1790). There is little evidence for root senescence (Fisher *et al.*, 2002), which stands to reason, as making limiting nutrients available to the rest of the plant by recycling them from the senescing root achieves a short term goal, but exacerbates the plant's situation, as the nutrient will become even more limiting as adsorption by roots is decreased. Thus, plants are likely to curb the effects of nutrient deficiency on root death (Fisher *et al.*, 2002). Root senescence would also compromise the stability of the plant. Stems do senesce, as seen in monocarpic senescence. However, availability of stem material, and the non-homogeneity of senescence across the whole organ being even more pronounced in stems than in leaves, make stems a less accessible model system for study. Stem senescence exclusively accompanies leaf senescence, as the leaves distal to the senescing section of stem will senesce prior to the stem (Grbic, 2003), so that the nutrients recycled from the leaves can travel along the stem and back to the plant. A case could be made that fruit comprise a fourth plant part, but these are derived from the floral tissues, normally the pericarp (ovary wall), as the enclosed seeds mature. Fruit are very diverse structures, differing greatly in type and precise floral origin between species. Thus, as floral parts are modified leaves, and fruit develop from floral parts, it could be argued that fruit are modified leaves and that the process of ripening is analogous to leaf senescence, as a late developmental event. Fruit ripening is in many respects similar to leaf senescence at the physiological and

biochemical level (Davies and Grierson, 1989), particularly in the case of dry fruits. Similar colour changes are seen in both processes, the photosynthetic apparatus is dismantled, there are increases in the activities of hydrolytic enzymes, starch and chlorophyll are degraded and respiration is stimulated (McGlasson *et al.*, 1975). Both processes are under genetic control (eg, Ambler *et al.*, 1987; Tigchelaar *et al.*, 1978) and there are changes in the pattern of mRNA and protein synthesis (Grierson *et al.*, 1985), with some evidence that there is some common gene expression involved (Davies and Grierson, 1989). However, ripening is intrinsically different from senescence in terms of the process and its purpose. Leaf senescence is a deteriorative process, with the purpose of recycling the nutrients within; whereas fruit ripening is a growth process, with the purpose of promoting seed spread. Indeed, a ripening fleshy fruit is a sink, taking nutrients, particularly sugars, from elsewhere to serve as an enticement to animals to help spread the seed, thus differing definitively from a senescing leaf, which acts as a source of sugars and other nutrients. The diversity of fruit types would also make it difficult to make a general comparison between fruit ripening and leaf senescence, without studying the events in a wide range of different species. Thus, with regard to senescence, the organs best studied as they develop and mature are leaves and flower petals. Interestingly, no studies to date have directly compared late developmental events in these two organs.

Leaves are known to senesce during late development, and much work has been done, and is continuing, in characterising this process in the leaves of many different plant species. Hence, they provide an invaluable point of reference with which to compare petals in order to address the question of whether or not petals senesce. An advantage of studying petals over leaves is that the process is irreversible and under tighter developmental control (Rubinstein, 2000) – so within a given species, it is possible to closely predict the timing of development from bud opening to senescence (Molisch, 1938). Although the progression of natural leaf senescence is predictable and conserved in most plant species, the timing of leaf senescence is more likely to be affected by external influences than that of petal death, including stresses such as extremes of temperature, drought, nutrient deficiency, pathogen infection, wounding and shading (eg. Rousseaux *et al.*, 1996); and the reversibility of the process adds another complicating factor (Molisch, 1938). Petal death is sometimes induced by flower pollination, or otherwise at the end of their predetermined life. As a

general rule, the larger and more elaborate the flower, the shorter its life will be (Ashman and Schoen, 1994), as more resources, from a limited floral reserve pool, are necessary for their preservation (Ashman and Schoen, 1997) and the opportunity for infection is greater (Shykoff *et al.*, 1996). Development also generally occurs over a shorter timescale in flowers compared to leaves. A combination of these factors means that the morphological and physiological changes that occur over time are more pronounced in petals than in leaves, making late petal development an easier process to document.

Direct comparison of late developmental events in leaves and petals should prove interesting, as these are comparable organs in many respects. It was first proposed more than 200 years ago, that petals are modified leaves (von Goethe, 1790). Since that time, mutations in the genes governing floral identity have produced plants with leaves where there should be petals and other floral organs (Bowman *et al.*, 1991) and more recently, the discovery of another class of floral identity genes has made it possible to convert vegetative leaves into petals (Pelaz *et al.*, 2001). Similarities between leaves and petals include their internal structure, as the cells of both organs are arranged similarly and contain similar organelles.

Plastids define the plant cells in which they are found, and the ways in which they interconnect and transdifferentiate is a key concept of developmental biology (Thomson and Whatley, 1980). Both leaves and young petals contain chloroplasts- which transdifferentiate into gerontoplasts as leaves senesce and into chromoplasts as petals mature; both of these processes result in the breakdown of chlorophyll, and the resulting plastids are closely related (Sitte, 1977). Chromoplasts form before flower opening, and so before the function of the petal in attracting pollinators starts. Similarly, gerontoplast formation is an early event in senescence, before the senescent leaf starts recycling nutrients. The conversion from chloroplast to gerontoplast is possible in lower plants, and so is an earlier event in evolutionary time than differentiation from chloroplast to chromoplast, and may be its evolutionary forerunner (Thomas *et al.*, 2003), or the two types of plastids may have derived from a common ancestor (Matile *et al.*, 1999). It is possible that reversibility is a feature that leaf senescence gained after the divergence of the two events, in order to increase

efficiency of nutrient use in an ever changing environment, alternatively it may be an ancestral quality, largely lost in petals (Thomas *et al.*, 2003).

Leaves often become yellow, orange or red as the partially retained carotenoids are unmasked as the chlorophyll is degraded, but can also turn a more striking yellow, red or purple as phenylpropanoid derivatives, such as anthocyanins, betacyanins and flavonoids, are synthesised *de novo* and accumulated in the vacuoles; finally turning brown on death, due to the formation of the oxidation products of the phenolics, which polymerise to give melanins (Matile, 2000). Similarly, petals accumulate anthocyanins, carotenoid derivatives and flavonoids in the vacuole and chromoplasts, which give them their vivid and attractive colours, and often also turn brown on death. Co-pigmentation with other phenolic glycosides and formation of complexes with metal ions in the vacuoles may contribute to the variation in shades of colour in senescing leaves, as is known to be the case in flower petals (Harborne, 1993). It is therefore reasonable to think of leaf senescence and petal maturation as homologous processes, and it could be argued that not only are petals modified leaves, but that they are modified senescing leaves (Thomas and Sadras, 2001; Thomas *et al.*, 2001). Indeed, the petals of *Poinsettia* are insignificant structures, and attention is drawn to the flowers through the involucre, a whorl of bracts surrounding an inflorescence, which turn bright red once the flowers are ready for pollination. Similarly, the 'fruit flagging' hypothesis suggests that senescing leaves may enhance the attractiveness of ripening fruit, increasing their chances of being noticed and dispersed by animals (Stiles, 1982).

There are, however, some important differences between leaves and petals. Mature leaves are source organs, photosynthesising to provide sugars that are translocated to all the other parts of the plant, whereas mature petals are sinks, taking nutrients from other parts of the plant. The increasing demands of sinks may function in triggering late developmental events in flowers (Nichols and Ho, 1975*a, b*); as trimming flowers from flowering spikes increases durability of the remaining flowers (Chanasut *et al.*, 2003). Similarly, removal of older flowers of *Gladiolus* reduces the size of the younger flowers when they open, indicating that material must be recycled from senescing to developing florets (Waithaka *et al.*, 2001). This provides evidence to

support Ashman and Schoen's (1994) assumption that there is a fixed pool of resources set aside for flowering.

There are parallels in chloroplast transdifferentiation in the two organ types, including chlorophyll breakdown, and the loss of the stromal components and the thylakoids; however, there are also differences which distinguish the two transdifferentiated plastids, gerontoplasts and chromoplasts. Gerontoplasts are distinct from chromoplasts, as carotenoids are only retained in gerontoplasts, whereas they are also newly synthesised in chromoplasts (Sitte *et al.*, 1980). Gerontoplasts are also distinct from all other plastids, in that their metabolism is entirely catabolic (Matile *et al.*, 1999). Gerontoplast development is under nuclear control, and the expression of plastid-encoded genes plays only a minor, if indeed any, part (Matile, 1992), as the ctDNA is extensively degraded before chlorophyll metabolism starts (Sodmergen *et al.*, 1991; 1989), although the reversibility of the process suggests that enough genetic information is retained to support regreening and chloroplast reassembly (Matile *et al.*, 1999). Chromoplast development differs from gerontoplast formation in this respect, in that the incorporation of a new set of proteins involved in the synthesis of secondary carotenoids is encoded in both the nuclear and plastid genomes (Camara *et al.*, 1995).

Returning to the 'wearing out' hypothesis of ageing, petals contain few nutrients by comparison to leaves and so the plant has little to lose by withdrawing its input of nutrients and merely allowing the petals to die, without any attempt at salvaging what is left in them. Indeed, the metabolic cost of implementing a senescence programme may be greater than the value of what can be recovered. Conversely, for leaves, there is considerable nutritional gain from senescing. Whether or not petals recycle their nutrients before abscission is an important consideration in defining whether or not petals senesce. Van Doorn (2004) puts forward three alternative models for the cause of petal senescence. Each involves the degradation of polysaccharides, proteins, lipids and nucleic acids, leading to the mobilisation of sugars and nitrogenous compounds, which occur before visible deterioration of the petal and subsequent cell death. This supports the model of a petal being a modified senescing leaf (Thomas and Sadras, 2001; Thomas *et al.*, 2001). However, no original research ascertaining that remobilisation is a feature of petal senescence is quoted as a basis for these

models. An important distinction in late floral development is between wilting and non-wilting species (van Doorn and Stead, 1997). Wilting occurs due to membrane breakdown, resulting in loss of turgor pressure (Noodén, 1988*a*). In wilting species, the perianth loses fresh weight and turgidity before abscission (Stead and van Doorn, 1994). Thus, wilting may be important in allowing remobilization of key metabolites from the senescing petal back into the plant (Wagstaff *et al.*, 2002; Rubinstein, 2000). The wilting of *Ipomoea* petals is accompanied by the breakdown of cellular integrity, with the mesophyll cells being autolysed first, followed by the epidermal cells; whilst the vascular tissue remains apparently healthy for much longer. This could be due to the recycling of the cellular contents of the autolysed cells, which needs the vascular tissue to remain functional for translocation (Matile and Winkenbach, 1971). Similar ultrastructural changes were seen in carnation petals (Smith *et al.*, 1992). Cycloheximide, an inhibitor of protein synthesis, prevents wilting in daylily petals (Lukaszewski and Reid, 1989), indicating that protein synthesis is needed for wilting (Stead and van Doorn, 1994). Studies of petal proteins and proteases with increasing age in *Sandersonia* petals have indicatd that protease action is important for normal progression of late developmental processes in petals, with protease-inhibitors increasing vase life (Eason *et al.*, 2002). This suggests that a certain level of control is required for late development in petals, and that it is not a passive process, which leads to the possibility that recycling of the amino acids may be one of the controlled processes. Similarly, in leaves, PCD and leaf browning precedes leaf abscission in some species, whereas in other species the shed leaves can remain yellow and turgescent for several days before browning and death occur (Matile, 2000).

By comparing the late developmental events of petals with leaf senescence, it will be possible to establish whether the two organs use similar processes, and thus whether or not petals actively senesce, as do leaves. However, one way in which both organs are known to be similar is in that their ultimate fate is death. Thus, it may be useful to compare different processes incorporating PCD in different plant tissues to establish whether the phenomenon is universal, or if there are differences in the process, depending on its purpose and location.

1.3 Death and senescence

In this section, different kinds of cell death are described, and their purpose, mechanisms and cellular processes are compared to those of senescence. Little data is available on the degree of overlap between the different types of death, but examples of similarities and differences in the processes are discussed. Parallels between PCD in plants and animals are drawn, with particular attention given to senescence in plants and apoptosis in animals, as comparisons are often made between these processes. Much headway has been made in apoptosis research in animals in recent years, and whether this bears any relevance to events in late plant development, such as senescence, is considered.

1.3.1 Defining programmed cell death

Cell death in animal systems can either be accidental or programmed, and the symptoms of accidental or necrotic death are very different to those of PCD. During necrosis, cell death is not controlled, resulting in cellular swelling and eventually explosion through gain of water and sodium, due to the failure of the membrane pumps. PCD is a broad term describing the actively regulated process of "cellular suicide" which occurs in both plant and animal cells in response to a wide variety of both internal and external stimuli, integrating death and survival signals (Jones, 2001; Leopold, 1961). Although necrosis has not been as clearly defined in plant cells as in animal systems, characteristics which occur during PCD distinguish it from necrosis, including the collapse of the vacuole, which is mediated by calcium flux, and chromatin degradation (Jones, 2001). Paradoxically, PCD is essential for the survival of the plant, as many different processes are accomplished through methods terminating in PCD, including developmental processes, environmental responses and responses to pathogen attack. Some are distinct from senescence, whereas others are very similar, and may serve to induce senescence prematurely, possibly by a different signalling pathway. The developmental processes are distinct, and, along with senescence, include xylogenesis, aerenchyma formation, reproduction, abscission and patterning. Many of the environmental responses appear superficially similar to senescence, and incorporate responses to abiotic stresses, such as extremes of temperature and water stress, as well as nutrient deficiency, wounding and changes in light conditions. The responses to pathogen attack include the hypersensitive

response, and bear some resemblance to senescence, although are likely to be distinct. To what extent the pathways governing different types of PCD overlap and share mechanisms is largely unknown.

1.3.1.1 Developmental PCD

Here, cell death is an integral part of plant development. It is necessary for the formation of certain types of tissue, such as the xylem and the aerenchyma tissue in waterlogged roots, and for normal reproductive development, as in sex determination in monoecious flowers and in the embryo suspensor, as well as for other developmental processes, like abscission and patterning. The recycling of nutrients is not a primary concern of these processes, distinguishing them definitively from senescence. Data on whether recycling may occur as a side effect of developmental PCD is limited, but it is unlikely considering the relatively small numbers of cells involved, by comparison to the large scale of leaf senescence. Despite the purposes being distinct, some of the mechanisms and cellular processes are shared with each other and with senescence (Jones, 2001; Jones and Dangl, 1996). Similar increases in the synthesis and activity of degradative enzymes are seen in each, and the implementation of calcium signalling controlling the action of the vacuole is also common (Jones, 2001; Cheng *et al.*, 1983). However, there are important differences in corpse management. During other forms of developmental PCD, there is little evidence for cytoplasmic degeneration prior to vacuole collapse, whereas during senescence, much of the cells' contents have been degraded before the collapse of the vacuole (Jones and Dangl, 1996). This difference is consistent with the idea that recycling does not occur during other forms of developmental PCD.

1.3.1.1.1 Abscission. In many species, abscission marks the end of an organ's life. In leaves, it can occur either after senescence, without senescence, or not at all, and similarly, in petals, after wilting, without wilting or not at all, depending on the species of plant. It requires PCD at the abscission zone: a morphologically distinct band of small, densely cytoplasmic, non-vacuolated cells, typically only a few layers thick. This is normally located at the base of the organ to be shed and created during the development of the associated organ system (Gonzalez-Carranza *et al.*, 1998; Osbourne, 1989; Addicott, 1982; Sexton and Roberts, 1982). Abscission is often induced by ethylene, and delayed by auxin (Abeles *et al,*, 1992; Sexton and Roberts,

1982), as is the case with senescence, and like senescence, abscission is a genetically determined event. Abscission in both leaves and flower petals involves the coordinated enlargement of the cell, breakdown of the middle lamella and cell wall matrix at discrete sites and specific stages (Roberts *et al.*, 2000; Bleeker and Patterson, 1997; Addicott, 1982; Sexton and Roberts, 1982). Once the organ is shed, the cells on the proximal face of the fracture plane continue to enlarge and differentiate into suberized scar tissue (Addicott, 1982). Upregulated genes include those that contribute to the process of cell separation, ones that act to protect the exposed fracture surface from pathogenic attack, and others whose function is presently unknown (Roberts *et al.*, 2002*b*). Thus, abscission differs considerably from senescence in purpose and scale, as well as notably in the absence of the action of the vacuole and in gene expression profile.

1.3.1.2 External stimuli resulting in senescence-like processes

Several external stimuli can affect the onset and progression of senescence, or processes resembling senescence and sharing some common pathways. These include environmental stresses, such as extremes of temperature, water stress, nutrient deficiencies, wounding and light conditions, as well as pathogen attack and the hypersensitive response (HR). This is because plants cannot move away from adverse environmental conditions, and senescence is one mechanism that they have evolved to cope with such problems. In these cases, it is likely that recycling of nutrients is an important concern wherever possible, thus resembling senescence; with possible exception when the speed of the response to changing conditions is of greater importance to the plant as a whole. However, the primary reason for cell death is, of course, a stress response; with the death of the organ being necessary or advantageous to the rest of the plant, even without nutrient recycling. Similarly, although many cellular and molecular events are shared between these responses and developmental senescence, with calcium induced vacuolar disruption again playing an important role and many similar genes being upregulated, there remain differences. Thus, these processes must be considered 'senescence-like', and distinct from true developmental senescence, despite it being likely that many of the pathways are shared.

1.3.1.2.1 Temperature. High temperature results in a decrease in leaf photosynthetic activity, chlorophyll levels and cytokinin levels – possibly a factor in

triggering the other symptoms (Caers *et al.*, 1985), as cytokinins are a negative regulator of senescence. However, some of the thylakoid proteins normally degraded during senescence, such as LHCPII, are protected from degradation (Ferguson *et al.*, 1993), suggesting either that normal senescence is disrupted, or that this process is distinct. Heat shock, however, can delay senescence in the petals of carnation (Verlinden and Woodson, 1998) and daylily (Panavas *et al.*, 1998*a*). This may be as the expression of senescence genes is delayed or prevented, or due to the protective effects of the heat shock proteins (Rubinstein, 2000; Vierling, 1991). It has been shown in oat that cold may induce senescence, but continued cold can reduce the rate of the process (Thomas *et al.*, 1980).

1.3.1.2.2 Water stress. Both drought (Mothes, 1928) and waterlogging (Belford, 1981) can induce leaf senescence. Drought also decreases the longevity of certain flowers, such as carnation, as ethylene is made prematurely and ion leakage increases, with protein synthesis necessary for this response (Beja-Tal *et al.*, 1995).

1.3.1.2.3 Nutrient deficiency. Deficiency of mobile nutrients such as nitrogen or phosphorus causes senescence of the older leaves so that these nutrients can be remobilized to the younger growing tissues (Simpson, 1986; Makino *et al.*, 1984; Goodwin *et al.*, 1978). Deficiency of non-mobile elements, such as calcium, results in only the younger tissue being affected (Sprague, 1964). As nutrient recycling is a primary feature of the response to mobile nutrient deficiency, it can be regarded as a senescence process.

1.3.1.2.4 Wounding often results in the stimulation of PCD, and as a wounded area is a likely point of infection, immediate removal of that organ is of benefit to the plant (Ichimura, 1998; Woltering *et al.*, 1997).

1.3.1.2.5 Light conditions. It would seem that natural selection is likely to have favoured the evolution of pathways that reallocate nutrients from the leaves when their photosynthetic capacity falls below their compensation point, as well as when the nutrients are needed elsewhere to support the development of other organs (Bleeker, 1998). Signalling may be through light quality (Rousseaux *et al.*, 1996), or reduced levels of photosynthate may act as a trigger. However, hard evidence in

support of this contention is difficult to find, and there are many examples of plants that maintain large numbers of 'inefficient' leaves. For example, a study of carbon dioxide flux at different levels in the leaf canopy of maize shows that more than 30% of the foliage of the canopy is located below a level where the compensation point is not crossed (Lemon and Wright, 1969). There must be a purpose to the plant of retaining such leaves, and storage is one plausible function (Roussaux *et al.*, 1997). Thus, the major leaf proteins, such as Rubisco, as well as having a role in photosynthesis, are also important as a nitrogen reserve (Thomas and Sadras, 2001). Shade leaves may also be useful for species growing where light conditions are variable, or as 'insurance' to replace leaves grazed by animals, eaten by insects or infected by pathogens. They may also act as cooled surfaces for condensation or as counterweights against top-heaviness and susceptibility to physical damage (Farnsworth and Niklas, 1995). No complete studies have been published on the effects of light quality on petal longevity (Rubinstein, 2000). Dark induced senescence is an extreme example of shading, and can be reversed by returning the plant to the light, but this reversal is increasingly less effective after longer periods in the dark.

Senescence in many species appears to be affected by changes in daylength, although this may sometimes act through the effect of daylength on the switch to flowering (Smart, 1994). Deciduous trees seem to be sensitive to decreases in daylength and temperature (Koike, 1990; Titus, 1989) and a relationship seems to exist between photoperiod and flowering, as many short-lived flowers (such as morning glory and daylily) open and begin to die at specific times of day. However, little work has been done with regard to this (Rubinstein, 2000).

Thus, it seems that changes in light conditions do induce true senescence in leaves, rather than being a senescence-like process; with recycling of nutrients a key feature and many of the processes shared. There are nevertheless differences in regulation and gene expression between developmentally-induced and light-induced senescence.

1.3.1.2.6 Pathogen attack can cause increased yellowing, either localised, mosaic or general, or the formation of "green islands" around the points of infection (Scholes

and Farrar, 1987; Mothes, 1970; Shaw, 1963). Cells infected with fungal hyphae show cytoplasmic changes similar to those seen in senescence, with calcium influx causing vacuole collapse (Jones, 2001). Some of the molecular changes seen are also similar in these two processes and certain genes are specifically expressed during both processes, indicating that some of the underlying molecular mechanisms may be shared (Jones, 2001), however, speed of response is a priority over nutrient recycling in responding to pathogen attack.

1.3.1.2.6.1 The hypersensitive response (HR). The HR is characterised by the rapid death of the plant cells directly in contact with, or close to the pathogen (Lorrain *et al.*, 2003). Following pathogen attack and signal transduction, ROS, particularly superoxide radicals and hydrogen peroxide (Mehdy, 1994; Tzeng and DeVay, 1993) are generated rapidly, along with the biosynthesis or release of potential microbial effector molecules. These are thought to contribute to the death of both the invading pathogen and the host. Lesion mimic mutants (LMM) mimic the HR in the absence of a pathogen, showing that the HR is an internally genetically regulated process (reviewed by Jones and Dangl, 1996). The variety of LMMs implies that there are a number of different initiating signals and cell death pathways, or that the mutant phenotypes are a result of disruptions to normal metabolic pathways (Dietrich *et al.*, 1994). Unlike in the examples of developmentally induced PCD described above, the corpse is not significantly autolysed during the HR, and is mechanically crushed as it is pushed together by the surrounding tissue as it grows and expands (Jones, 2001; Jones and Dangl, 1996), implying that nutrients are not recycled. Gene expression analyses indicate that there may be some overlap between the HR and senescence, with genes encoding products similar to the pathogenesis-related proteins associated with the HR, termed defence-related proteins, expressed during senescence (van Loon, 1994). These may protect from opportunistic infections in the senescent leaf and must be an integral part of the senescence programme, as they are still induced in senescent *Arabidposis* plants grown under sterile conditions (Quirino *et al.*, 1999). However, nutrient recycling is not a task for this rapid form of PCD, rather its purpose is to prevent spread of the pathogen by the death of the host cells nearby. Thus, although pathways may be shared between the two processes, the HR and senescence are distinct routes.

1.3.1.3 Overlap between developmental and externally induced senescence

Investigations have explored the overlap between developmental senescence and externally induced senescence-like processes, revealing a range of different SAG (senescence-associated gene) activation patterns (Weaver *et al.*, 1998). Some genes are expressed exclusively in one process, whereas others are common to more than one (Nam, 1997). Within the senescence-like processes, it is likely that the initiating signals vary, but in general, the processes are likely to be the same (Buchanan-Wollaston, 1997). Senescence enhanced expression of 40 different transcription factors has been reported, with most also showing induced expression in response to at least one stress treatment (Chen *et al.*, 2002*b*). Thus, there is evidence for sharing of pathways, but that the processes are not identical. Furthermore, it is likely that there is some variation in the pathways leading to death even within a specific type of cell death (Jones, 2001), as blocking of a particular pathway may not have a significant effect on the progression of senescence, indicating that there is a regulatory network rather than a single pathway (Gan and Amasino, 1997).

1.3.2 Parallels to animal cell death and apoptosis

As plant cells have unique features, such as cell walls and vacuoles, it is likely that they have unique mechanisms for cell death. This is particularly true of whole organ death, which has no analogous events in most animals (Rubinstein, 2000).

In animals and yeast, comparative studies have shown that insulin signalling, metabolic flux and free radicals are conserved strategies employed to regulate ageing. The insulin/IGF (insulin growth factor)-1 signalling system represents the conserved hormone regulation of lifespan (Kenyon, 2001; Finch and Ruvkun, 2001; Longo and Fabrizio, 2002). This pathway is also responsible for animals' ability to sense environmental cues, to adjust growth and development, and to control oxidative stress resistance, food utilisation and reproduction (Jing *et al.*, 2003). Plants do not posses this signalling pathway, but do employ phytohormones to regulate senescence, which differs from the insulin/IGF system, in involving many different hormones, all interacting with each other (section 1.4.2.3).

The role of metabolic flux in regulating ageing in animals was first shown by calorie restriction (30-60% of *ad libitum* intake) increasing lifespan and retarding the rate of

ageing in rodent studies, and it has since been shown in a wide range of species (Guarente and Kenyon, 2000; Merry, 2000; Pugh *et al.*, 1999). In fruit flies, calorie restriction has been shown to share overlap effects with insulin/IGF-1 signalling in regulating lifespan (Clancy *et al.*, 2002). Although similar results have been noted in plants as in animals with regard to metabolic flux, with increased leaf longevity as a result of restricting photosynthetic ability and accelerated senescence due to increased energy input (discussed in detail in section 1.4); the photoautotrophic nature of plants makes them fundamentally different from animals.

Increased oxidative damage accelerates ageing in animals, and enhanced resistance to oxidative damage can extend lifespan (Finkel and Holbrook, 2000), supporting the free radical theory of ageing (section 1.1.2). This also appears to be the case in plant senescence (Jing *et al.*, 2003), providing a direct parallel between the animal and plant systems.

The caspases, a family of cysteine proteases, play important roles in the signalling of apoptotic death in animals (reviewed in Salvesen and Dixit, 1997; Zhitvotovsky *et al.*, 1997), participating in both the initiation and execution of apoptosis. Although cysteine proteases increase in expression and activity during senescence in plants, no caspase homologues have yet been found. It is nevertheless possible that there are proteins that are structurally non-orthologous but functionally equivalent to caspases in plants, as caspase-like protease activity has been found in some types of plant PCD (Korthout *et al.*, 2000; del Pozo and Lam, 1998). The *Arabidopsis* genome contains more than 10 metacaspase homologues (Aravind *et al.*, 2001), which contain some, but not all, of the domains found in mammalian caspases (Uren *et al.*, 2000). In addition, poly-(ADP-ribose) polymerase (PARP), a specific substrate of caspase 3 during the apoptosis of mammalian cells, has been found during H_2O_2-induced PCD in plants (Amor *et al.*, 1998), further indicating that functional equivalents of caspases may be involved in plant PCD, and thus possibly senescence.

A significant difference between animals and plants is in the importance of telomere length and telomerase activity. Several yeast homologue genes involved in maintaining telomere length have been identified in *Arabidopsis*. Mutations in these generate either no abnormality in phenotype in the case of KU70 (Bundock *et al.*,

2002) and *mim* (Mengiste *et al.*, 1999), or sterile plants in which the senescence phenotype could only be examined using callus in the case of *rad50* (Gallego and White, 2001). In one extreme situation, a T-DNA knock out of *Arabidopsis At*TERT survived 10 generations without telomerase, albeit with severe cellular damage in the last five generations (Riha *et al.*, 2001). These results confirm earlier observations that telomere dynamics are not associated with plant longevity (Riha *et al.*, 1998). This is in striking contrast to animal response to telomere dysfunction.

The term 'apoptosis', first coned by Kerr (1971), describes a specific type of PCD in animal cells with several distinguishing features, and derives from the Greek, meaning 'a falling away', as used in the context of leaf fall. Thus, implicit in the word, there is a connection with leaf senescence in plants. However, although some of the characteristics of apoptosis can occur in plant cell death, such as DNA fragmentation (reviewed by Rubinstein, 2000), there is little evidence that the molecular and biochemical events responsible for the altered morphology and/ or cell death are the same in both systems. Apoptotic cells lose water, leading to cell shrinkage. The cells remain viable, with the membrane pumps continuing to function, energy production maintained and the production of new mRNA and protein species, as is also the case in senescent cells. However, a defining characteristic of apoptotic cells is that they exhibit a discrete fragmentation into physiologically intact spherical apoptotic bodies, which are immediately engulfed by near neighbours or macrophages. Thus, it could be argued that any nutrients contained within the apoptotic cell are recycled to those near neighbours, making apoptosis functionally akin to senescence. However, the mechanisms are quite different, as the cell walls make such a response impossible in plant cells. Thus, as well as controlling the decision to die, the signals that induce and carry out plant PCD must also instruct the cells how to process the corpse through the action of the vacuole (Jones, 2001). The fate of the corpse must be decided by the living plant cell before the point of no return, as corpse processing is autolytic. Dramatic changes occur in the vacuole prior to PCD as it is transformed into a large hydrolytic compartment containing sequestered hydrolases. Its collapse is regulated by the cell (by calcium flux) and is not a result of metabolic rundown. These events do not occur during necrotic death (Jones, 2001). With regard to the importance of the vacuole, plant PCD more closely resembles the non-apoptotic vacuolar or autophagic cell death in animals (Bowen *et*

al., 1998), where an increasing number of lysosomal and autophagic vacuoles appear (Bowen *et al.*, 1996) and the elevation of digestive hydrolases plays a significant role in final cell autolysis (Bowen and Lockshin, 1981). However, senescence in plants most strongly resembles the non-apoptotic method of differentiation to death in animals. Since most differentiated animal cells are postmitotic and have lost the ability to reproduce and divide, it can be argued that all differentiated somatic cells inevitably move along specialised channels to death, in contrast to meristematic-, stem-, and germ-cell lines (Wangenheim, 1987). The case has already been made for senescence in plants as a process of differentiation leading to death in section 1.1. In conclusion, the characteristic signs of apoptosis in animals have not been convincingly demonstrated during normal leaf senescence (Noodén and Guiamet, 1996).

1.4 Regulation of senescence

Senescence requires precise control of gene expression for it to be executed properly and to be coordinated with other physiological processes.

1.4.1 Regulation of senescence is multifactorial

Many genes and diverse biochemical pathways are involved in the regulation of leaf senescence. Regulation is organised on several levels, cascading from large scale signalling to ever finer levels of regulation. Developmental cues, like reproduction and age related signals, control the timing using plant growth regulators (PGRs) and metabolic flux to signal throughout the plant. G-proteins, calcium and protein phosphorylation are responsible for intra-cellular regulation, which in turn affect the regulatory genes and promoters governing gene regulation. It is possible that regulation in different plants may differ, as in *Arabidopsis*, leaf senescence does not seem to be affected by a signal from the developing fruit (Hensel *et al.*, 1993), whereas in tomatoes and other fruiting plants, such a signal accelerates senescence (Lanahan *et al.*, 1994). Regulation is complex, with multiple pathways forming a regulatory network (Gan and Amasino, 1997). Thus, although processes may look similar phenotypically, the underlying molecular basis could be very different, as a blockage of one pathway in the senescence network can be circumnavigated, and does not necessarily affect the overall appearance (He *et al.*, 2001).

1.4.2 Developmental cues

Both reproductive and somatic development can control the time that death is initiated in different plant organs. Flowering and seed set can induce leaf senescence to reallocate nutrients for reproduction, and can stimulate whole plant senescence in monocarpic species. Flower pollination can induce petal death, the petals having fulfilled their role and thus having no further reason to be maintained. The chronological age of an organ also appears to be an important factor in determining the timing of senescence onset, through the action of unknown "age related factors".

1.4.2.1 Reproductive development

In some species, flowering and seed development increase the number of senescing leaves so that nutrients can be recycled to the reproductive structures (Hayati *et al.*, 1995). This is known as 'correlative control'. Removal of developing sinks, such as flowers, and restriction of pod growth as well as the removal of younger leaves delay leaf senescence (Miceli *et al.*, 1995; Crafts- Brandner, 1991; Noodén, 1988*a*). Many hypotheses have been presented concerning the nature of correlative control signals (reviewed in Woolhouse, 1983; Kelly and Davies, 1998; Noodén, 1988*b*) but they have yet to be established unequivocally for a higher plant, although various PGRs have been implicated in different systems (section 1.4.2.3). Leaves of monocarpic species live longer when flowering and pollination are prevented (Molisch, 1938), but despite *Arabidopsis thaliana* being monocarpic, somatic tissue longevity is not governed by reproductive development (Hensel *et al.*, 1993), but appears to be an intrinsic age-related property of the organ system. Cytokinins can prevent or delay senescence (section 1.4.2.3.2), and cytokinin levels in plants may be influenced by signals from developing sinks with the shift in nutrient allocation from vegetative to reproductive organs starting early in development (Kelly and Davies, 1988). It has also been suggested that developing fruit produce a "death hormone" (Lindoo and Noodén, 1977). This may refer to ethylene, which is produced by many fruit as they ripen and has the effect of promoting senescence in some species (section 1.4.2.3).

Pollination accelerates flower senescence in at least 60 genera, most of which are suspected to be ethylene sensitive (O'Neill, 1997; van Doorn, 1997). The flowers of the orchid *Phalaenopsis* can live for up to three months, but die within a day of pollination (Halevy, 1998). Signalling is by means of ethylene evolution. Increased

sensitivity to ethylene has also been noted in response to pollination, possibly mediated by short chain fatty acids, in carnation (Whitehead and Vasiljevic, 1993) and *Phalaenopsis* (Halevy *et al.*, 1996). Thus, although ethylene is important in pollination-induced senescence in many species, the signal transduction pathway varies between species, and may be due both to an increase in ethylene production and to an increased sensitivity to ethylene. Unusually, in *Phalaenopsis* removal of the pollinia also results in the death of the flower (Curtis, 1943).

1.4.2.2 Age and "maturation factors"

Although age may be a factor in determining when the senescence process starts, this is distinct from 'ageing'. The chronological age of the leaf appears to be an important factor in determining the onset of senescence. For example, ethylene will only induce senescence in older mature dicot leaves (Buchanan-Wollaston, 1997; Grbic and Bleeker, 1995), and the rate of photosynthesis declines after full leaf expansion (Hensel *et al.*, 1993; Jiang *et al.*, 1993; Batt and Woolhouse, 1975), a response which may also be ethylene mediated (Grbic and Bleeker, 1995); with unknown "maturation signals" implicated in controlling the timing in both cases. It has also been suggested that physical stress in older leaves may cause senescence: for example, in soybean, the angle between the petiole and the main stem increases markedly for the older shoots as the auxiliary shoots grow out (Thomas and Stoddart, 1980). However, in some species, leaf age seems to be less noteworthy than such factors as location or orientation in influencing leaf photosynthetic capability (Ackerly, 1999; Murchie *et al.*, 1999; Hikosaka *et al.*, 1994) and thus the useful life of the leaf before senescence is initiated. In the leaves of other species, such as *Arabidopsis* (Noodén and Penney, 2001; Hensel *et al.*, 1993) and in the case of many flowers (Rubinstein, 2000), however, the chronological age of the organ is a determining factor in regulating lifespan, and is independent of the reproductive status of the plant, although in the case of flowers with rare and very specific pollinators, such as many orchids, age is not as important a factor as reproductive status in initiating senescence. This is dependent on the floral resource allocation strategy of the species in question, balancing the cost of maintenance of an existing flower with that of constructing a new flower as an adaptation to rates of pollen removal and receipt (Ashman and Schoen, 1994). In conclusion, chronological age appears to be an important factor in

governing senescence onset in both leaves and flowers, although in some specific cases, there may be reason for other factors to override age as determining factors.

1.4.2.3 Plant growth regulators

In some systems, there is evidence for PGRs acting as major regulators of PCD. All of the major phytohormone classes have been implicated, but only ethylene and cytokinins have been shown definitively to have a role in senescence regulation.

1.4.2.3.1 Ethylene. The role of ethylene as a promoter of late development and of fruit ripening has been revealed by many studies on ethylene-treated plants and ethylene mutants as well as on transgenic plants (Johnson and Ecker, 1998). Plants can either be sensitive to ethylene (climacteric plants) or insensitive (non-climateric). In climacteric plants, ethylene production and respiration decrease, then increase (termed the climateric) (van Altvorst and Bovy, 1995), initiating late developmental processes in flowers by co-ordinately inducing gene expression. However, ethylene is probably not directly involved in regulating gene transcription (Theologis, 1993; Borochov and Woodson, 1989). In the non-climateric plants of families such as the Amaryllidaceae, Liliaceae, Iridaceae and Asteraceae, there is no requirement for ethylene signalling (Rubinstein, 2000; Valpuesta *et al.*, 1995; Smart, 1994; van Doorn and Stead, 1994) and the coordinating signal, if it exists, remains obscure. Ethylene also enhances leaf senescence in many plants, especially dicots (Smart, 1994), as demonstrated by the effects of ethylene treatment in advancing visible yellowing and SAG induction (Weaver *et al.*, 1998; Grbic and Bleeker, 1995) and by *Arabidopsis* ethylene-insensitive mutants that display delayed senescence (Bleeker *et al.*, 1998; Oh *et al.*, 1997; Chao *et al.*, 1997). In the many flowers whose demise is ethylene-dependent (Stead and van Doorn, 1994), addition of ethylene initiates premature advancement of late developmental stages, and inhibitors of its synthesis or action prolong flower life (Podd and van Staden, 1998; Lee *et al.*, 1997; Midoh *et al.*, 1996; Serek *et al.*, 1995; van Altvorst and Bovy, 1995). However, transcription of senescence related genes is not activated by ethylene unless the leaf is prepared to senesce, in which case senescence is enhanced. Thus, 'age-related-factors' are also required (Buchanan-Wollaston, 1997; Grbic and Bleeker, 1995). Ethylene may increase the sensitivity of an interaction that takes place at some point in the signalling pathway, controlled by the presence of 'age-related-factors'. In the absence

of ethylene, transmission of the signal may occur later, as a higher concentration of 'age-related-factors' are required (Buchanan- Wollaston, 1997). It is also possible that plants become more sensitive to ethylene as they age, as a gene encoding a putative ethylene response sensor increases in expression in pea petals and can be repressed by STS – an inhibitor of ethylene binding (Orzazez *et al.*, 1999). The ethylene receptor gene in *Arabidopsis* is expressed only in 'early senescent' flowers (Payton *et al.*, 1996). Thus, it is possible that the 'age-related-factors' may be ethylene receptors.

Ethylene biosynthesis from its precursor, S-adenosyl methionine (SAM), involves two enzymes : ACC (1-amino-cyclopropane-1-carboxylic acid) synthase and ACC oxidase. Application of the ethylene precursor ACC to *Alstroemeria* flowers resulted in accelerated senescence (Chanasut *et al.*, 2003). The expression of the genes encoding both of these ethylene synthesis pathway enzymes is increased in ripening fruit, late petal development and during post-harvest degradation of broccoli (Jones and Woodson, 1999; van Altvorst and Bovy, 1995; Michael *et al.*, 1993; Park *et al.*, 1992; Rottman *et al.*, 1991; Hamilton *et al.*, 1990). Antisense expression of either of these genes delays senescence in climateric flowers (Aida *et al.*, 1998; Michael *et al.*, 1993) and leaves (John *et al.*, 1995) as less ethylene is produced, and overexpression results in premature senescence (Lanahan *et al.*, 1994). As well as being a precursor of ethylene, SAM is also a precursor of certain polyamines. Polyamines have a role in cell division and growth, and can delay senescence in some instances (Serafini-Francassini *et al.*, 2002). Inhibiting polyamine synthesis leads to increased ethylene production (Lee *et al.*, 1997).

In conclusion, ethylene is neither necessary nor sufficient to induce senescence; as senescence occurs eventually in the ethylene insensitive mutants and as ethylene will only induce senescence in older organs, and not in young. However, it is likely to act in modulating the timing and in coordinating late developmental processes in leaves, flowers and fruit.

1.4.2.3.2 Cytokinins are a class of phytohormones, comprising a number of closely related compounds. Although there may be subtle differences between the effects of different compounds and this may vary further with the concentrations used, as

cytokinin control of senescence is not a focus of this thesis and as each member has a similar effect on plant senescence, they will be discussed in this section as a class of compounds, rather than as individual chemicals.

Cytokinins inhibit PCD (van Staden and Joughin, 1988), inhibit the transcription of SAGs (Teramoto *et al.*, 1995) and can even cause regreening of yellow leaves in some species (Venkatarayappa *et al.*, 1984). They also delay loss of chlorophyll, proteins (Richmond and Lang, 1957) and nucleic acids (Dyer and Osbourne, 1971), stimulate protein, chlorophyll and RNA synthesis (Osbourne, 1962) and chloroplast differentiation (Harvey *et al.*, 1974). Endogenous levels drop markedly with age (Singh *et al.*, 1992*a*) and senescence can only be initiated when cytokinin levels fall below a certain threshold. Increased use of cytokinin precursors by other metabolic pathways in transgenic *Arabidopsis* are accompanied by a drop in cytokinin levels and a premature senescence-like response (Masferrer *et al.*, 2002). Thus, decrease in cytokinin levels may be the cause of senescence onset in some systems. Externally applied cytokinins also inhibit senescence (Richmond and Lang, 1957), and if cytokinins are applied to one leaf of a plant, the higher leaves senesce quicker than they would ordinarily (Smart *et al.*, 1991). Young tobacco leaves can synthesise their own cytokinins, whereas older leaves cannot do so (Singh *et al.*, 1992*b*) which is possibly a factor in the control of sequential leaf senescence. Transgenic overproduction of cytokinins under heat- (Smart *et al.*, 1991), wound-, light-, tissue- or development-specific promoters delays senescence, but causes developmental abnormalities due to an excess of the hormone. This problem was overcome by using the highly senescence specific *SAG12* promoter (Gan and Amasino, 1995), forming an autoregulatory loop. These transgenic plants showed no signs of leaf senescence, their photosynthetic activity remained high and more flowers were produced (although any effects on flower or petal longevity were not reported). Similar work in petunia has resulted in increased corolla longevity (Chang *et al.*, 2003). All aspects of leaf senescence are delayed in such plants, although a steady deterioration was noted (Wingler *et al.*, 1998). This indicates that when one senescence process is blocked in leaves, that other pathways or passive ageing remain functional. Control by cytokinins is likely to be at the transcriptional level, inhibiting the expression of SAGs. Fusion of the *SAG12* promoter to the *GUS* reporter gene transformed into tobacco, show high levels of the GUS protein in senescing leaves. However, co-

expression of this fusion and of cytokinin overproduction under the *SAG12* promoter in the same plant resulted in very low levels of *GUS*, indicating the *SAG12* induced cytokinin prevented the expression of *GUS* (Gan and Amasino, 1995). In general, cytokinins also increase petal longevity, possibly by preventing ethylene synthesis or decreasing sensitivity to ethylene (Borochov and Woodson, 1989).

Cytokinins are produced mainly in the roots and transported to the rest of the plant in the xylem; therefore stress applied to the roots results in less cytokinins, leading to leaf senescence (van Staden and Mallett, 1988). The molecular mechanisms of cytokinin involvement are not yet fully understood, but advances have been made recently in dissecting the components involved (Hwang *et al.*, 2002; Hutchison and Kieber, 2002). Among the genes characterised, only the receptor CKI1 and the response regulator ARR2 appear to be involved in regulating leaf senescence (Hwang and Sheen, 2001).

1.4.2.3.3 Jasmonic acid (JA) and its derivative, methyl jasmonate (JA-Me) promote senescence, with endogenous levels increasing 4-5 fold during leaf senescence (He *et al.*, 2002). The products of LOX (lipoxygenase) activity are components of the pathway leading to JA synthesis, and LOX expression often increases during senescence (Rosahl, 1996). Treatment of leaves or cell cultures with jasmonates results in loss of chlorophyll and reduced expression of photosynthesis-associated genes (reviewed by Creelman and Mullet, 1997) and exogenous application of JA-Me stimulates late flower development in petunia and *Dendrobium*, possibly by elevating ACC and promoting ethylene production (Porat *et al.*, 1993). Jasmonates also rapidly induce the expression of chlorophyllase (Tsuchia *et al.*, 1999) and several SAGs (Schenk *et al.*, 2000; Park *et al.*, 1998*a*). However, although ethylene production increases in response to exogenous application, it would seem that LOX activity and JA are unlikely to be involved in the endogenous regulation of the process (Porat *et al.*, 1995*a*). Cytokinins can reverse the effect of JA-Me, even though the jasmonate-induced proteins are still synthesised (Weidhase *et al.*, 1987). The nature of some of the JA-induced proteins studied indicates that jasmonates are more likely to be involved in stress-related responses, which may result in senescence (Becker and Apel, 1993). None of the JA biosynthesis or signalling mutants isolated (Berger, 2002), nor transgenic plants underexpressing enzymes involved in JA synthesis have

been reported to have aberrant senescence phenotypes, suggesting that JAs are not essential. However, this may be due to the plasticity of the senescence network, as He *et al.* (2002) report upregulation of endogenous JA, as well as components of its biosynthesis pathway, during senescence. Thus, the role of jasmonates in senescence remains to be fully understood.

1.4.2.3.4 Other plant growth regulators. There is no consistent pattern of endogenous auxin levels in relation to senescence (Noodén, 1988*a*). They inhibit PCD in the leaves of some species, but are usually not very powerful compared to cytokinins, so much higher concentrations are needed to achieve an effect (Smart, 1994). Generally, they do not inhibit senescence and there are reports that they promote the process (Mishra and Gaur, 1980), as they induce ethylene production (Thimann, 1980). Auxins stimulate senescence of some ethylene sensitive flowers, probably via ethylene (Stead, 1992), but delay petal senescence of daylily (Rubinstein, 2000).

Abscisic acid (ABA) can promote leaf senescence in high concentrations, but molecular genetic analysis has yet to show a crucial link between ABA and senescence (Fedoroff, 2002). Response is genotype-dependent in soybean, with different cultivars ranging from being very sensitive, to completely insensitive (Sloger and Caldwell, 1970). ABA levels increase in stress-induced senescence (Zeevaart and Creelman, 1988), and thus the ABA effect could be linked to a general stress response. In roses, ABA levels fall, then increase just before petal abscission (Le Page-Degivry *et al.*, 1991). In carnation flowers, ABA levels increase before loss of fresh weight. Application of ABA leads to early ethylene evolution and increased ethylene sensitivity (Borochov and Woodson, 1989). In daylily petals, which are not ethylene sensitive, ABA hastens events such as ion leakage, lipid peroxidation and the activities of proteinases and nucleases (Panavas *et al.*, 1998*b*). Thus, although the extent of its importance in senescence and the exact mechanism of action remain to be elucidated, ABA may have a role in senescence regulation.

Mutants deficient in brassinosteroids show altered leaf senescence, suggesting involvement in regulation (Clouse and Sasse, 1998; Yin *et al.*, 2002). However, a systematic study is required to assess any regulatory functions.

1.4.2.4 Metabolic flux

Altering the availability of the different components required for photosynthesis can alter the process of leaf senescence considerably. Elevated CO_2 levels can accelerate the developmental shift from an increase in photosynthetic levels to a decrease (Miller *et al.*, 1997), due to the premature onset of senescence (Ludewig and Sonnewald, 2000). When carbohydrate levels build up in the leaves of tobacco, *Arabidopsis*, and *Medicago*, the rate of photosynthesis drops and the leaves yellow prematurely (von Schaewen *et al.*, 1990; McKersie *et al.*, 1992). Similarly, mature Rubisco antisense tobacco plants had less dry weight and chlorophyll content than the wild type plants, without alteration to leaf ontogeny, but with the process of senescence markedly prolonged, thus increasing longevity (Miller *et al.*, 2000). A similar pattern was described in one of the stay-green mutants of pea (Thomas and Howarth, 2000). These results indicate that limiting photosynthetic productivity increases longevity, whereas overly efficient photosynthesis promotes senescence.

It has also been proposed that leaf senescence is initiated when photosynthetic activity drops below a certain threshold level, on or near the compensation point, due to the suppression of photosynthetic genes by 'maturation signals' (King *et al.*, 1995; Hensel *et al.*, 1993), which may be related to leaf sugar levels. Soluble sugar content increases with leaf age, possibly due to the breakdown of chloroplast and cell wall compounds (Quirino *et al.*, 2001), however, the sugar levels of transgenic tobacco overproducing cytokinin under a *SAG12* promoter and *SAG12-GUS* plants did not differ, despite senescence being substantially delayed in the former (Ludewig and Sonnewald, 2000). This suggests that increased sugar levels may be a consequence, rather than a cause, of senescence. However, exogenous sugar has an effect on the expression patterns of SAGs; enhancing some, such as *SAG21* and *SAG13*; but inhibiting others, such as *SAG12* (Noh and Amasino, 1999*a*; Xiao *et al.*, 2000). Similarly, sugar deprivation in *Arabidopsis* leaves induces SAG12 expression, thus indicating that reduced sugar levels due to decreased photosynthesis may activate the senescence pathway (Quirino *et al.*, 2000). Thus, the absolute levels of sugar appear not to be directly involved in leaf senescence regulation, although sugar sensing and signalling may influence the process. Several transgenic studies and work on leaf senescence mutants have shown a correlation between sugar and senescence (Rolland *et al.*, 2002; Yoshida *et al.*, 2002*a*; Xiao *et al.*, 2000; Quirino *et al.*, 2000; Dai *et al.*,

1999; Jang *et al.*, 1997; Bowling *et al.*, 1997). It is known that sugars can interact with several distinct signalling pathways, such as ABA, ethylene, light and cytokinins, all of which are implicated in the regulation of leaf senescence (Rolland *et al.*, 2002; Smeekens, 2000), thus sugars may mediate their effect on senescence through interacting with these pathways. Addition of sugars to the water of cut flowers has been noted to increase the vase life (i.e. delay late developmental processes) in many flower species (Chanasut *et al.*, 2003; Ichimura, 1998; Eason *et al.*, 1997; Serek *et al.*, 1994), although not in all species (Redman *et al.*, 2002; Ichimura, 1998). However, once detached from the plant, increasing sugar availability to cut flowers only mimics what would naturally be available on an intact plant. Despite increasing flower longevity, excess sugar accelerated leaf senescence in the same plants (Chanasut *et al.*, 2003), which supports the hypothesis that increased sugar can accelerate leaf senescence.

Decline in protein synthesis also leads to senescence. There are three peaks of amino acid incorporation during a plant's life cycle, the third of which occurs at early senescence. Cytokinins can only delay the process if applied before this peak, thus protein disassembly may not occur until rate the of protein synthesis falls below a threshold (Stoddart and Thomas, 1982).

1.4.3 Intra-cellular regulation

It seems that at the start of senescence a receptor system is activated at the cell surface or in the cytosol. This signal must be conveyed to the nucleus by intermediary messengers, to change the pattern of nuclear gene expression. Regulatory genes are expressed, which may act through a network of *trans*-acting factors, changing the expression of genes that directly influence the progression of senescence (Smart, 1994). This would be analogous to the regulation of cell death in animals. In conveying the extracellular signal to senesce to the intracellular environment, this relay system, or cascade, also serves to amplify the strength of the signal.

1.4.3.1 G-proteins and calcium

G-proteins are so-called because they bind the guanidine nucleotide GDP in their inactive state and GTP in their active state. There are several types of G-protein, but they are most often heterotrimeric GTPases associated with the inner surface of the

plasma membrane and often coupled to receptors spanning the membrane, G-protein coupled receptors (GPCRs). There is evidence to support the hypothesis that plants may use a similar system to the G-protein mediated lipid metabolism model established in animals.

In this model (figure 1.4.1; adapted from Assmann, 2002 and Ricart and Millner, 1997), the heterotrimer is bound to the transmembrane GPCR and the GDP nucleotide. Once the GPCR is activated by the binding of a hormone or other ligand, the GDP is replaced by GTP, activating the heterotrimer and inducing the Gα subunit to dissociate from the Gβ and Gγ dimer. The Gα activates an effector molecule, hydrolysing the GTP to GDP, and reassociates with the dimer, returning to their inactive trimeric form. The Gβγ dimer can also activate effector molecules in some cases (Hepler and Gilman, 1992). In plants only one type of Gα has been identified (Ma *et al.*, 1990), which activates phospholipase *c* (PLC), generating the secondary messengers inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG) by hydrolysing the membrane component phosphatidylinosotol 4,5-bisphosphate (PIP_2). IP_3 then acts as a second messenger for elevating calcium levels in the cytosol (Drobak, 1993; Poovaiah and Reddy, 1993). The calcium ions bind to calmodulin, a ubiquitous and highly conserved calcium binding protein, thus activating phospholipase *a* (PLA), which releases linoleic and linolenic acids from membrane phospholipids, which, by cleavage and oxidation by LOX, can be converted into precursors of JA (Vick and Zimmerman, 1984). JA and other proposed products of the pathway, such as ethylene and calcium ions, may recycle to the membranes, allowing more calcium ions to enter and generating a cascade. The DAG leads to the activation of protein kinases, such as phosphokinase c (in the presence of calcium) and the phorbol ester PMA which stimulates ethylene evolution (Borochov *et al.*, 1997). DAG is also involved in proton pump recharging. Thus, it seems that cytosolic calcium results in the calcium dependent phosphorylation of proteins that is necessary to upregulate senescence proteins (Rubinstein, 2000).

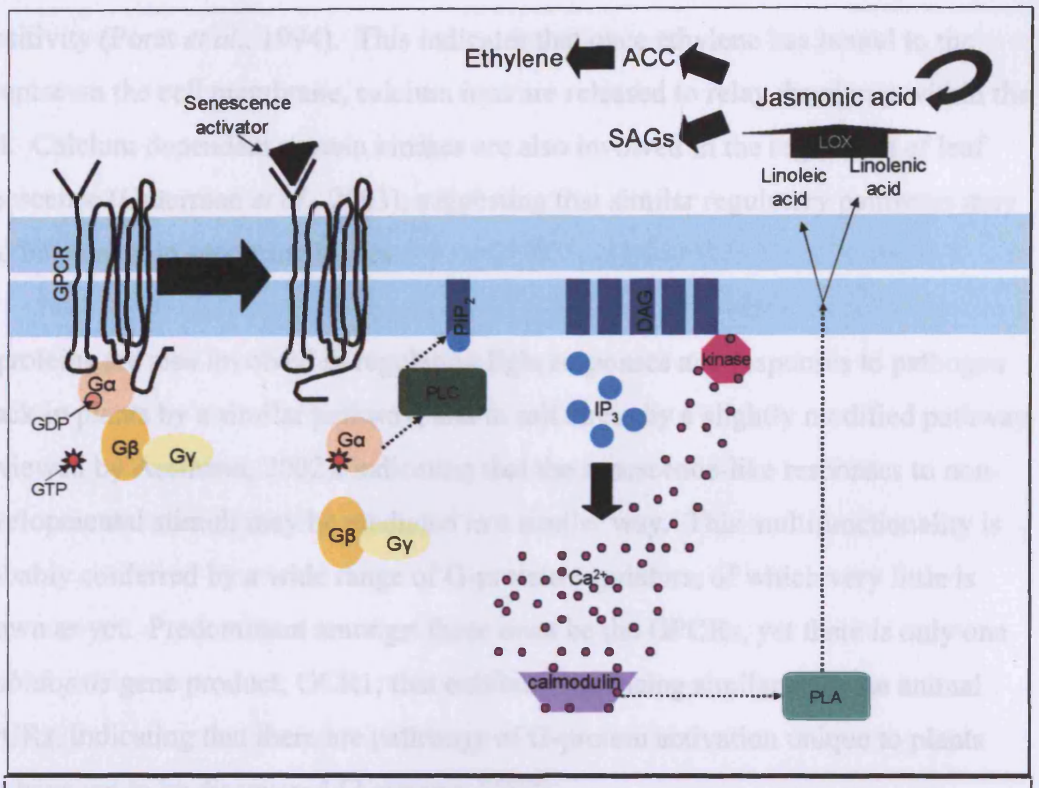


Figure 1.4.1. Model of G-protein signal transmission. Once a senescence activator binds to the GPCR, the G-protein heterotrimer is released and the GDP in the α subunit is replaced with GTP, whilst the β and γ subunits remain linked. Gα activates PLC, hydrolysing the GTP to GDP as it does so, thus returning to its inactive trimeric form. The PLC hydrolyses the membrane component PIP_2 to IP_3 and DAG. IP_3 releases calcium which binds to calmodulin, in turn, activating PLA. The PLA releases linoleic and linolenic acids from membrane phospholipids, which are precursors of JA. The JA stimulates its own production, along with that of ethylene via its precursor ACC as well as activating certain SAGs. The DAG activates protein kinases, some of which are also calcium dependent and is involved in proton pump recharging.

Evidence supporting the involvement of this model during senescence includes that binding of the GTP analogue GTPγS increases by 20% during pollination-induced senescence in *Phalaenopsis* flowers and reagents that maintain G- proteins in their active state accelerate the process by 20-30% in the presence of ethylene (Porat *et al.*, 1994), indicating that G-proteins are active. PLA and PLC increase in activity in rose and petunia petals before late developmental processes start (Borochov *et al.*, 1994) and DAG levels increase before the ethylene burst (Borochov *et al.*, 1997). A constitutively active tomato Gα increases cytosolic calcium levels (Aharon *et al.*, 1998). Furthermore, endogenous calcium levels increase during senescence, and the addition of calcium along with a calcium ionophore increases sensitivity to ethylene in the orchid *Phalaenopsis*, while addition of a calcium chelator decreases this

sensitivity (Porat *et al.*, 1994). This indicates that once ethylene has bound to the receptor on the cell membrane, calcium ions are released to relay the signal within the cell. Calcium dependent protein kinases are also involved in the regulation of leaf senescence (Guterman *et al.*, 2003), suggesting that similar regulatory pathways may also be present in senescing leaves.

G-proteins are also involved in regulating light responses and responses to pathogen attack in plants by a similar pathway, and to salt stress by a slightly modified pathway (reviewed by Assmann, 2002), indicating that the senescence-like responses to non-developmental stimuli may be mediated in a similar way. This multifunctionality is probably conferred by a wide range of G-protein regulators, of which very little is known as yet. Predominant amongst these must be the GPCRs, yet there is only one *Arabidopsis* gene product, GCR1, that exhibits convincing similarity to the animal GPCRs, indicating that there are pathways of G-protein activation unique to plants that have yet to be discovered (Assmann, 2002).

1.4.3.2 Protein phosphorylation

Protein phophorylation is often involved in controlling signalling cascades leading to developmental responses (Soporny and Munshi, 1998). This is controlled by kinases and phosphatases, which phosphorylate and dephosphorylate proteins, respectively; controlling their level of activation. An inhibitor of certain phosphatases stimulates wilting in *Phalaenopsis* petals, although an inhibitor of protein kinase C has no effect (Porat *et al.*, 1994). Preliminary evidence suggests that ion leakage of daylily petals is retarded by an inhibitor of some kinases and stimulated by an inhibitor of certain phosphatases (Rubinstein, 2000). Thus, it would appear that phosphorylation can activate certain senescence associated proteins. This is consistent with the activation of protein kinases by the G-protein pathway described above, and shows that post-translational modification of proteins may be important in controlling senescence.

1.4.4 Regulatory genes and promoters

No common *cis* elements have been found in the promoter regions of SAGs, suggesting that there are no common regulatory mechanisms controlling their expression. Comparison of the promoters of several SAGs did not show any recognisable regions of similarity (Gan and Amasino, 1997; Oh *et al.*, 1996), which

would be expected if there was a common *trans*-acting element. However, promoters of the same gene are conserved between species, and the promoters of senescence related genes show senescence specific expression when used as transgenes in other species (for example Gan and Amasino, 1995; Noh and Amasino, 1999*b*; Graham *et al.*, 1992), indicating a conservation of *trans*-acting factors controlling gene expression in the different species. Gel shift assays using the essential promoter regions of genes show that the extent of binding of a senescence-specific DNA-binding protein from *Arabidopsis* is proportional to the levels of expression of the genes, indicating that the expression levels may reflect the affinities of the senescence-specific DNA-binding protein for that promoter element (Noh and Amasino, 1999*b*). The fact that many SAGs have also been found to have a role in other biological processes and that gene mutations in diverse facets of plant growth and development can alter leaf senescence, indicate that there is an overlap between the senescence network and other biological networks (Jing *et al.*, 2003), or that genes that have evolved for one purpose have been recruited for other purposes. This would account for the lack of common *cis* elements in leaf senescence induced promoters.

The expression of some members of the WRKY family of proteins have been shown to be associated with senescence (Robatzek and Somssich, 2001), and also linked to senescence-like processes (Chen and Chen, 2000; Eulgem *et al.*, 2000; Hara *et al.*, 2000; Kim *et al.*, 2000). WRKY proteins constitute a large multigene family of transcription factors, specific to plants, and containing over 75 members in *Arabidopsis* (Eulgem *et al.*, 2000). Features of WRKY proteins shared with transcription factors include their ability to bind DNA specifically, nuclear localisation and transactivation capability (Eulgem *et al.*, 2000; Hara *et al.*, 2000; Eulgem *et al.*, 1999; de Pater *et al.*, 1996). However, little information is available concerning their spatial expression or regulatory function *in planta* (Robatzek and Somssich, 2001).

1.5 Key features of leaf senescence and their timing

Physiological, biochemical and molecular studies of senescence have shown that senescing cells undergo a series of highly co-ordinated changes in cell structure,

metabolism and gene expression. Certain organelles, like the chloroplast, due to its high protein and lipid content, are targeted early for resource reallocation (Quirino *et al.*, 2000); whereas others, such as the nucleus and mitochondria, which are needed for transcription and energy provision respectively, remain functional and intact until the late stages of senescence (Thomson and Platt-Aloia, 1987; Peoples *et al.*, 1980). Total RNA levels decrease and the expression of many genes is switched off (Hensel *et al.*, 1993; Lohman *et al.*, 1994) although certain genes are transcribed *de novo* (eg. Gepstein *et al.*, 2003; Buchanan-Wollaston and Ainsworth, 1997; Lohman *et al.*, 1994; Hensel *et al.*, 1993; Thomas *et al.*, 1992).

1.5.1 When does senescence start?

It is difficult to determine when senescence begins and to define its different stages. Parameters such as chlorophyll content and photosynthetic rate have often been used to characterise the stages (Smart *et al.*, 1995), and can be used to define the timing of expression of different genes, although some SAGs in *Brassica napus* are induced before chlorophyll levels fall (Buchanan-Wollaston, 1997). Difficulties in determining the start has resulted in the use of artificially induced systems, such as detaching leaves or dark treatment (Thimann, 1980). However, although the effects seen in the induced systems are superficially similar to those seen in natural senescence, the differences are significant (Noodén, 1988*a*). On detachment of senescing leaves, sinks for export of recycled nutrients are removed and the translocation of hormones or other signalling molecules to the leaves from other parts of the plant cannot occur (Woolhouse, 1987). Other problems with using artificial stimuli to initiate senescence include the sudden change from anabolism to catabolism, which contrasts with the subtle shift that occurs naturally (Buchanan-Wollaston, 1997). Also, after detachment, stress related genes are induced in addition to SAGs (Kawakami and Watanabe, 1988; Malik, 1987). Becker and Apel (1993) incubated barley leaf segments in the dark and found three mRNAs that increased in level. Two of these were also induced by wounding, drought stress, jasmonate and ABA and repressed by cytokinin, but were not detectable in naturally senescing leaves, indicating that they may be part of the stress response, rather than senescence. The third mRNA was present in naturally senescing leaves, but was unaffected by plant growth regulators or stress. Similarly, the gene *SAG12* is expressed during natural senescence only (Noh and Amasino, 1999*a*; Weaver *et al.*,1998; Gan and

Amasino, 1995; Lohman *et* al., 1994). Thus, of the genes only expressed during late development, there may be genes that are expressed exclusively under stress, exclusively during natural senescence, or expressed under both conditions. Another complexity in defining the start of senescence is that not all tissues in leaves and petals develop synchronously (section 1.1.1.2). Thus, senescence of the mesophyll tissue, which makes up the majority of these organs, will be discussed. However, even this one tissue type does not senesce synchronously (section 1.1.1.2), as the process is one of recycling and is not simply degenerative (Gan and Amasino, 1997). Although petal cell death is often described as being rapid and synchronous (eg. Rubinstein, 2000), asynchrony has been observed, again with the outer edges deteriorating first and the process working inwards (Wagstaff *et al.*, 2003; Bailly *et al.*, 2001; Stead and van Doorn, 1994). This may indicate that petals also recycle their contents.

Thus, due to the differences between natural and artificially-induced senescence, only studies of naturally senescing systems will be reviewed in looking at the cellular events and changes in gene expression during senescence, and their timing relative to each other.

1.5.2 Altered gene expression patterns during senescence

Several genes are preferentially or exclusively expressed during senescence, including proteases, nucleases, enzymes involved in lipid and carbohydrate metabolism and in nitrogen mobilisation, as well as genes of no obvious senescence related function (Buchanan-Wollaston, 1997). Many mRNAs with increased levels in senescent tissue appear to be induced by ethylene and repressed by cytokinin (eg. Azumi and Watanabe, 1991). Cycloheximide, a protein synthesis inhibitor and actinomycin D, an RNA synthesis inhibitor, both inhibit senescence by inhibiting the translation and transcription of SAGs, respectively (reviewed by Noodén, 1988*a*) proving that new gene expression is necessary for senescence. Some genes that are downregulated during senescence may also be of interest, for example the *DAD* (defender against death) gene, which may be involved in protecting cells against cell death, decreases in expression in pea petals during senescence (Orzaez and Granell, 1997), however, these are harder to identify when the expression of most genes is decreasing.

Ten broad classes of genes have been described with regard to development and senescence, on the basis of expression pattern and function (Buchanan-Wollaston, 1997; Smart, 1994) and are summarised in table 1.5.1.

Class	Description	Examples
1	housekeeping genes controlling the essential metabolic activities of the cell, expressed at a constant level throughout the life of the leaf.	
2	genes expressed in green leaves, active well before the start of senescence and switched off before any signs are present, but the encoded proteins are activated during senescence.	Vacuolar enzymes
3	genes expressed in green leaves, active well before the start of senescence and switched off before any signs are present, but the encoded proteins may cause the initiation of senescence by their absence.	Pigment breakdown
4	regulatory genes, expressed immediately before or at the start of senescence for a relatively short time only.	
5	genes involved with the mobilisation processes that occur specifically during senescence, expressed from the onset of senescence until the death of the leaf.	LSC 54, LSC222 (Buchanan-Wollaston, 1997)
6	genes involved with the mobilisation of storage products that may also function during other developmental stages, expressed from the onset of senescence until the death of the leaf.	Malate synthase (Graham, 1992)
7	genes that gradually increase in expression from very young leaves through to late senescence.	LSC7, LSC210, LSC212, LSC460 (Buchanan-Wollaston, 1997)
8	genes with the mRNA levels starting low early in development and increasing dramatically at a certain stage of senescence.	LSC94 (Buchanan-Wollaston, 1997)
9	genes whose expression are induced specifically during senescence, but the duration of expression is short and does not continue into the late stages.	LSC550, LSC680 (Buchanan-Wollaston, 1997)
10	genes expressed strongly early in development and during senescence, but also significantly expressed in mature leaves.	LSC8, LSC101 (Buchanan-Wollaston, 1997)

Table 1.5.2.1. Description of the ten gene expression classes from Buchanan-Wollaston (1997) and Smart (1994)

SAGs have also been classified into two expression types: genes expressed only during senescence eg. *SAG12*, *SAG13* and *LSC54* (class I SAGs), and genes that increase in expression from a basal level (class II SAGs) (Gan and Amasino, 1997).

The range of expression patterns, and the fact that these groupings are generalised, so not all genes of a given class will have exactly the same expression pattern, suggests that these genes are regulated in different ways, by different regulatory pathways. Also, a gene may not belong to the same expression class as its homologue from a different species. For example, the *Brassica LSC54* gene (Buchanan-Wollaston, 1994) has a class 5 expression pattern according the Buchanan-Wollaston (1997) and Smart (1994) classification or class I expression pattern according to Gan and Amasino (1997), whereas its *Arabidopsis* homologue, *SAG17* (Lohman *et al.*, 1994), shows class 8 or II expression, respectively.

1.5.3 Organelle breakdown

Each of the organelles in the plant cell has an important role to play in senescence, either in its implementation, or as a source of nutrients, which are released on its breakdown, and are, in turn, remobilised and recycled to other parts of the plant.

1.5.3.1 Chloroplasts. Initiation of senescence occurs before the leaf shows any visible signs of senescing, such as yellowing, which begins shortly after full expansion of the mature leaf. Transdifferentiation of the chloroplast, which contains 70-80% of the leaf protein (Makino and Osmond, 1991; Morita and Kono, 1974), and therefore leaf nitrogen, and 75% of leaf lipid (Forde and Steer, 1976) is an early event (Gan and Amasino, 1997; Smart, 1994). About 90% of the nitrogen exported from the leaves comes from the chloroplasts (Morita, 1980) with apoproteins of pigments in photosystems and light harvesting proteins accounting for approximately 30%, and Rubisco around 50% (Matile, 1992). Thus, photosynthesis as an energy source is replaced by the catabolism of macromolecules (figure 1.5.4.2.1). Chlorophyll, however, is not recycled after breakdown, but detoxified and stored in the vacuole (Matile *et al.*, 1999). These changes are accompanied (or driven) by changes in gene expression. There is also a reduction in the photosynthetic rate and capacity for electron transport (Grover, 1993) and photosynthesis related genes decrease sharply in their expression (Noh and Amasino, 1999*a*; Humbeck *et al.*, 1996; Lohman *et al.*, 1994; Jiang *et al.*, 1993; Hensel *et al.*, 1993; Bate *et al.*, 1991). Studies on *Festuca* leaves indicate that the stromal and extrinsic membrane proteins of the chloroplast are broken down by one proteolytic system, while intrinsic membranes are lysed by another system (Thomas, 1982*a,b*), explaining how the plastid envelope remains

intact until the final stages (Peoples, 1980). The chloroplast genome is active during plastid assembly, but is degraded before chlorophyll breakdown (Sodmergen *et al.*, 1991; 1989), thus chloroplast transdifferentiation must be under nuclear control (Matile, 1992). Indeed, only chloroplasts in nucleate protoplasts of *Elodea* can transdifferentiate, and RNA and protein synthesis inhibitors impede the process, demonstrating that new gene transcription is needed (Yu and Kao, 1981; Yoshida, 1961). Although the chloroplast DNA is degraded, some chloroplast encoded mRNAs are still maintained through senescence, such as large subunit of Rubisco in primary bean leaves (Bate *et al.*, 1991). Also, gerontoplasts are able to regreen and reform chloroplasts, so enough information must be retained from the chloroplast genome to support reassembly (Matile *et al.*, 1999), thus indicating that a few copies of the chloroplast DNA must remain intact.

In petals, the chloroplasts have already transdifferentiated into chromoplasts well before the petals are fully expanded, indicating that if they do senesce, the process is initiated earlier than in leaves, supporting the theory that petals are modified senescing leaves (Thomas and Sadras, 2001; Thomas *et al.*, 2001). A comparison of leaf and petal chloroplast transdifferentiation has already been made in section 1.2.

1.5.3.2 Microbodies. Peroxisomes and glyoxysomes transdifferentiate between one and the other during development. Glyoxysomes are present in germinating seeds, transdifferentiating into peroxisomes in green leaves, then as the leaf senesces, revert to glyoxysomes (also termed 'gerontosomes' by Vicentini and Matile, 1993). Glyoxysomes are sites of lipid metabolism by β-oxidation in leaf and petal cells, and of the glyoxylic acid cycle during leaf senescence (Vicentini and Matile, 1993; de Bellis *et al.*, 1990; Gut and Matile, 1988), channelling degradation products towards the synthesis of numerous carbon compounds (figure 1.5.4.2.1). The senescence enhanced expression of genes encoding enzymes required for β-oxidation and the glyoxylate pathway (reviewed by Graham and Eastmond, 2002) indicates the importance of these pathways, but the signals that induce these pathways have not been elucidated. Glyoxysomes also seem to be active in purine catabolism in senescing leaves, and an increase in the activity of uricase indicates that they are also involved in RNA breakdown (Vicentini and Matile, 1993). In petals they produce the ROS O_2^- and H_2O_2, especially in the absence of protective enzymes.

1.5.3.3 Mitochondria. Electron microscope studies in leaves show that these remain intact until a very late stage of senescence (Thomson and Platt-Aloia, 1987). There is a sharp increase in the rate of respiration in early senescence, possibly partially due to the respiration of amino acids and to a lesser degree, soluble sugars, as well as the uncoupling of respiration from phosphorylation (Tetley and Thimann, 1974). Fatty acids are also likely to be major respiratory substrates, as these can be converted into sugars by the glyoxylic acid cycle, along with β- oxidation and gluconeogenesis (figure 1.5.4.2.1). The fact that mitochondrial structure and function are maintained until late senescence further indicates that the process is organised.

1.5.3.4 Nuclei. Intact nuclei can also be seen by electron microscopy until the late stages of leaf senescence (Thomson and Platt-Aloia, 1987). These are still functional as new mRNAs from SAGs are transcribed, even in late senescence. Nuclear breakdown is a late event, as senescence is under direct nuclear control, as shown by enucleation studies using *Elodea*, where enucleated cells did not senesce (Yoshida, 1961).

1.5.3.5 Vacuole. The tonoplast remains intact until the final stage of senescence as indicated by ultra-structural studies (Thompson and Platt-Aloia, 1987). It is likely that nutrient salvage is quicker and more organised this way than would be possible if everything was targeted to the vacuole (Smart, 1994). The vacuole is involved in the final stages of chlorophyll breakdown, and is where the breakdown products are localised (Matile *et al.*, 1988; Hinder *et al.*, 1996). The vacuole contains a wide variety of hydrolytic enzymes, including nucleases, phosphatases, acid hydrolases and most of the endoprotease activity of the cells (Feller and Fischer, 1994; Huffaker, 1990; Heck *et al.*, 1981). Cysteine protease levels also increase notably in the vacuole (Jones and Dangl, 1996). There is evidence that some senescence related degradative enzymes are synthesised in younger leaves, stored in an inactive form in the vacuole and are activated by vacuolar processing enzymes during senescence (Yamada *et al.*, 2001; Smart *et al.*, 1995). It has further been suggested that vacuoles may act as lysosomes in plant cells, degrading proteins after invagination of the cytoplasm (Matile, 1982). In morning glory petals, the tonoplast invaginates during senescence in a way indicating an endocytotic uptake of cytosolic components. The vacuole increases in size as the cytoplasm becomes a thin layer, but later the vacuole

shrinks and the cytoplasm expands, indicating a loss of differential permeability of the tonoplast (Phillips and Kende, 1980; Matile and Winkenbach, 1971). Calcium flux has been documented in senescing cells, which is implicated in tonoplast breakdown (Huang *et al.*, 1997; Butler and Simon, 1971). The organelles in daylily petals have already degraded by the time the flower opens. The vacuole is large and the tonoplast appears to rupture in epidermal cells. Autolysis is obvious 24 hours after opening (Stead and van Doorn, 1994), which is at the very end of the daylily petal's life. Thus, it can be concluded that the vacuole plays an important role both in leaf senescence and late petal development, by remaining intact until the end of both processes then autolysing the corpse. Thus, the role of the vacuole is similar in both processes. However, this does not necessarily indicate that petals senesce, as the vacuole has a similarly important function in all forms of PCD (see section 1.3) which it accomplishes by comparable means.

1.5.3.6 Cellular membranes and the cell wall. During senescence, the structural and functional integrity of cellular membranes decreases as a result of the accelerated metabolism of membrane lipids (reviewed by Thompson *et al.*, 1998). In broccoli, the concentration of membrane fatty acids decreases rapidly as senescence begins, but there is no appreciable increase in lipid peroxidation products. This, coupled with an increase in expression of antioxidant-encoding genes, implies that the first stages of senescence involve an orderly degradation of cellular components using lipids as an energy source (Page *et al.*, 2001). Thylakoid membranes in particular provide an abundant source of energy during senescence, as their disintegration is an early event in senescence, whereas many other membrane systems remain intact until later in the process. Petal cell death is preceded by a loss of structure and function of the cellular membranes. The fluidity of the membranes of several species of flower petals decreases before senescence is obvious (Thompson *et al.*, 1997; Paliyath and Droillard, 1992). In carnations and roses, formation of gel phase lipid occurs, probably leading to water loss from the cells in the later stages of senescence (Faragher *et al.*, 1987; Itzhaki *et al.*, 1995). The phase change is, at least partially, due to the failure of the membrane to remove metabolites by blebbing of lipid-protein particles, as occurs during normal membrane turnover (Thompson *et al.*, 1997). In daylily, the cell membrane is still functional up to 12 hours after flower opening, but has degraded 12 hours later (Panavas and Rubinstein, 1998). Enzymatic changes in

the cell walls have also been detected in petals (Panavas *et al.*, 1998*a*), such as pectinesterase expression (Nari *et al.*, 1991).

1.5.4 Macromolecule breakdown and catabolism

As the organelles are dismantled, their component macromolecules and those stored within them are either detoxified so as not to cause the premature death of the cell, broken down for the nutrients to be recycled, or play a role in implementing PCD as sequestered hydrolases are released.

1.5.4.1 Chlorophyll. Leaf yellowing is due to the preferential degradation of chlorophyll over caroteniods (Matile, 1992). Carotenoids play a protective role, deactivating excited chlorophyll and so preventing photodynamic damage to the leaf by ROS (Matile, 2000). Carotenoids can also be newly synthesised in petals (Sitte *et al.*, 1980), where they are partially responsible for petal colour (Harborne, 1993) and may also prevent ROS damage, as in leaves. Chlorophyll breakdown occurs during differentiation both into gerontoplasts, and into chromoplasts (Sitte, 1977). The catabolic pathway (reviewed by Hörtensteiner and Feller, 2002 and by Matile *et al.*, 1999) extends over several subcellular and suborganellar compartments, starting in the thylakoids and ending in the vacuole (reviewed by Matile, 2000). None of the nitrogen from the chlorophyll is recycled (Tommasini *et al.*, 1998; Hinder *et al.*, 1996) as the purpose of the energy-expensive chlorophyll degradation steps is not for recycling, but to detoxify this highly reactive compound (Matile *et al.*, 1999), which is essential to maintain cellular viability during senescence. This is illustrated by the *Acd2* gene (accelerated cell death 2), which encodes one of the chlorophyll catabolism enzymes. In the absence of this enzyme's activity, phytotoxic chlorophyll products such as red chlorophyll catabolite are accumulated, causing rapid cell death (Mach *et al.*, 2001).

Stay-green mutants have been found in a number of different species, and can either be functional, retaining both their chlorophyll and their photosynthetic competence, or non-functional, retaining only their chlorophyll and degrading other components of the photosynthetic apparatus. However, the eventual loss of chlorophyll in these mutants again reveals the plasticity of the network and its ability to circumnavigate blockages. Thylakoid proteins, such as LHCPII, exist as pigment-protein complexes

with chlorophyll, and so require parallel detoxification of chlorophyll, as exemplified by the stay-green *Festuca* mutant where chlorophyll catabolism is blocked, stabilising the LHCP, which is not degraded (Thomas and Donnison, 2000). Thus, it would appear that the control of degradation of entire complexes is co-ordinated.

1.5.4.2 Proteins. Protein levels decrease progressively during leaf senescence, due to both an overall decrease in synthesis and an increase in degradation by proteases. Proteolysis is one of the most obvious enzymatic events of senescence and is important for nitrogen remobilization. Proteolysis may also be important for defence, as well as remobilisation (Thomas *et al.*, 2003). Proteolysis can be either selective, as in the case of the ubiquitin-mediated proteolysis pathway via the 26S proteasome; or non-selective, like vacuolar proteolysis. There is evidence to support the involvement of both pathways in leaf senescence, by the isolation of mutants (Jing *et al.*, 2003).

Several protease genes show *de novo* transcription during leaf senescence, and increased protease activity, along with increased transcription of genes with high homology to known protease genes during late petal development has been shown in both *Alstroemeria* (Wagstaff *et al.*, 2002) and *Hemerocallis* (Stephenson and Rubinstein, 1998; Guerrero *et al.*, 1998). This can be prevented in *Hemerocallis* by the protein synthesis inhibitor cycloheximide, indicating that the proteases are newly translated, or activated by newly translated proteins (Stephenson and Rubinstein, 1998). In both morning glory (Matile and Winkenbach, 1971) and *Alstroemeria* (Wagstaff *et al.*, 2002) petals, the drop in protein levels per unit fresh weight does not correlate with the increase in protease activity. Possible reasons include that the proteases may be released from the vacuole when the differential permeability of the tonoplast is lost, or as protein synthesis is occurring during this time to replenish the proteins which are degraded (Wagstaff *et al.*, 2002) or due to fluctuations in fresh weight. During late development in daylily and *Hibiscus* petals, protein synthesis declines (Woodson and Handa, 1987; Courtney *et al.*, 1994). After flower opening, attached daylily flowers change rapidly from a sink to a source, exporting carbohydrates and amino acids for many hours after wilting (Bieleski, 1995).

One of the mechanisms for differential protein degradation is ubiquitination. Ubiquitin is a highly conserved protein, found in all eukaryotes (Callis and Viestra,

1989). Once a protein is ubiquitinated, which occurs by a series of ordered events (Vierstra, 1987), it is prevented from interacting with its substrates (Baek *et al.*, 2001), or it is targeted for degradation by the 26S proteosome (an ATP-dependent protease complex) when four or more ubiquitin moieties are attached (Chau *et al.*, 1989). The ubiquitin system mainly seems to be a route for eliminating abnormal cytosolic proteins and for the rapid turnover of short-lived proteins, but may also be important during leaf senescence (Smalle and Viestra, 2004). Identification of an ubiquitin conjugating-type carrier protein gene which increases in expression during leaf senescence in *Nicotiana sylvestris* (Genschik *et al.*, 1994) and increased levels of polyubiquitin genes in senescing potato (Garbarino *et al*, 1995) and *Arabidopsis* leaves indicates that the system may be used for the breakdown of specific cytosolic proteins (Park *et al.*, 1998*b*). The recent identification of ORE9, an F-box protein involved in controlling selective ubiqutination (Woo *et al.*, 2001), and DLS1, involved in supporting the N-end rule pathway which targets proteins for ubiquitin-dependent proteolysis (Yoshida *et al.*, 2002*b*; Varshavsky *et al.*, 2000), further implicate the ubiquitin/26S proteasome system in senescence. ORE9 may play a part in ubiquitylating a key repressor of senescence (Woo *et al.*, 2001), like a positive regulator of cytokinin signalling (Frugis and Chua, 2002) and targeting it for degradation. It has also been shown that the 20S proteasome remobilises bulk proteins during leaf senescence in wheat (Roberts *et al.*, 2002*a*), with the endoproteolytic and chymotrypsin-like activities of the 20S proteasome remaining constant until the late phases of senescence. Analogous results were found in cucumber (Yamauchi *et al.*, 2002). There may also be a role for the ubiquitin-proteasome system in late petal development. In daylily petals some ubiquitin transcripts increased, others decreased and yet others did not change in their expression level during flower opening and senescence (Courtney *et al.*, 1994). The enzymes involved in the ubiquitin pathway decrease in expression with age, on a per petal basis, but remain about equal as a proportion of the total protein content (Stephenson and Rubinstein, 1998). Thus, the fact that they retain expression indicates a possible involvement, as there are less proteins remaining at this time, compared to earlier in development, for degradation. Furthermore, inhibitors of proteosome delay the ion leakage associated with senescence in daylily petals (Stephenson and Rubinstein, 1998), again indicating involvement of the ubiquitin/26S proteosome system in petals. Stephenson and Rubinstein (1998) suggest that

ubiquitination may be important for the fragments of the high-molecular-mass proteins already cleaved by other, less selective, proteases.

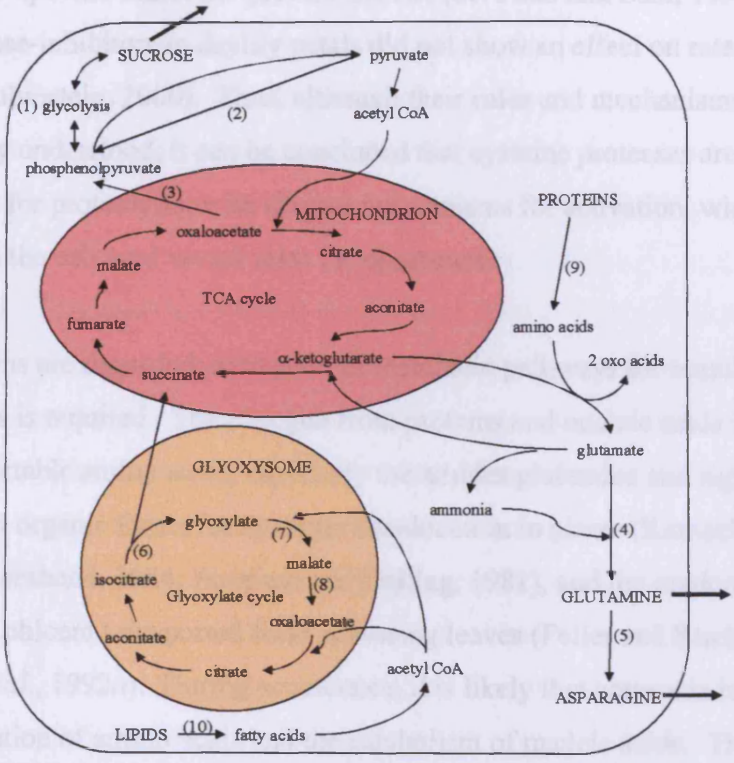


Figure 1.5.4.2.1 Possible metabolic scheme controlling senescence related mobilisation of macromolecule components, adapted from Buchanan- Wollaston (1997). 1. Fructose 1,6 bisphosphate aldolase and glyceraldehyde 3 phosphate dehydrogenase, 2. Pyruvate orthophosphate dikinase, 3. phosphoenolpyruvate carboxykinase, 4. Glutamine synthetase, 5. Aspargenine synthetase, 6. Isocitrate lyase, 7. Malate synthase, 8. NAD malate dehydrogenase, 9. Proteases, 10. Lipid degrading enzymes.

As well as ubiquitin mediated proteolysis via the proteosome, a plethora of proteases are expressed in senescing tissues. Of the protease genes identified, cysteine, aspartic and serine proteases are dominant classes expressed during leaf senescence. A cysteine protease from carnation increases after the pollination induced climacteric, and can be induced by the addition of ethylene and repressed by the inhibition of its action (Jones *et al.*, 1995). Another two cysteine protease genes from daylily petals (Valpuesta *et al.*, 1995; Guerrero *et al.*, 1998) have a peak in expression 12 hours after flower opening and again after 19 hours. These transcripts decreased in expression during leaf senescence (Guerrero *et al.*, 1998). Many cysteine proteases have an N-terminal hydrophobic region, thought to be involved with localisation to

the vacuole (Noh and Amasino, 1999*b*) and thus are only activated on tonoplast rupture in late senescence. Caspase-like activity has been detected in tobacco leaves and caspase-specific inhibitors prevent the HR (del Pozo and Lam, 1998), but a study using caspase-inhibitors in daylily petals did not show an effect on rates of ion leakage (Rubinstein, 2000). Thus, although their roles and mechanisms of action are not yet fully understood, it can be concluded that cysteine proteases are a prevalent mechanism for proteolysis, with diverse mechanisms for activation, wide-ranging locations in the cell, and varied roles in senescence.

Once proteins are degraded, activation of metabolic pathways for remobilisation of the products is required. The nitrogen from proteins and nucleic acids is converted into transportable amino acids, especially the amides glutamine and asparagine, which are the main organic forms for nitrogen translocation in plants (Kamachi *et al.*, 1991; Kar and Feierabend, 1984; Simpson and Dalling, 1981), and the predominant amino acids in the phloem transported from senescing leaves (Feller and Fischer, 1994; Kamachi *et al.*, 1992*a*). During senescence, it is likely that ammonia is released by the deamination of amino acids and the catabolism of nucleic acids. The ammonia can be converted into glutamine by the action of glutamine synthase (GS). The activity of the plastid GS (GS2) decreases with senescence, whilst that of the cytosolic form (GS1) increases in several different plant species (Buchanan-Wollaston and Ainsworth, 1997; Bernhard and Matile, 1994; Kamachi *et al.*, 1992*a*; Kawakami and Watanabe, 1988*b*). This confirms that GS1 must be important during senescence and predicts its role, as it is mainly found in the vascular bundles of senescing rice leaves (Sakurai *et al.*, 1996), indicating involvement in the translocation of transportable amino acids. Similarly, expression of an asparagine synthetase gene increases in senescing asparagus ferns (King *et al.*, 1995). Amino acids such as threonine and serine cannot be metabolised by transamination. However, a senescence specific threonine dehydratase has been identified in tomato, which may release ammonia from these amino acids during senescence (Szamosi *et al.*, 1993). The carbon skeletons of the amino acids can enter gluconeogenesis either as pyruvate, acetyl CoA or α- ketoglutarate (figure 1.5.4.2.1). Thus, the senescence-related expression of the pyruvate orthophosphate dikinase gene in maize (Smart *et al.*, 1995) and *Brassica napus* (Buchanan-Wollaston, 1997) is probably involved with converting the pyruvate

from amino acid breakdown into phosphoenolpyruvate for gluconeogenesis (Buchanan-Wollaston, 1997).

1.5.4.3 Nucleic acid degradation. Nucleic acids, especially rRNA, are important sources of carbon, nitrogen and particularly phosphorus for the senescing cell. The total level of DNA in leaves does not change dramatically during leaf senescence, although rRNA levels do decrease (Makrides & Goldthwaite, 1981), and the amount of extractable nucleic acids decreases during late flower development (Lesham *et al.*, 1986) and nuclear DNA is degraded (Rubinstein, 2000). New mRNAs are synthesised during senescence, encoding proteins that have an active role in the process. RNAse activity in leaves increases during senescence (Green, 1994). Three RNAse genes which increase in expression when phosphate is limiting have been identified in *Arabidopsis*. One of these, RNS2 is also expressed at high levels during senescence and is likely to function in remobilising inorganic phosphorus (Pi) in senescing leaves (Bariola *et al.*, 1994; Taylor *et al.*, 1993). It is not yet known what happens to the purines and pyrimidines resulting from nucleic acid degradation, but it can be presumed that they are further metabolised, to enable export and recycling of carbon and nitrogen. Glyoxysomes may be the site of purine catabolism (Vicentini and Matile, 1993) (section 1.5.3.2). The expression, activity and abundance of many nucleases increases as leaf senescence progresses (eg. Gepstein *et al.*, 2003; Blank and McKeon, 1991; Blank and McKeon, 1989). Endonuclease activity increases in the petals of morning glory (Lesham *et al.*, 1986) and daylily (Panavas *et al.*, 1998*b*). A single-copy, petal-specific gene increases in expression with senescence in daylily and its product degrades both ssDNA and RNA (Panavas *et al.*, 1999).

The breakdown of nucleic acids during senescence is therefore a balance between retaining what is needed to control the process and degrading what is not needed for the recycling of nutrients – particularly Pi. Both leaves and petals degrade their nucleic acids, indicating that petals may recycle the valuable phosphate, as do leaves, but there is evidence that the two organs express different genes to accomplish this.

1.5.4.4 Lipids. The membranes of plant cells are a valuable store of lipid molecules, which can be mobilised and used during senescence. The levels of total lipids along with levels of total fatty acids and of most polar lipids decrease during senescence of

tobacco leaves (Wanner *et al.*, 1991; Koiwai *et al.*, 1981). In *Alstroemeria*, phospholipids and galactolipids are extensively degraded during senescence in both sepals and petals and the ratio of saturated/unsaturated fatty acids increased (Leverentz *et al.*, 2002). Enzymes such as phospholipase D, phosphatidic acid phosphatase, lytic acyl hydrolase and lipoxygenase have been implicated, and genes that may encode enzymes with these activities show senescence enhanced expression (Gepstein *et al.*, 2003; He and Gan, 2002; Thompson *et al.*, 1998). Senescence-related expression of the gene encoding phospholipase D, which may play a part in hydrolysing membrane phospholipids, has been shown in dark induced senescence of castor bean leaves (Ryu and Wang, 1995). This enzyme may catalyse the first degradative step in the deterioration of membrane integrity during senescence (Thompson *et al.*, 1998). In petals, levels of all classes of phospholipids decrease whereas those of neutral lipids increase, due to the greater activities of phospholipases and acyl hydrolases (Leverentz *et al.*, 2002; Paliyath and Droillard, 1992). Phospholipid synthesis is blocked early in the senescence of rose and daylily petals (Itzhaki *et al.*, 1998; Bieleski and Reid, 1992), contributing to this decline. An acyl hydrolase initially identified in carnation petals (Hong *et al.*, 2000) causes delayed leaf senescence when reduced levels are expressed in transgenic *Arabidopsis* (Thompson and Okuyama, 2000). A similar study of antisense expression of *SAG101*, an acyl hydrolase gene from *Arabidopsis*, also resulted in delayed leaf senescence (He and Gan, 2002) and overexpression resulted in premature senescence. The sterol/ phospholipid ratio increases in petals (Thompson et al., 1998; Lesham, 1992; Adam *et al.*, 1983; Thompson *et al.*, 1982), which may contribute to the decreased fluidity of the membranes.

A lot of energy is required for senescence to take place, but the photosynthate supply is decreasing. Of the total thylakoid lipid, 40- 50% are the galactolipids mono- and digalactosyl diacylglycerol, which can be converted to sugars by gluconeogenesis and either respired or exported to provide energy for senescence (figure 1.5.4.2.1). β-galactosidase gene levels are also enhanced during the senescence of asparagus ferns and may be involved with the mobilisation of galactose during the degradation of the galactolipids (King *et al.*, 1995). The glyoxylate cycle is used only in germinating seeds to remobilise the oil stores for respiration, and during senescence, where it is likely that it performs the same function with membrane lipids. The succinate

produced by the conversion of acetyl CoA in the glyoxylate cycle can feed the TCA cycle, where it is converted to oxaloacetate for gluconeogenesis or α- ketoglutarate for transamination to glutamate (figure 1.5.4.2.1).

Membrane lipid degradation in petals may occur by the action of lipoxygenase (LOX), which oxidises the fatty acids released from membranes (Siedow, 1991). LOX activity increases before senescence becomes obvious in carnation, before the flowers open in daylily petals and prior to the onset of electrolyte leakage (a marker of loss of membrane semi-permeability) in both species (Sylvestre *et al.*, 1989; Panavas and Rubinstein, 1998) as well as in rose (Fukuchi-Mizutani et al., 2000). Oxidation of existing membrane components leads to loss of membrane permeability, and lipid peroxidation increases during carnation, daylily and rose petal senescence (Sylvestre *et al.*, 1989; Bartoli *et al.*, 1995; Panavas and Rubinstein, 1998). In two orchid species however, there is no increase in LOX specific activity and inhibitors of LOX had no effect (Porat *et al.*, 1995*b*). Similarly, in *Alstroemeria* petals, loss of membrane function was not found to be related to LOX activity (Leverentz *et al.*, 2002). Another two cDNA clones from daylily that increase in expression during senescence show similarity to an in-chain fatty acid hydrolase bound to cytochrome P450 and to an allene oxide synthase and may also play a part in oxidising membrane lipids (Panavas *et al.*, 1999).

1.5.4.5 Carbohydrates. The dry weight of petal cells decreases with senescence, in part due to the reduced sizes of starch bodies and loss of starch (Lesham *et al.*, 1986). The expression of two genes that appear to encode the carbohydrate hydrolases β-glucosidase and β- galactosidase increase during senescence in carnation petals (Woodson, 1994). In daylily, carbohydrates are the main export of the senescing petals (Bieleski, 1995), with 95% of carbohydrate lost after flower opening (Bieleski, 1993). Sugars are also lost from gladiolus florets (Yamane *et al.*, 1993), *Ipomea* petals (Hanson and Kende, 1975) and carnation petals (Trippi and Paulin, 1984; Nichols and Ho, 1975a,b) during late development.

1.5.4.6 Sulphur metabolism. Cysteine is converted to the tripeptide glutathione, which appears to be important in responding to many internal and external stresses (Foyer *et al.*, 1994; Wingate *et al.*, 1988), and may be important in the storage and

mobilisation of the sulphur released by macromolecule degradation during senescence (Buchanan-Wollaston, 1997). Genes encoding glutathione S transferases also increase in expression in senescing leaves (Smart *et al.*, 1995), which detoxify herbicides by conjugation with glutathione, amongst other diverse functions. Their expression during senescence may be a stress response or they may have a role in sulphur metabolism (Buchanan-Wollaston, 1997).

1.5.5 Other events

1.5.5.1 Antioxidant gene expression. Levels of ROS such as free radicals and reduced oxygen species increase during both developmental and stress induced senescence of leaves and of petals (Borochov and Woodson, 1989; Merzlyak and Hendry, 1994). Stromal proteins such as Rubisco and glutamine synthetase can be degraded non-enzymatically by ROS when chloroplasts are incubated in photo-oxidative conditions (Ishida *et al.*, 2002, 1999; Roulin and Feller, 1998), however, it is not clear whether photo-oxidation is the mechanism for ROS production during senescence. It is more likely that the increased ROS levels are a result, rather than a cause, of macromolecule degradation. The effect of ROS on senescence could be mediated through plant hormones and MAP kinase signalling (Jonak *et al.*, 2002; Meinhard and Grill, 2001; Delledonne *et al.*, 2001; Orozco-Cardenas *et al.*, 2001; Sharma *et al.*, 1996) or by directly damaging the DNA. Among several identified DNA repair mechanisms in response to oxidative stress, nucleotide excision repair appears to play a pivotal role in ensuring the normal progression of leaf senescence. In *Arabidopsis*, homologues to several yeast genes that are involved in nucleotide excision repair have been identified, mutations in which cause premature senescence in response to oxidative damage (Liu *et al.*, 2001; Costa *et al.*, 2001; Liu *et al.*, 2000), demonstrating a link between lack of DNA damage repair and senescence. However, increased levels of ROS promote senescence only in mature leaves (John *et al.*, 2001; Miller *et al.*, 1999), demonstrating that 'age-related factors' may also be important. ROS may also be a by-product of LOX activity (Siedow, 1991). Highly reactive oxygen radicals can be formed from H_2O_2 (Halliwell, 1989). In daylily, endogenous levels of H_2O_2 increase, along with lipid peroxidation, before the flower opens. Treatments that accelerate senescence in petals increase H_2O_2 and treatments that retard senescence decrease H_2O_2 (Panavas and Rubinstein, 1998). There are several enzymes that can protect against ROS and H_2O_2. SOD may protect against ROS

build-up, but the product of this reaction is H_2O_2. H_2O_2 levels can be lowered by the action of the enzymes APX and CAT, which reduce H_2O_2 by the oxidation of ascorbate and by conversion to H_2O and O_2, respectively (Asada, 1992). Catalases are present at all stages of leaf development, and a catalase gene with high levels of expression in late senescence has been found in *Brassica napus* (Buchanan-Wollaston and Ainsworth, 1997). This catalase may protect the cell at a time when the chloroplast APX system may become less active. SOD activity decreases in carnation petals (Sylvestre *et al.*, 1989) and increases in daylily (Panavas and Rubinstein, 1998) late in senescence. The activities of both APX and CAT increase in carnation petals (Bartoli *et al.*, 1995) and decrease in daylily petals (Panavas and Rubinstein, 1998). This is confusing, as each enzyme is doing the opposite in one species to what it is doing in the other. Peroxidase activity, however, increases as senescence progresses in both carnation (Bartoli *et al.*, 1995) and daylily petals (Panavas and Rubinstein, 1998), and uses H_2O_2 as a substrate for several reactions. GST transcription and activity also increase in carnation petals (Meyer *et al.*, 1991; Sylvestre *et al.*, 1989). It would seem that these increases are a response to oxidative stress from H_2O_2 (Rubinstein, 2000). Levels of natural antioxidants, such as ascorbate, glutathione and α- tocopherol, fall in both carnation and daylily petals as senescence progresses (Bartoli *et al.*, 1997; Pavanas and Rubinstein, 1998). However little is yet known about the involvement of these compounds or of the protective enzymes (Bartoli *et al.*, 1997), it seems that as ROS are produced by the breakdown of macromolecules, the cell must protect itself from their effect so that senescence can continue in an orderly manner.

1.5.5.2 Genes involved with pathogenesis response. Some pathogen response genes increase in expression during senescence in *Brassica napus* including PR1a, a chitinase and a gene showing similarity to an antifungal protein (Hanfrey *et al.*, 1996). It is possible that these are to protect the senescing leaf from potential pathogen attack whilst it is vulnerable to infection, or that they have a key part to play in senescence, as their role in defence is unclear (Buchanan-Wollaston, 1997).

1.5.5.3 Genes of unknown function. There are many genes whose senescence related function is not known. Sequence homology of a newly identified gene to a previously known gene does not necessarily help to assign a function to that gene in

the senescence process. In addition, a number of senescence related genes bear no sequence similarity to sequences within the databases. In these cases, antisense technology may help to determine gene function, as was done with the tomato fruit ripening associated gene *TOM13*, which encodes ACC oxidase, an enzyme required for ethylene biosynthesis (Hamilton *et al.*, 1990).

So far, many of the genes identified as having a function during senescence have confirmed what was already known from biochemical studies. Analysis of the genes whose function in senescence is not yet clear by transgenic analysis will probably generate new information that could not be gleaned from biochemical studies. Also, as more senescence related genes are identified, perhaps more information will be discovered regarding the regulation of senescence.

1.6 Comparative methods

Studying the molecular aspects of senescence provides a different approach to the problem to the biochemical studies. Problems with biochemical methods include the difficulties in identifying some enzymatic changes. This is either because of a high background of similar enzymes or where the proteins are only produced at very low levels. By identification of genes that are expressed exclusively or preferentially during senescence, information can be inferred on the site of activity and the function of the gene product and the timing of expression, as well as the regulation of the gene by studying the upstream regulatory regions and transcription factors that bind there (Buchanan-Wollaston, 1997).

Differential gene expression during leaf senescence was first demonstrated by *in vitro* translation and gel electrophoresis to detect changes in translatable mRNA populations (Watanabe and Imaseki, 1982). Most senescence related genes have been identified using differential screening to detect genes expressed specifically in senescing leaves and not in green leaves (eg. Smart *et al.*, 1995; Lohman *et al.*, 1994; Buchanan-Wollaston, 1994; Hensel *et al.*, 1993). However, this only detects genes expressed at a reasonably high level, as the message must be abundant enough to be labelled sufficiently by the probe. Subtractive hybridisation (see chapter 5 for fuller description of this method) is a technique which has been applied successfully in

order to identify senescence related genes expressed at a lower level (Gepstein *et al.*, 2003; Buchanan-Wollaston & Ainsworth, 1997). Northern hybridisation and RT-PCR (see chapters 4 and 7 for fuller description of these methods) can be used to study the pattern and timing of gene expression and to compare expression of the gene in different parts of the plant. Although the levels of mRNA detected by these differential techniques are usually indicative of the level of transcription, the amount of hybridisation or amplification also depends on the relative stability of the mRNA. However, it can be assumed that if the stability of an mRNA is high at a time when general RNA degradation is increased, that the product of that mRNA must be important (Buchanan-Wollaston, 1997). The availability of cDNA microarrays (chapter 6) and Affymetrix GeneChips has considerably increased the speed by which differentially expressed genes can be identified, particularly in *Arabidopsis*.

1.7 Wallflowers as a model species for senescence studies

The reasons for the choice of wallflowers for this study are outlined, along with a description of the plant and a brief account of its taxonomy, particularly of its relationship to the other model species *Arabidopsis thaliana* and *Brassica* species.

The wallflower variety *Erysimum linifolium* 'Bowles Mauve' was chosen for this study for several reasons. It is taxonomically close to both *Arabidopsis thaliana* and *Brassica napus* (all members of the Brassicaceae family), both of which have been studied extensively regarding leaf senescence, and there is much sequence information available for them, from the *Arabidopsis* genome initiative (2000) and the UK *Brassica* genome project (The John Innes Centre, http://brassica.bbsrc.ac.uk; and Texas A&M, http://hbz.tamu.edu). *Erysimum linifolium* 'Bowles Mauve' is easier to work with, on account of its relatively large petals, and is more convenient, being a perennial evergreen with a long flowering season, making it possible to collect material all year round. It is also a commercially relevant ornamental plant. In addition, Bowles Mauve is sterile, and so petal senescence is unaffected by pollination and seed set, which are factors that can affect the initiation and rate of senescence in some flowers (van Doorn, 1997; O'Neill, 1997; Woltering *et al.*, 1994; Stead, 1992), and distinct differences have been noted between natural and pollination-induced petal senescence (Havely *et al.*, 1984; Zieslin and Gottsman, 1983; Gärtner, 1844).

Comparison of wild type *Arabidopsis* with delayed flowering and sterile flower mutants showed no difference in the timing of senescence in individual leaves (Hensel *et al.*, 1993), thus, it is doubtful that the sterility of Bowles Mauve will result in an altered leaf senescence phenotype. *Arabidopsis* is also a poor model for late petal development as the flowers are so short-lived, with the abscission occuring soon after opening, and so late petal development in *Arabidopsis* remains poorly characterised.

Of the three senescence patterns described by Smart (1994), monocarpic, sequential and autumnal, the pattern followed by this variety of wallflower leaves is that of sequential senescence, which results from competition for resources between the growing parts of the plant and the lower, older leaves. The petals wilt prior to abscission.

1.7.1 Description

Erysimum linifolium 'Bowles Mauve' grows as a perennial bush about 60cm tall with a 90cm spread. This differs from *Arabidopsis*, a much smaller winter annual and *Brassica*, which is of similar size, but is biennial. The long, narrow leaves (growing to about 5cm long and 0.5cm wide) are evergreen, grey-green in colour, are sessile and spirally arranged, with the youngest leaves growing from the centre of the rosette. The inflorescence is racemose, emerging from the centre of the leaf rosette (figure 1.7.1.1 b) in early spring and is distinct from other varieties within the species in having longer flower heads and a deeper purple flower colour. The opened flowers are 2cm across, vivid purple in colour and, like all other flowers of the Brassicaceae, actinomorphic with the four petals arranged in a cross. They have six anthers, four of which are long and two short, and a bicarpellate gynoecium with a short style (figure 1.7.1.1a).

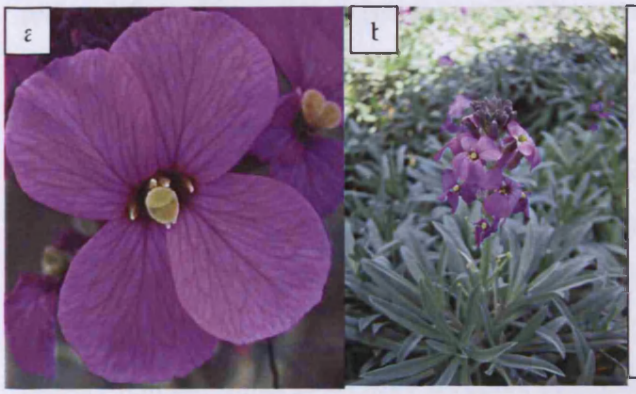


Figure 1.7.1.1 a) A single *Erysimum linifolium* flower, showing the cross-shaped arrangement of the petals and the six anthers (the four long ones are seen two above and two below the stigma and the two short ones are seen either side of the stigma). b) The racemose inflorescence growing from the centre of a rosette of leaves.

1.7.2 Taxonomy

Despite the Brassicaceae being a large family (with 390 genera and 3,000 species), they are also rather uniform (Cullen, 1997). They form a natural group, based on flower morphology and glucosinolate profiles; however, inferring phylogenies within the family is complicated by a significant degree of convergent evolution and highly uniform traits (Al-Shehbaz *et al.*, 1999*a*; Al-Shebaz, 1984). All of the species within the family have diverged from a common ancestor over a period of approximately 40 to 50 million years, as a result of numerous speciation events (Koch *et al.*, 2001). Cross species comparisons of genomes within the Brassicaceae have primarily been limited to plants in the genus *Brassica*, aiming to identify genes useful for crop improvement (Hall *et al.*, 2002). These studies have relied on *Arabidopsis* as a reference genome, and have demonstrated that the genomes diverged 16 to 21 million years ago (Yang *et al.*, 1999). Since this time (Lagercrantz and Lydiate, 1996), there appears to have been a genomic triplication event in the genus *Brassica*, as both physical and genetic mapping studies have indicated that *Arabidopsis* chromosomal segments are often present in triplicate in the diploid *Brassica* species genomes (Jackson *et al.*, 2000; O'Neil and Bancroft, 2000; Lagercrantz, 1998). Comparisons of the two genera indicate that the conservation of gene sequence, content and order are common, facilitating cross species mapping and identification of syntenic regions and gene homologues (Quiros *et al.*, 2001; O'Neil and Bancroft, 2000). Sequence comparisons of the two SAG 12 orthologues in *Brassica napus* show over 80% homology to each other, and to *Arabidopsis thaliana* SAG 12, at nucleotide level, with slightly lower homology at the amino acid level (Noh and Amasino, 1999*b*). Gene density is also high in both genera, and repetitive DNA content is low (Kumar and Bennetzen, 1999). The genus *Arabidopsis* has recently been redefined and

reduced from 59 species to just 9 (Al-Shehbaz *et al.* 1999*b*). *Erysimum* (Stevens, 2001 onwards), *Arabidopsis* (Mitchell and Heenan, 2000) and *Brassica* (Stevens, 2001 onwards; Yang *et al.*, 1999) are members of the same sub-family: Brassicoideae. In a phylogenetic analysis of the Brassicaceae based on a strict consensus of 13 equally parsimonious trees constructed from nuclear ribosomal internal transcribed spacer sequence data, *Erysimum* and *Arabidposis* species came out in sister clades (Mitchell and Heenan, 2000), indicating that the two genera are closely related. Indeed, another species of *Erysimum* (*E. hedgeanum*) was previously placed in the genus *Arabidopsis*, as *A. erysimoides* (Schulz, 1924).

1.8 Aims of the project

The objective of the project is to compare the late developmental events in the leaves and petals of wallflowers, to establish similarities and differences in their execution. The hypothesis being tested is that leaf senescence and late petal development are distinct processes, but may have certain features in common. Thus, the aim is to establish the extent of the similarities between the two processes (if any), comparing:

- Physiological processes
- Genes expressed
- Temporal gene expression patterns relative to each other, and to other developmental events

Although the hypothesis being tested is based on accounts in the literature concerning gene expression and remobilisation of metabolites during leaf senescence and during late petal development in various species, no direct comparisons of events in the two organs have yet been made in the same species. Thus, the parameters outlined above need to be compared in the petals and the leaves of the same species in order to be able to more accurately determine the extent of the overlap in gene expression and the extent of physiological similarities between the two organs. Direct comparison in this way, with reference to what is already known in other species, therefore may make it possible to determine where the regulation and execution of late developmental stages in these two organs follow a similar pattern, involving active recycling of their cellular contents; and where these patterns diverge, indicating a different mechanism in petals more related perhaps to ageing, i.e. a passive deterioration with time.

2 Materials and methods

This general materials and methods chapter describes the common materials and methods used in more than one of the subsequent chapters of this thesis. Specific materials and methods will follow as part of each of the subsequent chapters. Optimisation of the methods are described in appendices.

All solutions and chemicals were obtained from Sigma (Gillingham, UK), unless otherwise stated. All centrifugation steps were carried out in a Du Pont instruments Sorvall RC-5B refrigerated superspeed centrifuge with an SS-34 rotor or a Beckman Coulter Allegra 2IR microcentrifuge with the stated rotor.

2.1 List of buffers and solutions used

SOC medium

2% w/v Bacto-tryptone, 0.5% w/v Bacto-yeast extract, 10mM NaCl, 2.5mM KCl, 10mM Mg^{2+}, 20mM glucose, pH 7.0

LB agar

10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl, 15g agar, dH_2O to 1L, pH 7.0

LB broth

10g Bacto-tryptone, 5g Bacto-yeast extract, 5g NaCl, dH_2O to 1L, pH 7.0

amp 100

100μg/ml ampicillin was added to LB agar or LB broth to prevent the growth of contaminating bacteria

50X TAE buffer

242g Tris base, 57.1ml glacial acetic acid, 100ml EDTA pH 8.0, dissolved in dH2O to final volume 1L and autoclaved.

Gel loading buffer

0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol in 50% glycerol

Hot borate buffer

0.2 M sodium borate (borax), 30 mM EGTA, 1% w/v SDS and 1% deoxychloate, sodium salt, dissolved in warm dH_2O, pH 9.0, autoclaved. 10mM DTT, 1% Nonidet P-40 (NP-40, Igepal), 2% w/v PVP-40 and 0.1% v/v antifoam A were added to the buffer immediately before use.

20X SSC

350.6g of sodium chloride, 176.4g sodium citrate, dissolved in dH_2O to a final volume of 2L, pH 7.0.

10X SSPE

584.4g sodium chloride, 1560.1g of NaH_2PO_4, 10mM EDTA (pH 7.4) dissolved in dH_2O to a final volume of 1 L.

10X E buffer

41.9g of MOPS, 4.1g of sodium acetate, 3.7g of EDTA dissolved in dH_2O to a final volume of 1L, pH 7.0, autoclaved.

5X Denharts solution

0.1% Ficoll (v/v) (type400, Amersham-Pharmacia, Little Chalfont, UK), 0.1% PVP (v/v), 0.1% BSA (w/v) (fraction V)

2.2 Methods

2.2.1 Filter sterilisation

Solutions to be filter sterilised were taken up into a plastipak sterile syringe (Becton Dickinson, Madrid, Spain) to which a 0.2μm minisart sterile filter (Sartorius, Epsom, Surrey) was attached. The solution was passed though the filter, the mesh of which was fine enough to remove any impurities (0.2μm).

2.2.2 Growth conditions

Two field-grown, mature bushs of *Erysimum linifolium* "Bowles Mauve" was used as source material. The bushes were grown at the nurseries at Tal-Y-Bont, Cardiff University and were maintained by Lyndon Tuck. One bush was grown from cuttings taken from the other, original bush, ensuring genetic homogeneity of the material. Petal samples were collected throughout the flowering season of the bush, from April until October and leaf samples were collected throughout the year.

2.2.3 Extraction of RNA

Tri reagent is an improvement on the single step RNA isolation reagent developed by Chomczynski and Sacchi (1987). It is a mixture of guanidine thiocyanate and phenol in a mono-phase solution, allowing simultaneous extraction of RNA, DNA and protein. The guanidine thiocyanate is a potent denaturing agent, disintegrating

cellular structures and dissociating nucleoproteins from nucleic acids as protein secondary structure is lost. It is also an effective inhibitor of RNAses, probably for the same reason. The properties of the guanadine thiocyanate as an inhibitor of RNAses are particularly important in senescing tissue, as levels of nucleases increase up to 10 fold. However, endogenous endonucleases are ubiquitous to all developmental stages, and although they are normally compartmentalised, the homogenisation of the tissue allows their release. Its feature as a chaotropic agent, as well as that of phenol, also facilitates cell breakage by weakening the cross-linking of the cell wall matrix (Wilkins and Smart, 1996), making it particularly useful for RNA extraction from plant material. Details of the optimisation procedure followed to arrive at the methods described are given in appendix 1.

All of the equipment and surfaces were sterilised and treated with RNAse ZAP prior to use. 1g of plant material (both petals and leaves of each developmental stage) was ground in liquid nitrogen to a fine powder. A total of 10ml of Tri reagent was added to each sample and homogenised using an IKA labortechnik T25 basic polytron motorised homogeniser to fully disperse the plant material, disrupt cellular integrity and shear genomic DNA. The samples were incubated at room temperature for 15 mins and centrifuged at 4°C and 11 K rpm for 20 mins to remove the cellular debris. The supernatant was removed and retained, 2ml of chloroform added, vortexed for 30 seconds and centrifuged at 4°C and 11 K rpm for 20 mins, retaining the top, aqueous layer which contains the RNA (the phenolic interphase contains DNA and the organic lower phase contains protein, thus it was important not to contaminate the RNA layer with these). The chloroform layer was back extracted with 1 ml of sterile, distilled water, and the two resulting aqueous layers mixed. The RNA was precipitated from solution by the addition of an equal volume of ice-cold isopropanol, incubation at room temperature for 10 mins and centrifugation at 4°C and 11 K rpm for 30 mins. The resulting pellet was dried and resuspended in 2 X 150µl of sterile distilled water (sdw) and stored overnight at -80°C.

2.2.4 Phenol: chloroform purification

An equal volume of iso-amyl alcohol: phenol: chloroform (1:25:24) was added to each of the samples, vortexed for 30 seconds, and microcentrifuged at 4°C at 13 K rpm for 20 mins, using the F2402H rotor, retaining the top, aqueous layer. The

organic layer was back extracted with 50µl of sdw, and the two resulting aqueous layers mixed. The iso-amyl alcohol: phenol: chloroform extraction step was repeated until no white residue formed at the interface between the aqueous and organic layers. An equal volume of chloroform was added to the aqueous layer, vortexed for 30 seconds, and microcentrifuged at 4°C and 13 K rpm for 30 mins, using the F2402H rotor, retaining the top, aqueous layer.

2.2.5 Ethanol precipitation

Nucleic acids were precipitated from solution by overnight incubation at -20°C with 0.1 volumes of 3M sodium acetate (pH 5.5) and 2.5 volumes of ice-cold ethanol. The samples were microcentrifuged at 4°C and 13 K rpm for 1 hour, using the F2402H rotor, the supernatant removed and the nucleic acid pellet was washed (optional; section 2.2.6) and redissolved in a minimal volume of sdw (50µl).

2.2.6 Ethanol washing

Once the precipitated nucleic acids had formed a pellet, the supernatant was removed and 80% ethanol was added to the pellet carefully, so as not to disturb the pellet. This was microcentrifuged again at 13 K rpm for 10 mins at room temperature using the F2402H rotor and the supernatent carefully removed and discarded before redissolving the pellet.

2.2.7 Butanol extraction

Aqueous solutions of nucleic acids can be concentrated to smaller volumes with the use of butanol to extract the excess water. 3.5 volumes of n-butanol were added to the solution to be concentrated, and vortexed to mix. The mixture was microcentrifuged at 14 K rpm for 1 minute at room temperature using the F2402H rotor and the organic upper phase of n-butanol discarded.

2.2.8 Column purification

Both Qiagen (Qiagen Ltd., Crawley, UK) and NucleoSpin RNA II (Macherey-Nagel, Duren, Germany) RNA purification columns were used according to the manufacturer's instructions. The RNA was bound to the column membrane, washed and eluted the RNA in 60µl of RNAse free water (supplied).

2.2.9 Quantification of RNA

The concentration of the RNA was determined by running dilutions of the RNA samples on an ethidium bromide stained agarose gel and comparing the intensity of the bands when viewed under UV to the intensity of standards of known concentration (Hyperladder 1; 36ng/µl; Bioline, London, UK). This was confirmed by spectrophotometry, using 1µl of the RNA, diluted 1 in 50 in water in a 50-200µl RNAse-, DNAse- and protein-free UVette (Eppendorf, Cambridge, UK) using an Amersham Pharmacia Biotech Ultrospec 2100 pro UV/visible spectrophotometer. If the samples were too dilute for the application for which they would be used, they were concentrated using YM-100 microcon columns (Millipore, Bedford, MA, USA), according to the manufacturer's instructions or by vacuum centrifugation at 60°C in an Eppendorf Concentrator 5301 until the desired volume was reached.

2.2.10 Gel electrophoresis

1%-2% agarose gels were used to separate and identify DNA fragments, as described by Sambrook *et al.* (1989). Agarose (multi-purpose; Bioline, London, UK) was dissolved in 1X TAE buffer by microwaving and allowed to cool slightly, before adding ethidium bromide (final concentration 0.5µg/ml). Samples containing loading buffer, at 10% of the sample volume, were pipetted into the wells, alongside 5µl Hyperladder 1 (36ng/µl; Bioline, London, UK) to enable size estimation, and run in 1X TAE buffer at 100V until fragments were adequately separated. DNA was visualised using the SynGene Gene genius bioimaging system and images captured using the GeneSnap software from SynGene (Synoptics Ltd, Cambridge, UK).

2.2.11 DNAse treatment of RNA

Total RNA was DNAse treated using 2µl RQ1 DNAse (Promega UK Ltd., Southampton, UK) with 1/10 total volume of the supplied RQ1 buffer and incubated at 37°C for 30 mins. The reaction was terminated by the addition of 2µl RQ1 DNAse stop solution (Promega UK Ltd., Southampton, UK) and incubation at 65°C for 12 mins.

2.2.12 cDNA synthesis

1µl of oligo dT random primer (Promega UK Ltd., Southampton, UK) was added to 1µg of DNAse treated total RNA in a 12µl reaction volume and denatured by heating

to 70°C for 10 mins before being chilled on ice. DTT was added to a final concentration of 10mM and dNTPs to a final concentration of 0.5mM in first strand buffer supplied with the enzyme, incubated at 42°C for 2 mins and 1 unit of Superscript II MMLV reverse transcriptase (RT) (Gibco, Paisley, UK) added before further incubation at 42°C for 50 mins. The reaction was terminated by heating to 70°C for 15 mins to inactivate the enzyme. An additional control reaction was performed for each sample, differing only in the lack of the RT enzyme, to verify the complete removal of any contaminating genomic DNA during the DNAse step.

2.2.13 Primer design

Primers were designed from flanking sequences of the DNA fragment of interest according to the following guidelines (C. Wagstaff and D. Chrimes, pers. comm):
Primers should ideally be about 20nt in length with a high melting temperature (Tm; 55-60°C).
Primers should not be complementary, to prevent self-annealing. This is particularly important at the 3' end, where complementarity could lead to primer dimers.
There should not be a T at the extreme 3' end. This should be a C or G if possible
It is advisable to have an A or a T within the extreme 3' triplet, to prevent mismatch of primers with consecutive Cs or Gs.
The Tm of a pair of primers to be used in the same reaction should be as close as possible. An estimate of Tm can be given by Tm= 4(G+C)+2(A+T), where G, C, A and T are the numbers of each of those nucleotides present in the primer.

2.2.14 Cleaning PCR products

The QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK) was used to remove enzymes, nucleotides and salt from PCR and other enzymatic reactions according to the manufacturer's instructions, with the DNA eluted in 30µl of EB buffer (supplied) or sterile distilled water. The DNA binds selectively to the silica-gel membrane within the spin column in the presence of high salt, impurities are washed away, and the DNA is eluted in a low-salt buffer.

2.2.15 Sequencing and sequence analysis

The clones were sequenced on an ABI Prism 3100 capillary sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing reactions were performed using the

BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using M13 forward primers (5'-GTAAAACGACGGCCAGT-3'). The sequences were viewed using the CHROMAS software (version 2.2.3; Technelysium, Tewantin, Qld, Australia) to verify the quality of the sequence and exported into the DNAStar package (DNASTAR, Madison, WI) to edit the sequence and remove the areas of sequence corresponding to the plasmid which the gene of interest was ligated into. The gene of interest was ligated into the multiple cloning site of the pGEM-T Easy vector using the *EcoR1* restriction site (5'-GAATTC-3'), thus the plasmid sequence flanking the gene of interest on either side of the two *EcoR1* sites was removed. The resulting sequence was used to search the BLAST Network Service database (National Centre for Biotechnology Information, NCBI website, 2003) of known sequences. The translated BLAST search was used (tblastx), which converts the nucleotide sequence into a protein sequence in all six reading frames, and compares each of the resulting protein 'products' with the nucleotides translated in all six reading frames on the on-line database. The results give a list of the closest matches to the submitted sequence, enabling a putative function to be assigned to the gene. The DNAStar package (DNASTAR, Madison, WI) was again used to align the wallflower sequence to those found in the BLAST database.

2.2.16 CL6B DNA purification

The CL6B was prepared by shaking the slurry as bought from the supplier and transferring the contents to an autoclavable bottle. The slurry was allowed to settle, the liquid removed and replaced with a volume of TE buffer (10mM Tris, 1mM EDTA) equal to that of the remaining slurry. This mixture was shaken and allowed to settle and the process repeated twice, the second time using half the previous volume of TE buffer, and autoclaved.

A CL6B column was prepared by making a hole in the bottom of a 0.75mL centrifuge tube using a ½ inch needle, and placing the tube in a 2mL collection tube. A drop of glass beads in TE buffer was placed in the bottom of the 0.5mL tube and 500µL of the autoclaved and shaken CL6B slurry added on top. The column was microcentrifuged for 2 mins at 3,000G to pack the column. The DNA (20-100µl) was added to the top of the column and centrifuged again under the same conditions in the same instrument, with the eluate containing the cleaned DNA.

2.2.17 Ligation

The plasmid vector used for all ligation reactions was the pGEM Easy T cloning vector kit (Promega UK Ltd., Southampton, UK). Both the plasmid and the insert were quantified and the volume of insert to be used for the ligation reaction for a ratio of 3:1 of insert:vector decided using the equation :

$$\frac{\text{ng of vector} \times \text{size of insert (Kb)}}{\text{size of vector (Kb)}} \times \text{ratio} = \text{ng of insert}$$

The ligation reaction was set up using 0.5µl of pGEM EasyT vector (Promega UK Ltd., Southampton, UK), 5µl of 2X buffer (supplied), 1µl of ligase enzyme (supplied) and the calculated amount of insert DNA in a total reaction volume of 10µl. The reaction was incubated overnight at 4°C.

2.2.18 Transformation

50µl of Epicurian Coli ZL1-Blue subcloning grade competent cells (Stratagene Ltd., Cambridge, UK) were aliquoted into pre-chilled 15ml Falcon polypropylene tubes, 2µl of the plasmid was added and the tube swirled gently to mix. Tubes were incubated on ice for 20 mins, heat pulsed in a 42°C water bath for 1 minute, and incubated on ice for a further 2 mins, before adding 0.9µl of SOC medium to each. They were incubated at 37°C for 30 mins with shaking at 225-250 rpm.

LB agar plates containing 100µg/ml ampicilin (Sigma-Aldrich Ltd., Dorset, UK), were prepared for blue white colour screening by spreading 100µl of 2% X-Gal (5-bromo-4-chloro-3-inodlyl-b-D-galactopyranisode, Sigma-Aldrich Ltd., Dorset, UK) and 100µl of 10mM IPTG (isopropyl-1-thio-β-D-galactopyranoside, Sigma-Aldrich Ltd., Dorset, UK) on the surface of the plates one hour prior to plating the transformations. The β-galagtosidase gene contained in the pGEM T vector when expressed in a host bacterium results in the formation of blue colonies in the presence of X-gal and IPTG. This gene is disrupted by the insertion of the DNA fragment in the multiple cloning site of the vector, which is located within the gene. Hence, white colonies are selected as those containing plasmids with a cloned product. The IPTG induces β-galagtosidase production in the bacteria, as a chemical analog of galactose which cannot be cleaved by β-galagtosidase, by binding to and inactivating the lac operon repressor. The X-gal is hydrolysed by β-galactosidase, resulting in the

formation of a blue-green precipitate. 200µl of the transformation reaction was plated using a sterile spreader. The plates were incubated at 37°C overnight or for a maximum of 17 hours. 2 ml liquid cultures (LB-broth containing100µg/ml ampicillin) were grown overnight with shaking at 37°C, from single, white colonies, so that the plasmid could be isolated. 2ml of each culture was used for plasmid isolation (section 2.2.19)

The remainder of the liquid culture was used to make glycerol stocks of the transformed bacteria. Cells were pelleted by centrifugation at 8000rpm in a microcentrifuge for two mins using the F2402H rotor. The supernatant was discarded and the cells re-suspended in LB broth media:glycerol (4:1) and stored at –70°C.

2.2.19 Plasmid isolation

The QIAprep Miniprep kit (Qiagen Ltd, Crawley, UK) was used to isolate plasmids from bacterial cultures, according to the manufacturers' protocol. The procedure is a three step protocol, based on the alkaline lysis of the bacteria (modified from Birnboim and Doly, 1979) and clearing of the lysate by centrifugation, followed by the selective binding of the plasmid DNA to the silica-gel membrane within the spin column in a high-salt buffer, then washing the DNA to remove impurities and eluting the DNA in 30µl of a low-salt buffer. The optimised lysis time allows maximum release of plasmid DNA without release of chromosomal DNA and minimises the exposure of the plasmid DNA to denaturing alkaline conditions (Birnboim and Doly, 1979).

2.2.20 Colony PCR

A PCR master mix using 0.625 units of Qiagen HotStarTaq polymerase and Qiagen buffer (Qiagen Ltd., Crawley, UK) with 1.25mM $MgCl_2$, 0.2mM dNTPs, and 10mM each of M13F and M13R (5'-GGAAACAGCTATGACCATG-3') primers per 25µl reaction was made up, which was then aliquoted into the appropriate number of PCR tubes. A pipette tip was used to touch the bacterial colony or liquid culture before being placed in the PCR tube containing the reaction components and mixed thoroughly. Reactions were given a 15 minute 95°C hotstart (which also served to lyse the bacterial cells) and cycled in a Perkin Elmer 2700 thermocycler for 35 cycles of {95°C 1 min, 50°C 1 min, 72°C 2 mins}.

2.2.21 Colony PCR following the lysis of bacterial cells

200µl of bacterial cells in liquid culture were transferred to a 96-well plate and microcentrifuged at 2500rpm for 10 mins at 4°C using the S2096 rotor. The supernatant was removed and the cells resuspended in 50µl of sdw. The cells were ruptured by a 10 minute incubation at 95°C in a waterbath, and debris removed by centrifugation at 2500rpm for 10 mins at 4°C. 5µl of this lysate was used for PCR amplification using 0.625 units of Qiagen HotStarTaq polymerase and Qiagen buffer (Qiagen Ltd., Crawley, UK) with 1.25mM $MgCl_2$, 0.2mM dNTPs, and 10mM each of M13F and M13R primers per 25µl reaction. Reactions were given a 15 minute 95°C hotstart and cycled in a Perkin Elmer 2700 thermocycler for 35 cycles of {95°C 1 min, 50°C 1 min, 72°C 2 mins}.

3 Physiology

3.1 Introduction

Before a comparison of late developmental events in the petals and leaves of wallflowers can be conducted, the stages of development in the two organs need to be defined. This will allow comparison of events in each organ between young tissue and old tissue, to see what processes are important in their late development. It will also permit comparison of these processes between the two organs, so that the late developmental events in petals can be related to leaf senescence and the extent of the similarities and differences between the two organs can thus be identified.

In order to define the developmental stages, petal and leaf morphology were studied, so that the developmental stages defined could be quickly and easily distinguished by eye for subsequent tests. A variety of physiological parameters were also investigated, pertaining to the breakdown and remobilisation features of leaf senescence (section 1.5). The physiological features chosen were the chlorophyll levels of leaves, fresh and dry weight determination of petal and leaf tissue and the protein and RNA content of petal and leaf tissue. These are all easy parameters to measure, giving reliable data and have been measured in other systems, so the results obtained in wallflowers can be compared to results from other species (e.g. Wagstaff *et al.*, 2002; Wagstaff *et al.*, 2001; Stephenson and Rubinstein, 1998; Celikel and van Doorn, 1995; Jones *et al.*, 1995; Smart, 1994; LayYee *et al.*, 1992; Stead and Moore, 1977). They are also all indicators of senescence in their own right, as chlorophyll levels, fresh and dry weight, and protein and RNA levels are all known to decrease during leaf senescence (section 1.5), and a similar drop during late petal development would indicate that similar breakdown processes might be occuring in both organs, leading to the possibility that petals may also recycle the constituents of the catabolised macromolecules, and thus senesce.

Measurement of comparable events in both organs will also make it possible to define 'old' petals and 'old' leaves in an equivalent and meaningful way for comparisons of late developmental events in the two organs. It will also be interesting to compare the physiological results to the molecular results obtained in subsequent chapters, as the

measurement of these physiological parameters will help put the molecular events and their timing into context. It may therefore be possible to correlate, for example, the timing of the expression of protease genes at the molecular level with a decrease in protein levels at the physiological level.

3.1.1 Morphological studies

Petal and leaf development will be studied morphologically with the aim of finding defining morphological characteristics of each developmental stage, allowing quick and easy staging of plant material by eye.

Petal death is sometimes induced by pollination, otherwise petals die at the end of their predetermined life (sections 1.2 and 1.4.2.1). As *Erysimum linifolium* 'Bowles Mauve' is sterile, petal development and death is only under developmental control and therefore should not vary between individual flowers. Although the progression of natural leaf senescence is also predictable and conserved, the timing of leaf senescence is more likely to be affected by external influences than that of petal death (section 1.2); and the reversibility of the process in leaves adds another complicating factor (Molisch, 1938). Development also generally occurs over a shorter timescale in flowers compared to leaves. A combination of these factors means that the morphological and physiological changes that occur over time are more pronounced in petals than in leaves, making late petal development an easier process to document. Thus, morphological changes alone will be enough to document the different stages of petal development, however, chlorophyll levels of leaves will also be measured and compared to the results of the morphological analysis to define the stages of leaf development.

3.1.2 Chlorophyll levels

Yellowing of the leaf, the visible sign of senescence, is due to chlorophyll degradation whilst carotenoids are retained (Matile, 1992). The former is detoxified, whereas the latter play a protective role in deactivating excited chlorophyll and so preventing photodynamic damage to the leaf by ROS (Matile, 2000), which would compromise leaf viability and therefore the ability to senesce. Chlorophyll levels therefore give a reasonable estimation of the stage of senescence and can be used to define late developmental stages in leaves, and parameters such as chlorophyll content and

photosynthetic rate have often been used to characterise stages of leaf development (Smart *et al.*, 1995). It is likely that, as yellowing is due to degradation of chlorophyll, measured chlorophyll concentrations will correlate well with visible yellowing. This means that once the stages have been defined, staging the leaves can be done by eye using the corresponding morphological criteria, and will be quick and easy. Clearly, it is likely that senescence is initiated before the leaf shows any visible sign of yellowing, and thus senescing.

Although chlorophyll is present in very young petals prior to bud opening, chlorophyll levels will not be investigated during petal development. This is because it is unlikely that older petals contain much chlorophyll, and because petal development is easier to document morphologically than leaf development, and so additional information is not needed to help define the stages of petal development.

3.1.3 Fresh and dry weight determination

Fresh weight of petals is expected to increase from the closed bud stage to when the flower is fully open and to decrease again during senescence as the flower wilts, as is observed in wilting flower species (e.g. Wagstaff *et al.*, 2001; LayYee *et al.*, 1992; Woltering and van Doorn, 1988; Lovell *et al.*, 1987). In species showing petal wilting, loss of fresh weight and turgidity before abscission has been noted (Stead and van Doorn, 1994). For example, a drop of 45% in fresh weight has been noted in carnation petals during senescence (de Vetten and Huber, 1990) and of 96% in daylily (Lay-Yee *et al.*, 1992). As the majority of the fresh weight of petals is accounted for by water, this magnitude of weight loss can only be due to rapid gain and subsequent loss of water. However, rapid cell division or growth during opening, resulting in gain of dry weight, and loss of dry weight by tissue breakdown and remobilisation during senescence may also account for some of the weight difference noted, with daylily petals falling to 33% of their maximum dry weight towards the end of their life (Lay-Yee *et al.*, 1992), but in *Alstroemeria*, only a very small difference in dry weight is seen with increasing age (Wagstaff *et al.*, 2001). The measurement of these parameters in wallflowers therefore allows some understanding of uptake and disposal of resources by the two different organs.

As senescing leaves become yellow, they also become visibly drier, indicating loss of water, and therefore fresh weight. As so many of the constituents of leaf cells are being broken down and remobilised to other parts of the plant during senescence, it would therefore also be expected that the dry weight should also fall with increasing age.

3.1.4 Protein content

Protein degradation has been observed during late development in both petals and leaves (Wagstaff *et al.*, 2002; Stephenson and Rubinstein, 1998; Celikel and van Doorn, 1995; Jones *et al.*, 1995; Smart *et al.*, 1995). The reason for this breakdown in leaves is to facilitate mobilisation from the senescing organ for recycling to other parts of the plant (Fischer *et al.*, 1998) and for energy production (Buchanan-Wollaston, 1997). Most of the protein for remobilisation from leaves originates from the chloroplasts (Thomas and Donnison, 2000). In petals, protein breakdown may also be for mobilisation and recycling, as there is direct evidence for protein remobilisation in daylily flowers (Bieleski, 1995), which would indicate that petals, like leaves, senesce.

3.1.5 RNA content

Nucleic acids, especially rRNA, are important sources of phosphorus, as well as of carbon and nitrogen for the senescing cell. In leaves, overall RNA levels fall during senescence, because the rRNA levels decrease (Makrides & Goldthwaite, 1981), as do the levels of most mRNAs, although mRNAs encoding SAGs are transcribed *de novo* (Buchanan-Wollaston, 1997). The amount of extractable nucleic acids also decreases during late flower development (Lesham *et al.*, 1986). Establishing the amount of extractable RNA in each developmental stage of wallflower leaves and petals is important for subsequent chapters, as much of the molecular work is on gene expression, and thus RNA extraction is key to its success. It will also be useful to compare gene expression patterns to total RNA levels, as some genes may increase in expression even as levels of RNA fall during late development.

3.2 Materials and Methods

3.2.1 Morphological studies

Flowers were tagged on the first day of opening and changes in their morphology were noted daily until abscission of the calyx, corolla and androecium. The timing of development was measured several times, including at different times of the year, to ensure reproducibility and to ascertain if there were any seasonal differences.

There was no convenient stage at which a leaf could be tagged, so they were classified based on the degree of expansion of the young leaves compared to the maximum expansion on the same rosette, and the degree of yellowing in older leaves that have already expanded fully.

3.2.2 Chlorophyll levels

The fresh weight and surface area of 2-3 leaves of each developmental stage were recorded. The leaves were ground in 0.5ml of 70% acetone and centrifuged at 4 K rpm for 10 mins in a Eppendorff (Cambrige, UK) minispin microcentrifuge to remove the leaf material. The pellet was re-extracted twice with fresh 70% acetone, to extract as much of the chlorophyll as possible. The supernatants were combined and made up to 10ml with 70 % acetone. Measurements were repeated twice for each developmental stage and the results averaged.

The absorbance of the chlorophyll extracts was measured at 645, 652 and 663 nm, against a 70% acetone blank using a Cecil Instruments Visible/UV spectrophotometer. Chlorophyll concentrations in μg of chlorophyll per ml of extract were calculated using the equations used by Bruinsma (1963):

Total chlorophyll (μg/ml) = 27.8 x [652 nm reading]

Chlorophyll *a* (μg/ml) = (12.7 x [663 nm reading]) – (2.7 x [645 nm reading])

Chlorophyll *b* (μg/ml) = (22.9 x [645 nm reading]) – (4.7 x [663 nm reading])

These results were used to derive the chlorophyll content of leaves in μg per leaf.

3.2.3 Fresh Weight and Dry Weight Determination

The fresh weight of a known number of petals or leaves of each developmental stage was determined using a fine balance. The same petals and leaves were dried at 60°C

for 5 days to determine dry weight. The weights were divided by the number of petals or leaves weighed, to obtain mean weight per petal or leaf.

3.2.4 Protein content

The amount of protein present was established using the dye-binding assay of Smith *et al.* (1985). This is based on the alkaline reduction of the cupric ion (Cu^{2+}) to the cuprous ion (Cu^{+}) by protein, followed by chelation with BCA (bicinchoninic acid), resulting in a bold blue/purple colour which can be quantified spectrophotometricaly (Smith *et al.*, 1985). The amount of Cu^{+} produced (and therefore the optical absorbence of the sample) is proportional to the amount of protein present, however, the presence of other reducing agents, such as reducing sugars or ammonium, can interfere with this reaction. The SDS used in the assay releases membrane bound proteins and denatures proteins, thus increasing reproducibility.

A crude extract was prepared by weighing and grinding 20 petals or 4 leaves from each developmental stage in 600µl of 50mM Tris-HCl pH 7.5. The samples were transferred to microcentrifuge tubes and centrifuged for 10mins at 12,000G, 4°C. The supernatant was retained and stored at -80°C until required. 10µl of 1% (w/v) aqueous SDS and 200µl of a freshly prepared solution of 50:1 BCA : 4% (w/v) copper (II) sulphate penhydrate were added to 10µl of a 1/10 dilution of each extract in a microtitre dish. The samples were incubated at 60°C for 1 hour and the OD measured at 590 nm in a Molecular Devices Emax precision microplate reader, using the Molecular Devices SOFTmax Pro software (version 3; Molecular Devices, Wokingham, Surrey, UK). Sample concentration was related to a standard curve consisting of 0.1-2.5 mg/ml solutions of BSA in 50mM Tris-HCl pH 7.5. Each sample and standard were done in triplicate on each plate and the assay was repeated on five different extracts made from independently harvested material.

3.2.5 RNA content

RNA was extracted from both petals and leaves of each developmental stage using the Tri reagent method with phenol: chloroform purification and ethanol precipitation and washing, as described in sections 2.2.3 to 2.2.6.

3.3 Results

3.3.1 Morphological studies

Flowers were studied daily for morphological changes. One flower on the raceme opened each day, taking seven days to complete its development from opening to abscission of the calyx, corolla and androecium (figure 3.3.1.1). This timetable assumes that each of the buds on the raceme is developing normally and is not damaged. If a bud failed to develop, the older flowers below that bud developed more slowly. No difference in morphology or in rate of development was noted for the flowers at different times of year. Stages of development were assigned based on number of days after opening (figure 3.3.1.1), as morphological features changed daily, making it easy to assign a developmental stage to each flower based on these features, as well as on the position of the flower on the raceme.

The flower stages chosen for analysis were stages -2 to 5, and can be characterised as follows:

-2 Third lowest unopened bud. Petals (within bud) still green

-1 Second lowest unopened bud. Petals (within bud) starting to turn purple

0 Lowest unopened bud. Petals (within bud) dark purple

1 First open flower. Petals pale purple, only four anthers visible, all undehisced.

2 Second open flower. Petals darker purple, all anthers visible, two dehisced.

3 Third open flower. Flower not as tightly held together as previously. Petals darker purple again and slightly wilted, all anthers visible and dehisced.

4 Fourth open flower. Flower loosely held together, with deteriorated appearance. Petals dark purple and wilted at the edges.

5 Fifth open flower. Flower loosely held together, appearance deteriorated. Petals dark purple and completely wilted. Last stage at which all petals are fully attached to the flower.

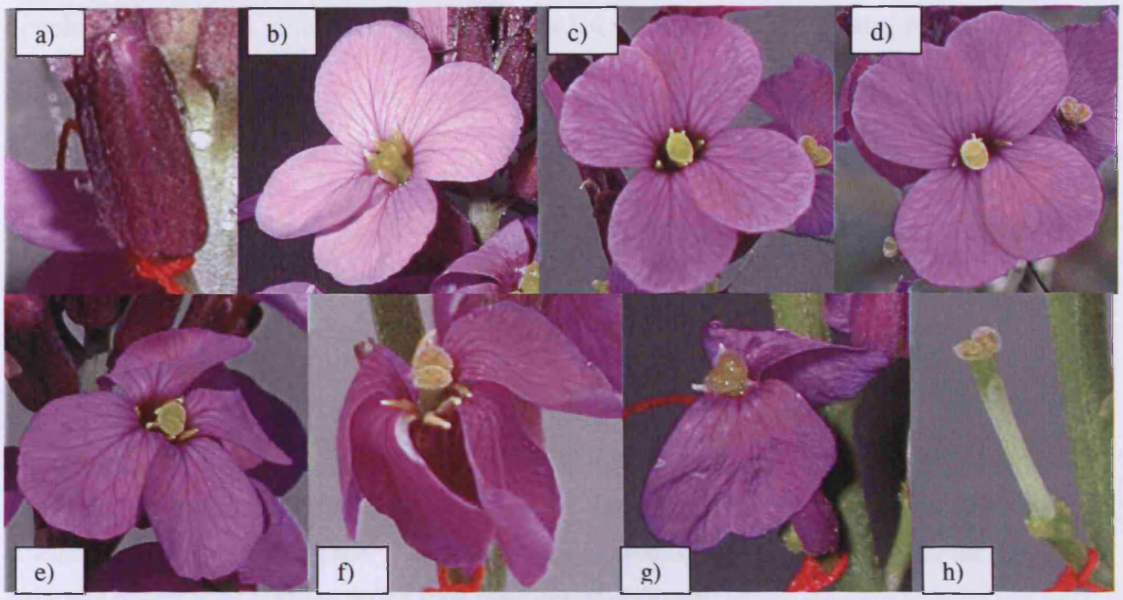


Figure 3.3.1.1 Stages of wallflower development. a) Stage 0. Lowest unopened bud on raceme. Petals dark purple in colour, tightly curled within sepals. b) Stage 1. Flower fully opened. Petals pale purple, with sepals folded back midway along their length (not visible). Stigma yellow and fuzzy in appearance, 4 of 6 anthers visible, all undehisced, positioned close to the stigma with the tips curled over the stigma. c) Stage 2. As stage 1, but petals darker in colour. All 6 anthers visible, 2 newly emerged anthers dehisced and curled back from the stigma. d) Stage 3. Flower not as tightly held together as previously. Petals wilting slightly and darker again in colour. Fuzz on stigma is not as fine as previously. All 6 anthers dehisced and curled back form the stigma. e) Stage 4. Flower loosely held together. Petals limp and curled over at the tips. Flower appearance deteriorated. f) Stage 5. As stage 4, but more extreme. Petals wilted, stigma discoloured with dark purple areas. g) Stage 6. Sepals, petals and stamens beginning to abscise. Remaining petals looked withered and dry. h) Stage 7. All sepals, petals and stamens abscised - only the stigma remains.

The leaf stages chosen for analysis could be characterised as follows within one whorl of leaves:

1. Very young leaves, ≤ 50% expanded
2. Very young leaves, 50 -> 75% expanded
3. Young leaves, 75 -> 100% expanded
4. Mature green leaves
5. Older mature leaves, green with signs of yellowing on the tip
6. Old leaves, up to 50% of leaf area yellow
7. Very old leaves, mostly or all yellow

Leaves were divided into seven stages which could be easily distinguished by eye (figure 3.3.1.2). Data from young leaves are needed to compare processes throughout

development, but as young leaves are not the focus of this study, so stages 1 and 2 were grouped together.

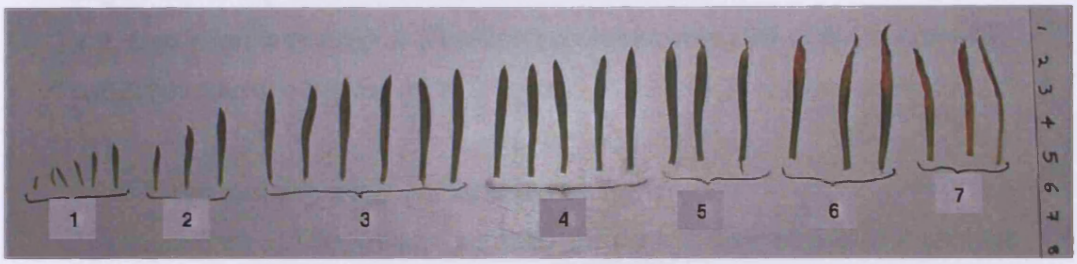


Figure 3.3.1.2. Stages of wallflower leaf development. The leaves from one whorl were removed and arranged in order of increasing age. The stages of development assigned are shown below the leaves and a size marker (cm) is shown on the right hand side of the image.

3.3.2 Chlorophyll levels

Chlorophyll concentration measurements were used to confirm the degree of leaf yellowing seen by eye. The average chlorophyll concentration in leaves of different developmental stages are shown in figure 3.3.2.1.

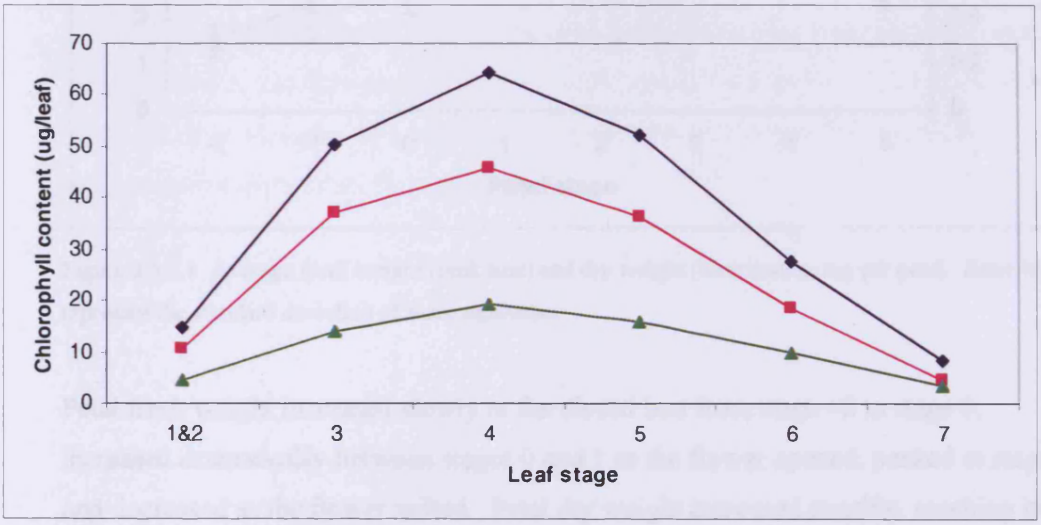


Figure 3.3.2.1 Average chlorophyll content of leaves expressed as μg of chlorophyll per leaf. Blue line indicates total chlorophyll, pink line represents chlorophyll *a* and green line denotes chlorophyll *b*. Error bars on total chlorophyll line show the difference between the measured total chlorophyll and the sum of chlorophyll *a* and chlorophyll *b* for each developmental stage. (n=2)

Chlorophyll measurements increased in early development to a peak at stage 4, then gradually decreased through the later developmental stages and senescence. Maximum chlorophyll levels were seen in stage 4 (mature green leaves), 20% less chlorophyll was present in stage 5 (signs of yellowing at tip) compared to stage 4, stage 6 leaves (up to 50% yellow) contained 40% of the maximum chlorophyll levels and stage 7 leaves (mostly or all yellow) contained only 10% of the chlorophyll content of mature green leaves.

3.3.3 Fresh and dry weight determination

The mean fresh and dry weights per petal and per leaf were calculated from three replicates. These replicates contained between 50 and 100 petals (figure 3.3.3.1) or 20 leaves (figure 3.3.3.2). The standard deviation was used as the measure of error as only three replicate measurements were taken.

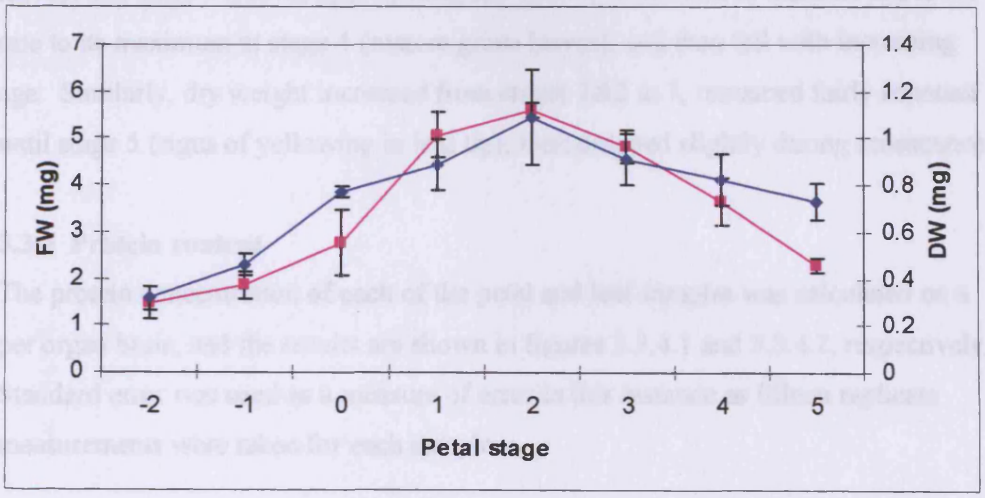


Figure 3.3.3.1 Average fresh weight (pink line) and dry weight (blue line) in mg per petal. Error bars represent the standard deviation of three replicates.

Petal fresh weight increased slowly in the closed bud from stage –2 to stage 0, increased dramatically between stages 0 and 1 as the flower opened, peaked at stage 2 and decreased as the flower wilted. Petal dry weight increased steadily, reaching its maximum shortly after flower opening at stage 2, then decreased as the flower wilted.

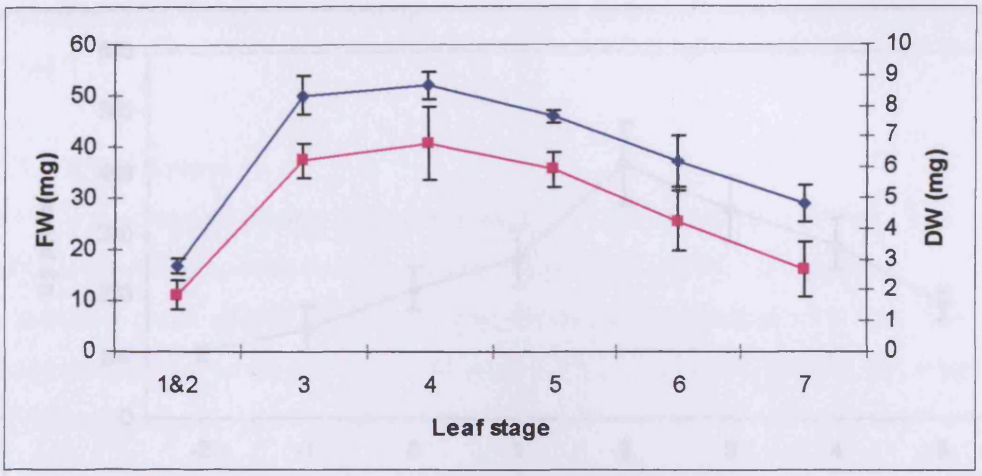


Figure 3.3.3.2 Mean fresh weight (pink line) and dry weight (blue line) in mg per leaf. Error bars represent the standard deviation of three replicates.

Leaf fresh weight increased sharply between the combined stages 1&2 (very young leaves) and stage 3 (leaves approaching full length), continued to increase at a lower rate to its maximum at stage 4 (mature green leaves), and then fell with increasing age. Similarly, dry weight increased from stages 1&2 to 3, remained fairly constant until stage 5 (signs of yellowing in leaf tip), then dropped slightly during senescence.

3.3.4 Protein content

The protein concentration of each of the petal and leaf samples was calculated on a per organ basis, and the results are shown in figures 3.3.4.1 and 3.3.4.2, respectively. Standard error was used as a measure of error in this instance as fifteen replicate measurements were taken for each sample.

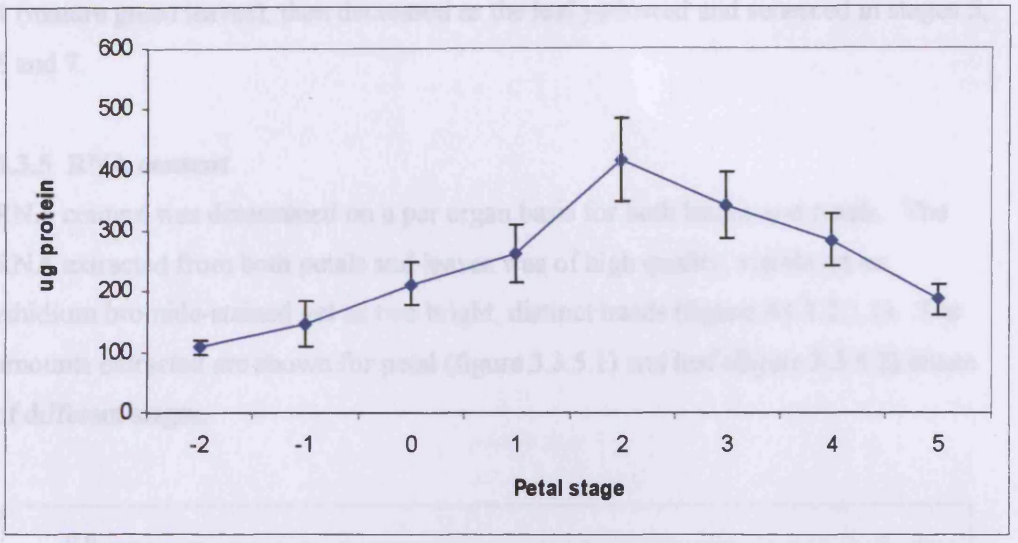


Figure 3.3.4.1 Mean protein content per petal (μg). Error bars represent the standard error of the mean for 15 replicates.

The petal protein content (figure 3.3.4.1) rose gradually, reaching a peak at stage 2 (shortly after flower opening), then dropped in late development.

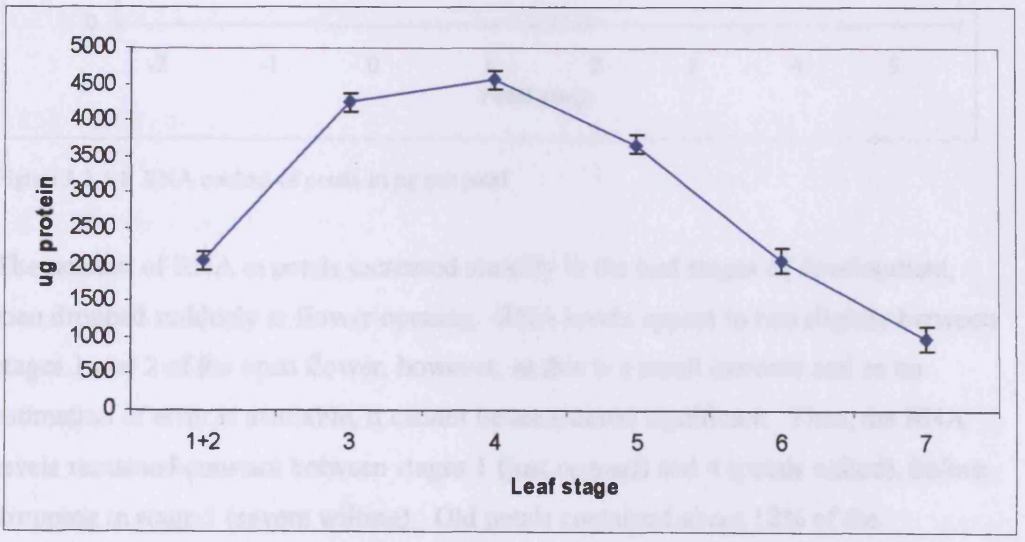


Figure 3.3.4.2 Mean protein content per leaf (μg). Error bars represent the standard error of the mean for 15 replicates.

The leaf protein content increased dramatically between stages 1&2 (very young leaves) and stage 3 (leaves approaching full length), continued to rise slightly to stage

4 (mature green leaves), then decreased as the leaf yellowed and senesced in stages 5, 6 and 7.

3.3.5 RNA content

RNA content was determined on a per organ basis for both leaves and petals. The RNA extracted from both petals and leaves was of high quality, visible on an ethidium bromide-stained gel as two bright, distinct bands (figure A1.3.2.1.1). The amounts extracted are shown for petal (figure 3.3.5.1) and leaf (figure 3.3.5.2) tissue of different stages.

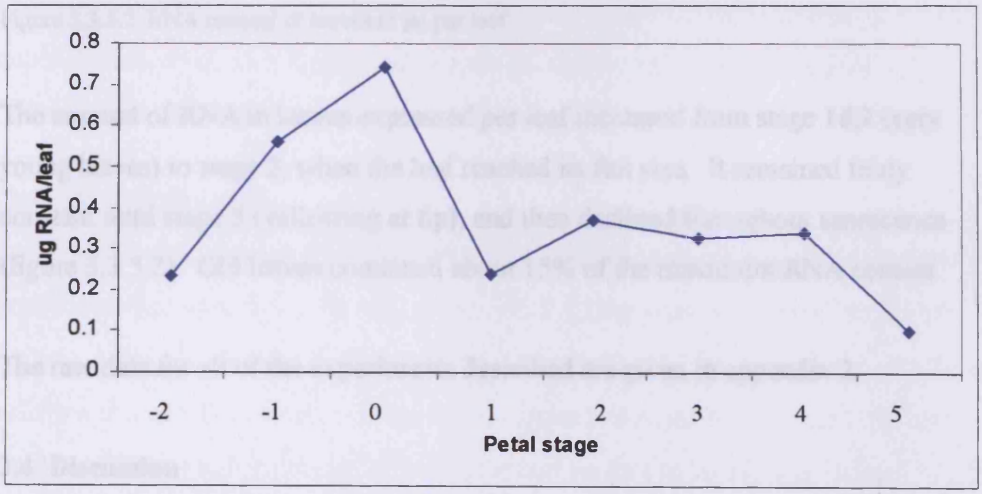


Figure 3.3.5.1 RNA content of petals in μg per petal

The amount of RNA in petals increased steadily in the bud stages of development, then dropped suddenly at flower opening. RNA levels appear to rise slightly between stages 1 and 2 of the open flower, however, as this is a small increase and as no estimation of error is available, it cannot be considered significant. Thus, the RNA levels remained constant between stages 1 (just opened) and 4 (petals wilted), before dropping in stage 5 (severe wilting). Old petals contained about 12% of the maximum RNA content.

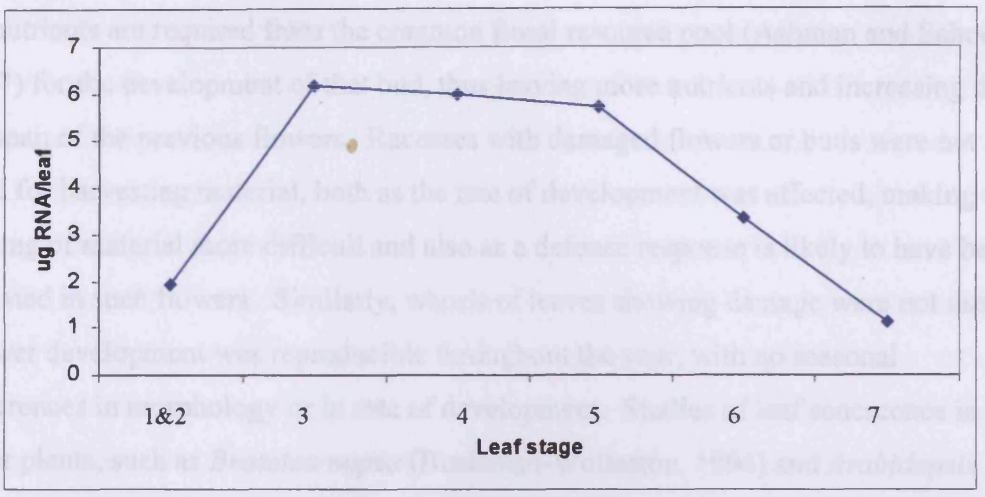


Figure 3.3.5.2 RNA content of leaves in µg per leaf

The amount of RNA in leaves expressed per leaf increased from stage 1&2 (very young leaves) to stage 3, when the leaf reached its full size. It remained fairly constant until stage 5 (yellowing at tip), and then declined throughout senescence (figure 3.3.5.2). Old leaves contained about 15% of the maximum RNA content.

The raw data for all of the experiments described are given in appendix 2.

3.4 Discussion

3.4.1 Morphological studies

The parameters chosen to define the stages of petal and leaf development are easy to distinguish by eye, making staging of material quick and easy for subsequent tests. They also show changes between stages for each of the physiological analyses, indicating that the stages are sufficiently different from each other to be useful. Three stages before flower opening (-2, -1 and 0) were chosen as many changes occur in the petal before flower opening (Stead and van Doorn, 1994; Faragher *et al.*, 1987; Suttle and Kende, 1980; Matile and Winkenbach, 1971). Stages 6 and 7 were not included for analysis as the petals have already abscised by these stages (although some petals remain on the flower at stage 6, they are not attached, and are very easily removed), and as they no longer have any connection to the rest of the plant, they cannot recycle nutrients and therefore are of no interest to this study. The slower development of older flowers on the raceme following damage to a bud could be due to the fact that

no nutrients are required from the common floral resource pool (Ashman and Schoen, 1997) for the development of that bud, thus leaving more nutrients and increasing the lifespan of the previous flowers. Racemes with damaged flowers or buds were not used for harvesting material, both as the rate of development was affected, making the staging of material more difficult and also as a defence response is likely to have been initiated in such flowers. Similarly, whorls of leaves showing damage were not used. Flower development was reproducible throughout the year, with no seasonal differences in morphology or in rate of development. Studies of leaf senescence in other plants, such as *Brassica napus* (Buchanan-Wollaston, 1994) and *Arabidopsis* (Buchanan-Wollaston *et al.*, 2003) have also used degree of expansion to characterise young leaf developmental stages and degree of visible yellowing to define stages of leaf senescence.

3.4.2 Chlorophyll levels

Chlorophyll levels, or degree of yellowing, have often been used to characterise stages of leaf senescence (eg Hensel *et al.*, 1993; Lohman *et al.*, 1994; Buchanan-Wollaston, 1994; Buchanan-Wollaston and Ainsworth, 1997). The pattern of change in wallflower leaf chlorophyll content shows a peak at stage 4, once the leaf has reached its full size and is likely to be photosynthesising at its most efficient. This is followed by a steady, yet rapid decrease in chlorophyll levels as the leaf yellows and senesces, reaching very low levels (10% of the maximum chlorophyll content) at stage 7. This matches the definitions of the later stages of leaf development from the morphological studies: stage 5 is defined as old leaves with signs of yellowing on the tip, and these contain 80% of the maximum chlorophyll content per leaf. Stage 6 is defined as the leaves being up to 50% yellow, and has a chlorophyll content of 40%. The stage 7 leaves, defined as mostly or all yellow, contain only 10% of the maximum chlorophyll levels. In *Brassica napus* leaves, Buchanan-Wollaston and Morris (2000) defined early senescent leaves as containing 90% of the maximum amount of chlorophyll and mid-senescent leaves as containing 50% chlorophyll, however, chlorophyll loss was only reported as being visible in late senescent leaves. Similarly, also in *Brassica napus* leaves, three senescent stages containing 98%, 60% and 35% of the maximum chlorophyll content have been defined (Buchanan-Wollaston and Ainsworth, 1997; Buchanan-Wollaston, 1997), with yellowing only visible after loss of 65% of the chlorophyll (Buchanan-Wollaston, 1997). These

results indicate that the extent of chlorophyll degradation is much greater than is visible by eye. This is different to what is seen in wallflowers, as yellowing is visible at the tips of stage 5 leaves, which contain 80% of the maximum amount of chlorophyll. A possible explanation for this discrepancy between the wallflower and *Brassica* results is that the chlorophyll loss from *Brassica* leaves may be an even loss from the entire leaf area, whereas in wallflowers the loss may be concentrated at the tip of the leaf, so that yellowing is visible in that region. However, in keeping with the *Brassica* results, stage 5 wallflower leaves do not appear, by eye, to have lost as much chlorophyll as they have (20%). In *Arabidopsis*, the relationship between degree of yellowing and chlorophyll content is more similar to that seen in the present study. At the time of full leaf expansion, *Arabidopsis* leaves contain the maximum amount of chlorophyll, falling to about 85% six days after full expansion, when the leaves are visibly yellow around the edges, then falling dramatically over the next two days to about 45% of the maximum chlorophyll content and about 50% of leaf area being yellow (by eye) and again over the next two days to 0% chlorophyll with the leaf being completely yellow (Hensel *et al.*, 1993). These results were corroborated in a similar study by Lohman *et al.* (1994), also in *Arabidopsis*. These results also correspond with the findings of the Buchanan-Wollaston group (2003), which show that Rubisco large subunit protein levels start to decline once the leaf has reached full size in *Arabidopsis*. Results such as these have led to the suggestion that *Arabidopsis* is not an ideal model system for senescence study, as the life of the leaf is short, and senescence begins immediately after full expansion is reached (Stessman *et al.*, 2002) and as reversibility of leaf senescence in *Arabidopsis* has not been shown, making this model system atypical. However, this can be refuted, because the life cycle of *Arabidopsis* is short, changes during development are sharper and so can be seen more easily, and unlike many other species, regreening has yet to be achieved experimentally in *Arabidopsis* leaves, which reduces the complexity of studying senescence in *Arabidopsis* compared to in other species. It would appear from the results for chlorophyll content in wallflower leaves that symptoms of senescence in this species also start being detectable at stage 5, which is the stage immediately after full expansion has been reached (stage 4). However, as the time taken for leaves to complete their development in wallflowers has not been measured, then the time taken for leaves to develop from fully expanded (stage 4) to start of visible yellowing (stage 5) is unknown.

3.4.3 Fresh and dry weight determination

The pattern of increase and decrease in the fresh weight of petals was very similar to that of dry weight, indicating that a combination of changes in water content and changes in dry weight were responsible for the overall change in fresh weight. On flower opening (between stages 0 and +1), there was an increase in both fresh (40%) and dry weight (15%), with that of the fresh weight being more pronounced than that of the dry weight. This would indicate that although there is an increase in dry weight, possibly due to cell division or growth, which accounts for a certain degree of the increase in fresh weight, the vast majority of the increase is due to rapid water uptake. This agrees with the findings of Weston and Pyke (1999), whose electron microscopy studies in *Erysimum cheiri* could not find evidence of cell division in expanding wallflower petals, only cell expansion and the similar findings of Pyke and Page (1998) in *Arabidopsis*. Loss of fresh weight after stage 2 and throughout late development is consistent with wilting species losing fresh weight and turgidity before abscission (Stead and van Doorn, 1994). A 60% drop in fresh weight was noted between the maximum at stage 2 until the petals were fully senesced at stage 5; which is intermediate between the 45% weight loss noted in old carnation petals (de Vetten and Huber, 1990) and the 96% drop in daylilies (Lay- Yee *et al.*, 1992). This water may be lost due to increased transpiration or decreased uptake of water, or as it is being exported to other parts of the plant, possibly taking nutrients with it in solution. The 33% drop in wallflower petal dry weight during late development, simultaneous with the drop in fresh weight, again indicates that the majority of the drop in fresh weight was accounted for by loss of water. However, the fact that some dry weight was lost indicates that there may be tissue breakdown and, therefore, there may also be some remobilization, as loss of fresh weight was found to correlate with export of soluble sugars in the petals of daylily (Bieleski, 1995), petunia (Verlinden, 2003) and in gladiolus florets (Yamane *et al.*, 1993). However, metabolism of macromolecules may also provide an explanation for this drop in dry weight, which may occur with or without recycling. The timing of influx and efflux of both water and nutrients appears to be linked, but further work would be necessary to ascertain the relationships between the mechanisms and signalling of these processes.

Leaf fresh weight increases as the young leaves expand, reaches a peak at stage 4 (mature green leaves), then falls as the leaves senesce. Similarly, leaf dry weight increases with leaf expansion, remains fairly constant between stages 3 (leaves approaching full length) and stage 5 (yellowing at tip), before falling during senescence. Differing from wallflower petals, the changes in leaf fresh weight are of a similar degree to those for dry weight, with fresh weight increasing by 70% as leaves reach full expansion then falling by 60% by the time the leaf is fully senescent, and dry weight increasing by 75% then falling by 70% at the same times, although as the majority of leaf fresh weight is made up of water, again the majority of the changes in weight are made up by gain and loss of water. The fresh weight of a stage 7 leaf (mostly or all yellow) is about a third of the maximum fresh weight, which is a similar drop to that seen in petals, indicating that these stages are physiologically comparable and that the leaves also wilt. As it is known that during leaf senescence macromolecules are broken down and transported to other parts of the plant in solution, it is possible, although not conclusive, that some of the drop in fresh weight seen during senescence can be accounted for by the breakdown and export of nutrients and that some of the water loss is as a solvent for their export. This does not preclude the possibility that some of the losses may also be due to such factors as increased transpiration and metabolism of macromolecules for energy to power the process. The fact that the changes in both fresh and dry weight seen in petals are very similar to those in leaves, where the changes can, at least in part, be attributed to the senescence process, would indicate that similar changes may be occurring in both organs, and that petals may also, therefore be a predominantly senescent system. However, as so many factors can be responsible for the change in weight of an organ, clearly it is not possible to draw any real conclusions on the similarity of late developmental processes in petals and leaves based solely on these results.

The changes in fresh and dry weight match the pattern of the results for chlorophyll content of the leaves, which also peaks at stage 4. As leaf dry weight falls from 8 to 5mg, between full expansion and full senescence, and chlorophyll content falls from 63 to 8μg per leaf, it can be seen that only a small proportion of leaf dry weight, even in mature green leaves, is accounted for by chlorophyll. However, there is proportionally more chlorophyll present in mature leaves per unit dry weight (0.78%), and in young leaves (0.47%) than there is in senescent leaves (0.16%).

3.4.4 Protein content

The values obtained for protein content of both petals and leaves were remarkably high, being an order of magnitude higher than published accounts of protein content in both petals and leaves in other species (e.g. Wagstaff *et al.*, 2002; Orzaez *et al.*, 1999; Abu-Shakra *et al.*, 1978). In order to try to establish the cause of these unusually high values, a series of controls were tried, including trying the reactions either without the wallflower extract (petal or leaf) or without the copper (II) sulphate penhydrate, to establish whether the colour of pigments was interfering with the readings of the optical absorbance. An *Alstroemeria* petal extract was tested, to establish whether there was something present in the wallflower tissues that interfered with the chemistry of the protein assay, and commercially bought standard solutions of BSA were also tested, to ensure that no error had been made in making up the original standard solutions from powdered BSA. The controls lacking an active reagent resulted in similar readings to blank wells, indicating that pigments from wallflowers do not interfere with the accurate reading of the optical absorbance. The *Alstroemeria* petal extract also appeared to contain more protein than published accounts of *Alstroemeria* petal protein content (Wagstaff *et al.*, 2002), indicating firstly that wallflowers do not contain any secondary metabolites that interfere with the chemistry of the protein assay, and secondly that there is a systematic error in the results of the protein assay. This error was not due to the standards being made up to the wrong concentrations, as the commercial standards gave the same results. The calculations were rechecked, and no error could be found in the calculation of petal and leaf protein content from the standard curves. Thus, it is acknowledged that the results of the protein assay as they are presented are flawed. However, as the error is systematic, it applies equally to all of the samples tested within this experiment, and thus the pattern of protein content over development in both organs still holds true and will be discussed further in this section. The relative protein content of wallflower petals and leaves can similarly be compared to each other.

Petal protein content reached its maximum at stage 2, matching the pattern of the results for petal fresh and dry weight, which also peak at stage 2. This is followed by a dramatic fall in petal protein content in late development. Thus, protein degradation is occurring, as has been observed in many senescing organs (Verlinden, 2003; Serafini-Fracassini *et al.*, 2002; Wagstaff *et al.*, 2002; Stephenson and Rubinstein,

1998; Collier, 1997; Jones *et al.*, 1995; Smart, 1994; Celikel and van Doorn, 1995), indicating that the protein may be being mobilised from the dying petal, which is a feature of senescence. This does not preclude the idea that protein synthesis is still occurring at stages 4 and 5 to some extent, only that protein breakdown is progressing at a greater rate. Wagstaff *et al.* (2002) expressed the protein levels of *Alstromeria* petals in relation to their fresh weight, showing them to drop dramatically at flower opening, then remain constant until perianth abscission with a slight rise in protein levels at the very end of development. A similar pattern is seen in wallflower protein levels when expressed in relation to petal fresh weight (data not shown). Wagstaff *et al* (2002) suggest that the late rise in protein concentration is a reflection of the loss of fresh weight at this time. To confirm that this is the case, wallflower petal protein content was also looked at in relation to petal dry weight, with a very similar pattern seen with a drop in protein content at flower opening, followed by a rise, but rather than rising until the end of the developmental series, as happened when expressed in relation to fresh weight, protein levels fell again from stage 3 until the end of development. Thus, despite the fact that dry weight is also falling, protein content is falling at a faster rate, indicating that these nitrogen-rich molecules are being degraded in preference to some of the other macromolecules within the organs, perhaps for remobilisation of the nitrogen.

Leaf protein reached its maximum at stage 4 (mature green leaves), again matching the patterns seen in both fresh and dry weights and also in chlorophyll content. The decrease in protein concentration later in development is consistent with that seen in other senescing systems, such as *Arabidopsis* (Lohman *et al.*, 1994), as the protein is broken down and recycled to other parts of the plant (Hensel *et al.*, 1993; Fischer *et al.*, 1998). Again, there may also be protein synthesis at this late stage, but protein breakdown is the predominant process. In leaves, the fact that chlorophyll and protein levels both began to drop at the same time is interesting, as most of the protein for remobilization from the leaves is from the chloroplasts (Thomas and Donnison, 2000), thus it may be hypothesised that protein levels should also fall at this stage. Thus, breakdown of both of the major chloroplast macromolecules occur in parallel, concordant with the findings of Thomas and Donnison (2000) in *Festuca*, which showed the control of breakdown of pigment-protein complexes to be coordinated (section 1.5.4.1).

The patterns of protein content between petals and leaves are similar, with the protein content of both organs rising until the maximum fresh and dry weights are reached, then decreasing throughout late development. This is interesting, as it again implies that similar processes may be at work in both organs. Comparing the amount of protein present in wallflower petals and leaves relative to the dry weight of the organs reveals that on a mass for mass basis, there may be equal value in reclaiming nutrients from both petals and leaves during senescence. However, each leaf is about 10 times heavier than a petal (for both fresh and dry weighs), meaning that on a per organ basis leaves contain far more nutrients. The fact that nitrogen-rich proteins are broken down during late petal development imply that the purpose of this breakdown may be remobilisation

3.4.5 RNA content

The amount of RNA in wallflower petals increased steadily in the bud stages of development, then dropped suddenly at flower opening, and dropped again at the very end of development, with old petals containing only about 12% of the maximum RNA content. These results are concordant with the amount of extractable nucleic acids being known to decrease during late flower development (Lesham *et al.*, 1986). Nucleic acids, especially rRNA, are important sources of carbon, nitrogen and particularly phosphorus for the senescing cell, and thus overall RNA content of leaves falls with senescence (Lohman *et al.*, 1994; Buchanan-Wollaston, 1997; Makrides & Goldthwaite, 1981). The fact that levels of these nutrient-rich macromolecules also fall during late petal development therefore again implies the possibility of remobilisation, and therefore senescence, in petals as well as leaves. In wallflower leaves, RNA levels did not begin to fall until stage 5 (yellowing at tip, 80% chlorophyll). This is intermediate between results reported in *Brassica napus*, where RNA levels did not begin to fall until chlorophyll levels were as low as 35% of the maximum levels (Buchanan-Wollaston, 1997) and in *Arabidopsis*, where RNA levels started to fall before any yellowing was visible (Lohman *et al.*, 1994). It is interesting that in all of the previous tests reported in this chapter: chlorophyll levels of leaves, fresh and dry weights of petals and leaves and the protein content of petals and leaves, that all of the parameters reached their maximum at stage 2 of petal development or stage 4 of leaf development. However, RNA content differs from all of the other parameters in both organs, reaching its peak earlier than the other parameters (stage 0)

in petals, and in leaves (stage 3), and starting to fall earlier than the other parameters in petals (stage 0, with a further drop at stage 4), but later than the other parameters in leaves (stage 5). This is interesting, as levels of RNA must be maintained in both organs to a certain extent, to allow the transcription and translation of SAGs, but at the same time it represents an important source of nutrients for recycling as a part of the senescence process.

As the peak for most of the parameters measured is at stage 2 for the petals and stage 4 for the leaves, these two stages can be assumed to be equivalent, being 'mature' tissues. Thus, old tissues are defined as stages 3, 4 and 5 for the petals and stages 5 and 6 for the leaves, with young tissues defined as stages -2, -1 and 0 for the petals and stage 3 for the leaves.

4 Nucleic acid hybridisation between species

4.1 Introduction

The main reason for the choice of wallflowers in this study is the close taxonomic relationship between the genus *Erysimum* and the model plant *Arabidopsis thaliana* and the crop plant *Brassica napus* (see section 1.7.2). A large number of genes involved in leaf senescence have been identified in these two better-studied species, and these can be used to study senescence in wallflowers. Firstly, they can be used to identify the expression of homologues in wallflower leaf senescence, and if this is successful, to compare expression during late petal development, to establish whether the same genes are involved in the two processes.

The first step is to use the senescence-associated *Arabidopsis* clones for northern hybridisation to wallflower RNA. This would establish whether cross-species hybridisation is possible between wallflower and *Arabidopsis*, and whether a homologous gene is expressed in wallflowers, at what level, during what stage of development and in which tissue. If this is successful, *Arabidopsis*-derived SAGs could be spotted on a microarray and probed with wallflower RNA-derived probes, and this information could be provided on a large number of genes simultaneously. It is also anticipated that a putative identity could be assigned to genes identified in senescent wallflower tissues by comparing the sequence to the fully sequenced *Arabidopsis* genome. However, in order for such approaches to be used, it must first be established that there is sufficient gene homology between wallflower and *Arabidopsis* for cross-species hybridisation to be possible. It is likely that this will be the case, as previous studies have shown that mRNA probes from *Thellungiella halophila*, a salt-tolerant close relative of *Arabidopsis thaliana*, will hybridise to *Arabidopsis* DNA on the GeneChip ATH1 microarray (Volkov *et al.*, 2004). When hybridising an *Arabidopsis* microarray with seed and leaf mRNA samples, the correlation coefficients between *Arabidopsis* and *Brassica* experiments varied between 0.73 and 0.83 for ratios and 0.76 and 0.83 for intensities, which are only slightly lower than those for repeated *Arabidopsis* experiments, which varied between 0.86 and 0.87 for ratios and 0.96 for intensities (Girke *et al.*, 2000). It was also found that most seed-specific signals identified with the *Arabidopsis* probes also gave seed-

specific signals with the *Brassica* probes. However, 80% of genes on the array gave signals at least 2-fold over background with *Arabidopsis*, with this figure falling to 50% with *Brassica*, indicating that signals from some weakly expressing genes are likely to be lost in experiments with heterologous probes (Girke *et al.*, 2000). Similarly, transcription mapping of the *Brassica* genome against *Arabidopsis* has shown both extensive colinearity between the two genomes and that 89% of the sequenced cDNAs from *Brassica* show similarity to genes reported in *Arabidopsis*. This indicates that not only are the gene sequences similar, but that the order of genes on segments of chromosomes are also conserved (Li *et al.*, 2003). Furthermore, the 11,522-element Functional Genomics Consortium *Arabidopsis* microarrays have been successfully hybridised to cDNAs from several different species, comprising wild oat (*Avena fatua*), poplar (*Populus deltoides*) and leafy spurge (*Euphorbia esula*). Between 23-47% of the genes on the array were detected by these other plant species (compared to 70% detected by probing with *Arabidopsis*) and conserved expression of genes involved in cell division, stress responses and development was reported between these distantly related species (Horvath *et al.*, 2003).

Thus, as cDNA from distantly related species can be used to probe *Arabidopsis* microarrays with a significant degree of success and the results of cross-hybridisation experiments between *Arabidiopsis* and closely related species are even more productive, it is hypothesised that *Arabidopsis* probes should hybridise well to cDNA and thus also RNA from wallflowers. It would also seem likely that PCR primers designed to conserved regions between *Brassica* and *Arabidopsis* genes should also work in wallflowers if a small amount of degeneracy is included as required.

To test this hypothesis, three genes from *Arabidopsis*, *LSC460*, *SAG12* and *RBCS*, each showing different expression patterns during development (see table 4.1.1), were used to probe northern blots of wallflower RNA. These were chosen due to the high level of homology between the homologues of these genes in different species. At the nucleotide level, *LSC460* shows 98% identity between *Arabidopsis* and *Brassica napus* (Buchanan-Wollaston and Ainsworth, 1997), whereas *Arabidopsis SAG12* shows 86% and 83% homology to the two *Brassica napus SAG12* homologues (Noh and Amasino, 1999*b*). *RBCS* is, of course, highly conserved in all plant species (Devos *et al.*, 1998). Northern analysis tests whether the *Arabidopsis* probe binds to

the wallflower RNA specifically, as the expression pattern over developmental time can be compared, and the size of the mRNA that the *Arabidopsis* probe binds to can be estimated from the northern and compared to the known size of that mRNA in *Arabidopsis*. RNA from young, mature and old leaves (see chapter 2) were run initially, to establish whether cross hybridisation would occur. RNA from each of the leaf developmental stages (see chapter 2) was subsequently used with *SAG12* and *RBCS* probes to obtain a more detailed expression pattern. Finally, both flower and petal RNA from each developmental stage was used with a *SAG12* probe to test whether probes would bind across different tissue-types as well as cross-species. *SAG12* was chosen to probe these preliminary northerns because it is highly conserved between *Arabidopsis thaliana* and *Brassica napus* (Noh and Amasino, 1999*b*), and is also expressed in flowers during late development (Clark *et al.*, 2004; Chang *et al.*, 2003; Grbic, 2002; Schroeder *et al.*, 2001). It is also only expressed during natural leaf senescence, and not during induced senescence or other forms of leaf cell death (Noh and Amasino, 1999*a*; Weaver *et al.*, 1998; Lohman *et al.*, 1994; Becker and Apel, 1993) and has therefore been used as a molecular marker for senescence (Noh and Amasino, 1999*a*).

Gene	Function	Expression	Size of mRNA	Reference
LSC460	Glutamine synthetase	Early senescence	1.4kb	Buchanan- Wollaston and Ainsworth, 1997
SAG12	Cysteine protease	Late senescence, strong	1.36kb	Lohman *et al.*, 1994
RBCS	Small subunit of Rubisco	Young and mature, strong	0.9kb	Kawaguchi *et al.*, 2003

Table 4.1.1. Table showing the *Arabidopsis*-derived genes used to probe wallflower northerns, their encoded protein, their expression patterns in *Arabidopsis*, and the size of the corresponding mRNA in *Arabidopsis*.

4.2 Materials and Methods

4.2.1 Northern analysis

All buffers used are described in the list of buffers, section 2.1.

4.2.1.1 Membrane preparation and prehybridisation

RNA was extracted from each developmental stage of both leaves and petals using the Tri reagent method with phenol: chloroform purification (as described in sections 2.2.3 and 2.2.4). 15µg of RNA from each stage was suspended in sample buffer (1X E buffer, 10% (v/v) ficoll, 0.5% (w/v) bromophenol blue, 30µg/ml ethidium bromide, 50% (v/v) formamide, 18% (v/v) formaldehyde), heated to 50°C for 5 minutes, cooled on ice and loaded in equal amounts onto a denaturing gel. The gel was prepared using 0.8 % (w/v) multi-purpose agarose (Bioline, London, UK) and 16% formaldehyde made up in 1X E buffer. The gel was run in a solution of 8% formaldehyde in 1X E buffer at 75 volts for about 3 hours before being visualised under UV and photographed. The gel was trimmed to size and the RNA was blotted onto a Hybond-N GS+ nylon membrane (Amersham Pharmacia, Little Chalfont, UK) overnight with 10X SSC. The wells were marked onto the membrane before it was removed from the gel, and the RNA fixed to it by baking at 80°C for 2 hours. The membrane was stained with a solution of 0.02% (w/v) methylene blue and 0.5M sodium acetate (pH 5.2) for 5 minutes and destained in 1X SSPE for 10 minutes, to confirm that the RNA had blotted successfully. The membrane was wetted in 2X SSC and prehybridised in a rotisserie tube with 15ml of prehybridisation solution (50% formamide, 5X SSPE, 5X Denharts solution, 0.5% SDS, 100µg/ml herring sperm DNA) and rotated for 8 hours at 37°C.

4.2.1.2 Preparation of the probe and hybridisation

Plasmids containing DNA inserts from *LSC460*, *SAG12* and *RBCS* from *Arabidopsis thaliana* were provided by V. Buchanan-Wollaston (HRI Wellesbourne), and 2µl of each was transformed into competent *E. coli* cells. Liquid cultures were grown and the plasmid purified, as described in sections 2.2.18 and 2.2.19.

DNA was amplified by PCR using M13 F and R primers. The PCR mixture consisted of 5µl of 10X PCR buffer (Qiagen Ltd., Crawley, UK), 200µM concentrations of each deoxynucleotide triphosphate, 0.3µM concentrations of each primer, 1U of HotStarTaq DNA polymerase (Qiagen Ltd., Crawley, UK), approximately 1ng of plasmid DNA and sdw to a final volume of 50µl. The following thermocycling pattern was used: 95°C for 15 minutes, 35 cycles of {95°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute}, 72°C for 7 minutes, 4°C hold.

5µl of the 50µl PCR reaction were analysed by agarose gel electrophoresis (1% w/v) with ethidium bromide staining to confirm that the product was a single fragment of the correct size (as described in section 2.2.10). The remaining 45µl was purified to remove unincorporated dNTPs and primer dimers, using the QIAquick PCR purification kit (Qiagen Ltd., Crawley, UK), following the manufacturer's instructions. The purified plasmids were eluted in 30µl of warm EB buffer (Qiagen Ltd., Crawley, UK).

3µl of the purified DNA was used to make the radioactively labelled probe, diluted with sdw to a volume of 47µl. The DNA was denatured for 3 minutes on a heat block at 100°C, incubated on ice for 2 minutes and pulse centrifuged to collect any condensation. The DNA and 3µl of Redivue [α-32P] dCTP (approx. 3000Ci per mM; Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK) were added to a Ready-to-go DNA labelling (-dCTP) bead containing the remaining three dNTPs, the random hexamer primer and the Klenow polymerase (Amersham Pharmacia kit, Piscataway, NJ, USA). The reagents were mixed by gentle pipetting and vortexing, pulse centrifuged to remove bubbles and incubated at 37°C for about 1 hour. The probe was fractionated on a CL6B column (as described section in 2.2.16) to remove unincorporated nucleotides and hexamer from the probe, denatured by heating to 100°C for 2 minutes, cooled on ice and carefully added to the prehybridisation solution in the rotisserie tube. This was rotated overnight at 37°C.

4.2.1.3 Washing and visualisation

The membrane was washed to remove unbound probe, using three changes of wash solution (0.1% SDS, 2X SSC) at 60°C for 20 minutes each with shaking. The membrane was sealed in clingfilm and visualised on Biomax MR scientific imaging film (Kodak, Rocheter, NY, USA) in a cassette with intensifying screens at –70°C for between 12 hours and two weeks, depending on the strength of the signal.

The blot was stored at -20°C and could be stripped of its probe by washing with three changes of boiling 0.05% SDS, 0.05X SSC, 0.1M Tris- HCl pH 7.4 for 2 minutes each with shaking, and could then be reused.

4.3 Results

In the case of all three genes, probes from *Arabidopsis thaliana* cross-hybridised well to northern blots of wallflower RNA (figure 4.3.1), each giving clear bands of the appropriate size and showing the expected expression pattern. *LSC460* shows a class 7 expression pattern, increasing throughout development, from very low in young leaves to high in old leaves. *SAG12* is exclusively expressed in old leaves, showing a class 5 expression pattern. *RBCS* is present in young leaves, has maximum expression in mature leaves, and then declines sharply in senescing leaves.

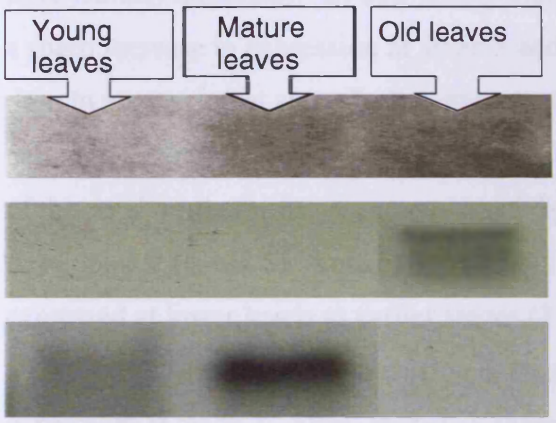


Figure 4.3.1

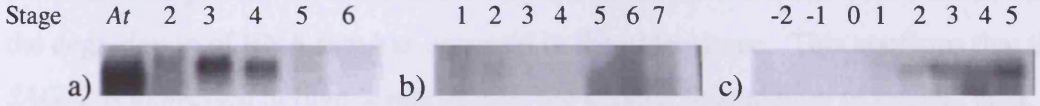
a) *LSC460*. 1.4 kbp. Expression increases as development progresses

b) *SAG12*. 1.38kbp. Only expressed in senescing leaves

c) *RBCS*. 0.96kbp. Maximum expression in mature leaves.

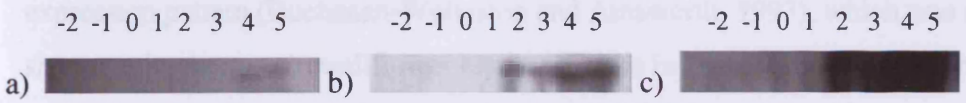
Probes made from leaf senescence-related genes were also used successfully to probe northerns of flower (figure 4.3.2) and petal (figure 4.3.3) RNA.

Figure 4.3.2



a) *RBCS* in leaves of stages 2 to 6, with mature *Arabidopsis* leaf (*At*) control. Maximum expression is in leaves of stage 3 (young, nearly fully expanded leaves), then falling with increasing age.
b) *SAG12* in leaves of stages 1 to 7. Very low expression in young and mature leaves, with a sudden increase at stage 5.
c) *SAG12* in flowers of stages -2 to 5. Again, very low expression in buds and newly opened flowers. Some expression in stage 2, increasing sharply with age until stage 5.

Figure 4.3.3



SAG12 in petals of stage -2 to 5.
a) 2 day exposure of the film only shows expression during very late senescence (stages 4 and 5).
b) 5 day exposure of film additionally shows expression at stages 2 and 3.
c) 9 day exposure of film shows some low level expression even in very young stage –2 petals.

Figures 4.3.1 and 4.3.2 show that *SAG12* is strongly expressed late in leaf senescence in *Erysimum linifolium*. *SAG12* is only expressed in leaves of stage 5 and older, with a sharp increase in expression at stage 5 and a further increase at stage 6, but then a drop in expression at stage 7. It is expressed in both leaves and flowers (figure 4.3.2), and also specifically in the petals (figure 4.3.3). A short exposure (52 hours at –70°C) of the petal northern shows expression of the gene only in the very late stages of development (4 and 5). Longer exposure (5 days at –70°C) reveals that it is also expressed at lower levels at earlier stages (2 and 3) and a longer exposure still (9 days at –70°C) shows very low expression at stages –2 to 1 and a sudden increase in expression at stage 2. Thus, there is a sharp increase in expression of *SAG12* at stage 2 and another at stage 4. This suggests that senescence may start very early in the petals compared to leaves, as this gene is not expressed until late leaf senescence.

It is interesting to note that the bands in the younger tissue of leaves, flowers and petals are sharp, whereas those of the very old tissue have a smear below them, due to the degradation of RNA that has occurred in the older tissue. This confirms that the *SAG12* is expressed at high levels despite the general degradation of nucleic acid, reinforcing that it is an important gene in senescence.

4.4 Discussion

The expression pattern found in wallflower leaves for each of the three genes was the same as is found in *Arabidopsis*, and all three wallflower genes were the same size (as estimated from the northern) as their *Arabidopsis* counterparts (table 4.1.1).

In *Arabidopsis* leaves, *LSC460* is expressed early in senescence, showing a class 7 expression pattern (Buchanan-Wollaston and Ainsworth, 1997), which was also shown to be the case in wallflower leaves. This is in keeping with its role in senescence as a cytosolic glutamine synthase (GS1), the activity of which increases during senescence, in contrast to the activity of chloroplast-located glutamine synthase (GS2), which decreases (Kamachi *et al.*, 1992*a*). Through the activity of GS1 proteins, such as LSC460, the nitrogen present in proteins and nucleic acids can be converted, via ammonia, into the transportable amino acid glutamine, and from there, also to asparagine (see figure 1.5.2), which are the predominant amino acids in the phloem transported from senescing leaves (Kamachi *et al.*, 1992*b*).

In the leaves of *Arabidopsis*, *SAG12* expression rises sharply from being undetectable in young leaves, to showing very strong expression during senescence and increasing throughout senescence even in the very late stages (Lohman *et al.*, 1994), an identical situation to that seen here in wallflower leaves. The drop in expression seen at stage 7 in wallflower leaves may be due to there being less intact RNA present at this stage, due to the increased activity of nucleases. *SAG12* encodes a cysteine protease. Many members of this family of proteases increase in expression during senescence, and they are likely to be an important mechanism for protein turnover during senescence, although the details of their involvement are not yet fully understood. *SAG12* is highly specific to developmental senescence in leaves, and is not expressed in response to induced senescence or during senescence-like processes (Noh and Amasino, 1999*a*; Weaver *et al.*, 1998; Lohman *et al.*, 1994; Becker and Apel, 1993). Thus, the fact that *SAG12* is expressed in flowers and in petals would indicate that these tissues are also senescing. It is interesting to note that despite *SAG12* levels being undetectable in non-senescent leaves, even with long exposures of the autoradiograph; increasing the exposure time of the petal blots showed that although it is only expressed strongly during late development in petals, it is also expressed (albeit at very low levels) even in very young petals. This implies that senescence processes start very early in wallflower petals, as was also found in *Alstroemeria* petals (Wagstaff *et al.*, 2003). This also fits with the data of Wagstaff *et al.* (2002), where cysteine protease expression was detected in unopened *Alstroemeria* flowers and increased gradually throughout the development of the flower. This provides

evidence to support the hypothesis that a young petal is already analogous to a senescing leaf (Thomas and Sadras, 2001; Thomas *et al.*, 2001).

RBCS expression increases in wallflower leaves as they develop from young to mature leaves, peaking in expression in mature leaves as the leaves photosynthesise actively. Levels then drop dramatically during early senescence, as is also the case in *Arabidopsis* leaves (Hensel *et al.*, 1993), as during this time the photosynthetic activity of a leaf declines, and so the levels of the transcripts encoding the proteins required for photosynthesis also decrease (Bate *et al.*, 1991; Hensel *et al.*, 1993; Jiang *et al.*, 1993). RBCS is likely to be broken down and its constituent nutrients recycled to other parts of the plant.

The successful hybridisation of probes across species and across tissue types on northern blots means that known senescence-related genes isolated from *Arabidopsis* can be used on the microarray. This will establish whether there are homologues to the *Arabidopsis* genes in wallflower leaves or in wallflower petals, and whether these have the same pattern of expression, both across species and between the two organs. However, the signal strength will clearly depend on the conservation of the genes across species. The successful hybridisation of probes derived from one organ-type to RNA from another organ also validates the spotting of both leaf and petal wallflower clones from the SSH libraries on the same array, to be probed with RNA from both organs.

5 Suppression subtractive hybridisation

5.1 Introduction

This technique (Diatchenko *et al.*, 1996) was used to identify genes specifically expressed during petal ageing and leaf senescence in wallflowers. Using this technique, it is possible to subtract the housekeeping genes which are expressed during all developmental stages from the pool of genes which are expressed during late development, leaving only those genes which are expressed specifically during late development. SSH has the added advantage of equalising genes with different expression levels, so that senescence-specific genes with low expression are equally likely to be detected as those with high expression, thus increasing the chance of identifying novel genes involved in the senescence process. By creating subtractive libraries of both petal and leaf late developmental genes, the genes expressed during both processes can be compared to establish whether the same genes, and therefore the same processes are at work in both organs.

The basic theory underlying subtraction is simple. mRNA from both young and old tissue is used to make cDNA. The cDNA derived from old tissue, which contains the specific (differentially expressed) transcripts of interest, is referred to as 'tester', and the reference cDNA (derived from young tissue) as 'driver'. The driver cDNA is subtracted from the tester cDNA by hybridising the two cDNA populations. The sequences from mRNAs that are expressed in both populations form hybrid molecules, which are then removed. Thus, the remaining transcripts represent differentially expressed genes specific to the tester, which are selectively amplified by PCR.

The SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, USA) used allows production of a large amount of cDNA from a small amount of mRNA and enriches for full-length cDNAs. First strand synthesis is primed by a modified oligo dT primer (figure 5.1.1). When the reverse transcriptase (RT) enzyme reaches the 5' end of the mRNA, a few additional nucleotides, particularly deoxycytidine, are added to the 3' end of the cDNA by the enzyme. These additional nucleotides base pair with the SMART oligonucleotide, which has an oligo dG sequence at the 3' end, thus

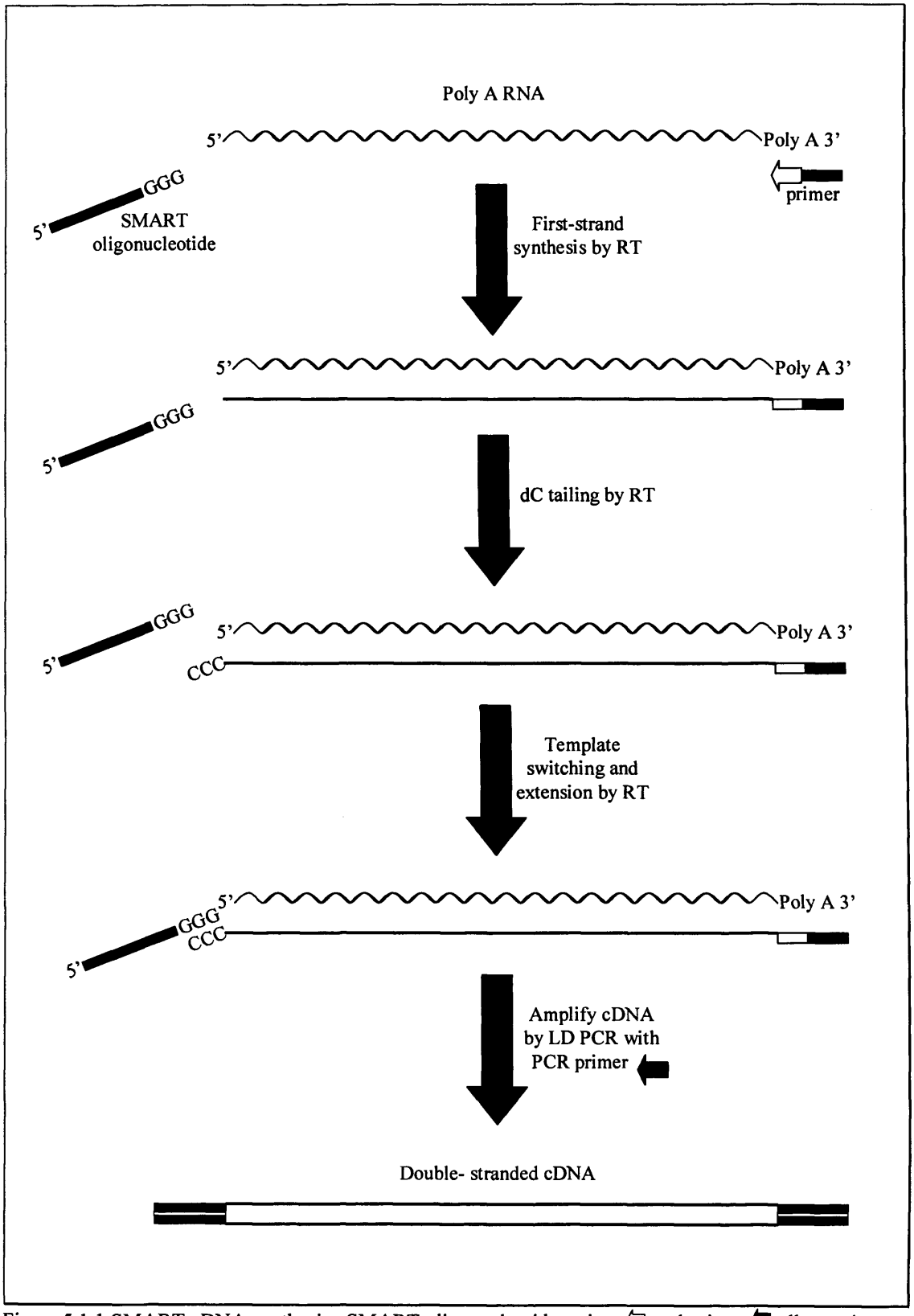


Figure 5.1.1 SMART cDNA synthesis. SMART oligonucleotide, primer⇦ and primer ⬅ all contain a stretch of identical sequence.

extending the mRNA template. The RT switches template and continues to replicate to the end of the oligonucleotide (figure 5.1.1). The resulting ss cDNA molecules are full-length copies of the original mRNAs with 5' oligo A sequences and 3' sequences complementary to the SMART oligonucleotide. Should the RT not transcribe the entire mRNA sequence, perhaps due to its length or persistent secondary structure, the addition of the deoxycytidine nucleotides is much less efficient, thus preventing base pairing with the SMART oligonucleotide. The SMART oligonucleotide sequence and the poly A sequence serve as primer sites for long distance PCR (LD PCR) amplification of the full-length ds cDNAs. DNAs lacking these sequences, such as contaminating genomic DNA or cDNAs resulting from incomplete RT activity, or transcription from non-polyA RNA, will therefore not be amplified exponentially. This methods was used to make both the tester and driver cDNAs.

Using the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA, USA), both tester and driver cDNAs are digested with *Rsa* 1, a four base cutting restriction enzyme yielding blunt ends. (Both the SMART oligonucleotide and the modified poly-A primers have an *Rsa* 1 restriction site, to facilitate removal of these identical sequences from the cDNA molecules before subtraction). The tester is divided in two, and each portion ligated with a different cDNA adaptor (figure 5.1.2). The ends of the adaptors lack a phosphate group, so only one strand of each adaptor can attach to the 5' end of the cDNA. Both adaptors have stretches of identical sequence, which allow annealing of the PCR primer once the recessed ends have been filled in. The driver cDNA has no adaptors ligated to it. Two rounds of hybridisations are performed. In the first, an excess of driver is added to each sample of tester. The samples are heat denatured and allowed to anneal, generating four types of single-stranded or hybrid molecules: types a: single stranded tester, b: double stranded tester hybrid, c: tester and driver cDNA hybrids, and d: single stranded or double stranded hybrids of driver cDNAs (figure 5.1.2). The single-stranded type a molecules are significantly enriched for differentially expressed sequences, as cDNAs that are not differentially expressed form type c associations with the driver. Reannealing to form type b molecules is more efficient between the more abundant cDNAs, so the concentration of high- and low- abundance sequences is equalised within the type a molecules. Driver cDNA molecules may also reanneal, forming type d molecules. During the second hybridisation step, the two primary hybridisation samples are

mixed together, without denaturing, in the presence of fresh denatured driver (figure 5.1.2). The addition of an excess of driver further enriches for differentially expressed sequences and the mixing of the two initial hybridisations allows the equalised and subtracted type a single-stranded tester cDNAs to reassociate, forming new, type e molecules: hybrids of tester cDNAs from each of the two ligation reactions (figure 5.1.2). These new hybrids are double-stranded tester molecules with a different adapter sequence ligated to each end. The DNA polymerase fills in the ends of the molecules, and the entire population of molecules is subjected to PCR using nested primers, which anneal to different sequences on the two adaptors, to amplify the differentially expressed sequences (type e). Type d molecules will not be amplified as they lack any primer annealing sites. The suppression PCR effect (figure 5.2.3) causes type b molecules, which have complementary sequences on either end of the ss DNA, to form pan-like secondary structures, thus preventing primer annealing, and therefore, exponential amplification. Occasionally, a primer will anneal and extend, but the resulting strand will also have inverted terminal repeats, and so will also preferentially form pan-like structures. Type a and c molecules will be amplified linearly as they only have one primer annealing site. Only type e molecules, which have two different adaptors, can be amplified exponentially. These are the equalised, differentially expressed sequences. As the 5' ends of both adaptors have an identical stretch of 22 nucleotides, a slight suppression PCR effect is introduced for the type e molecules (figure 5.1.3). However, since the identical sequences are the same length as the PCR primer, the suppression effect becomes significant only for very short cDNAs (under 200 nucleotides), as the formation of pan structures for these short molecules is more efficient. This enrichment for longer molecules balances the inherent tendency of the subtraction procedure to favour short cDNA fragments, which are more efficiently hybridised, amplified and cloned than longer fragments. A secondary PCR reaction using nested primers further reduces background and enriches for differentially expressed sequences.

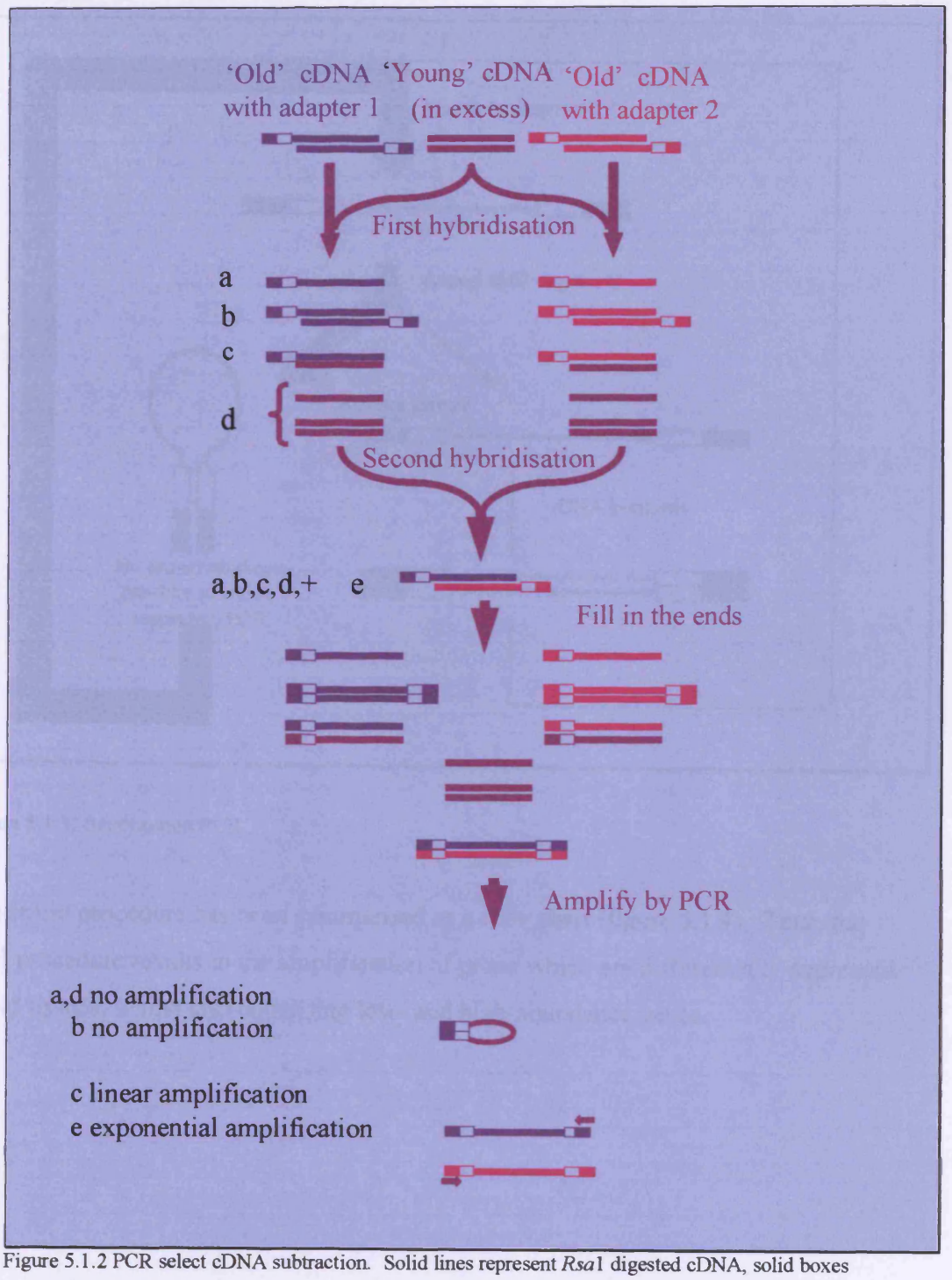


Figure 5.1.2 PCR select cDNA subtraction. Solid lines represent *Rsa*1 digested cDNA, solid boxes represent the outer part of the adaptor- sequence corresponding to PCR primer 2, white boxes represent inner part of the adaptor-sequence corresponding to nested PCR primers

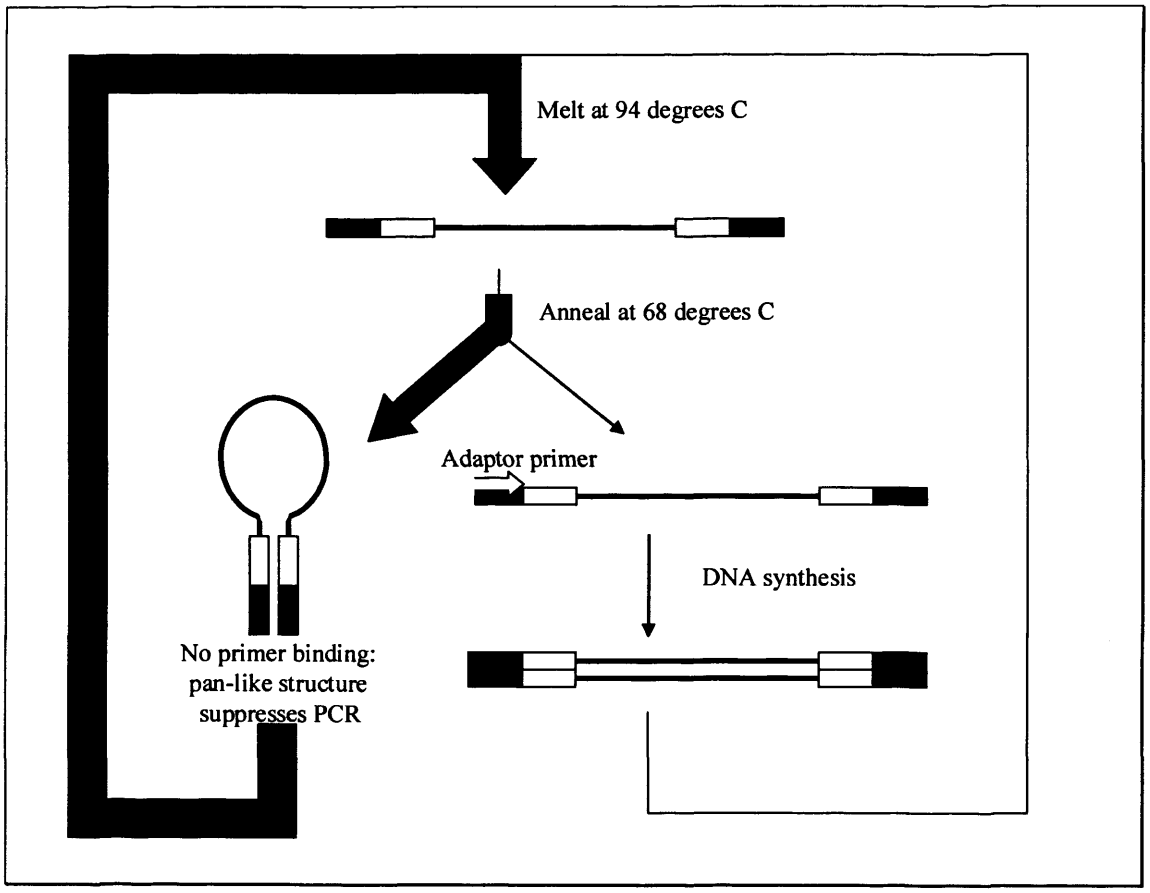


Figure 5.1.3 Suppression PCR

The entire procedure has been summarised as a flow chart (figure 5.1.4). Thus, the SSH procedure results in the amplification of genes which are differentially expressed in old tissues, whilst also equalising low- and high-abundance genes.

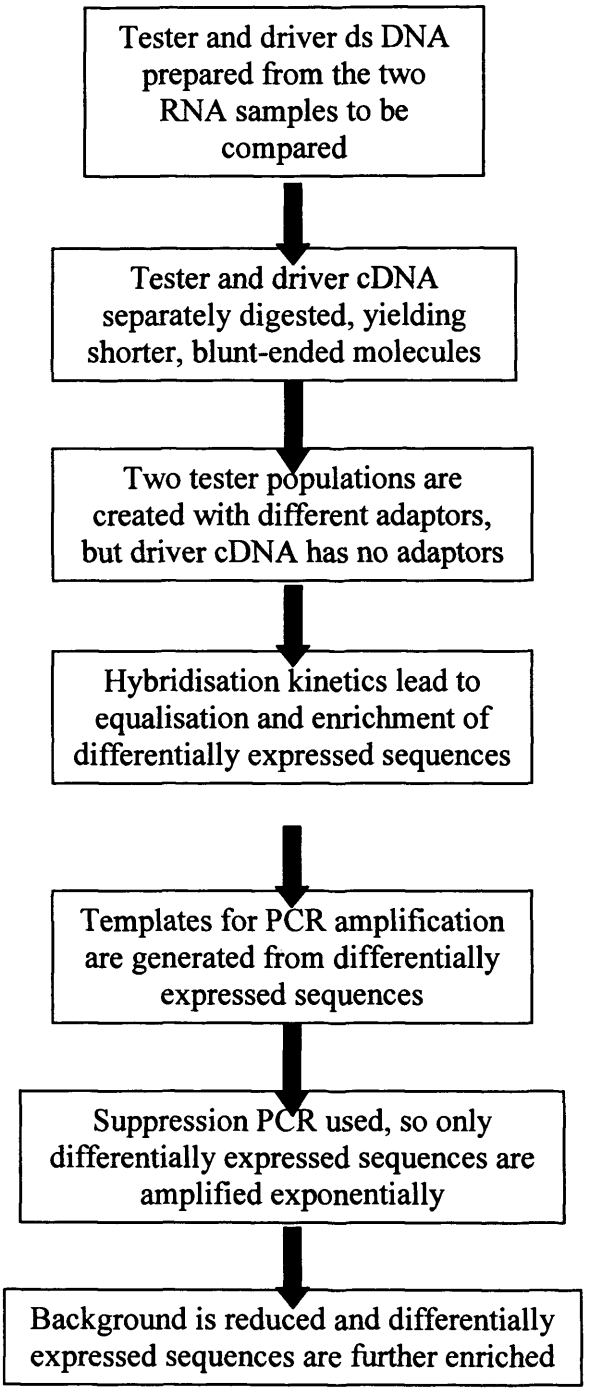


Figure 5.1.4 Summary of the SSH procedure.

5.2 Materials and Methods

Total RNA was extracted from leaves and petals of each developmental stage, using the Tri reagent extraction method (section 2.2.3). Equal amounts of RNA from petal stages –2, -1 and 0 were mixed to give young petal RNA and RNA from petal stages

3, 4 and 5 were combined to give old petal RNA. Leaf stage 3 was used as young leaf RNA and RNA from leaf stages 5 and 6 were mixed and used as old leaf RNA (section 3.3.1 defines stages of petal and leaf development). These four samples were converted into cDNA using the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA), following the manufacturers' protocol. All incubation steps and PCR reactions were carried out in a Perkin Elmer 2700 thermocycler, unless otherwise indicated. Incubations at sub-ambient temperatures were performed in a cooled waterbath or a refrigerator (4°C). All centrifugation steps were carried out in a Beckman Coulter Allegra 2IR microcentrifuge with a F2402H rotor.

5.2.1 SMART cDNA synthesis

For the first strand synthesis, 1µg of each total RNA sample was incubated with 2µM modified poly A primer (5'-AAGCAGTGGTATCAACGCAGAGTAC$T_{(30)}N_{-1}$N-3', where N = A, C, G or T; and N_{-1} = A, G or C) and 2µM SMART oligonuclotide (5'-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3', where underlined sequence = *Rsa*1 restriction site) in a volume of 5µl at 72°C for 2 mins then on ice for 2 mins, before addition of the 1st strand buffer, 2mM DTT, 1mM dNTPs and 1 unit of PowerScript RT enzyme, giving a total reaction volume of 10µl, and was further incubated at 42°C in an air incubator for one hour. After completion of first strand synthesis, the samples were placed on ice, diluted 5X with TE buffer (10mM Tris, 1mM EDTA, pH 7.6) and the reaction was terminated by heating to 72°C for 7 mins.

Second strand synthesis and cDNA amplification was done by LD PCR. Two 100µl reactions were performed for each sample, to be combined, as well as an additional tube for optimising the cycle numbers, to achieve the maximum number of cycles whilst still in the exponential phase of the amplification, thus achieving maximum yield, whilst minimising non-specific product formation. 1µl of single-stranded cDNA was used in each 100µl reaction with 0.2µM 5' PCR primer II A (5'-AAGCAGTGGTATCAACGCAGAGT-3' and, 0.2mM dNTPs, using the buffer and Advantage 2 Polymerase mix (a combination of a primary polymerase and a proofreading polymerase, leading to improved amplification over long distances) from the Advantage 2 PCR Kit (Clontech, Palo Alto, CA, USA). After 1 minute at 95°C, the samples were cycled at 95°C for 5 seconds, 65°C for 5 seconds, 68°C for 6 mins. All reactions were left for 15 cycles, at which time all reactions apart from the

cycle number optimisation reactions for each sample were removed from the thermocycler and stored at 4°C. A 15µl aliquot was removed from the optimisation reactions at 15, 18, 21 and 24 cycles and visualised on an ethidium bromide gel to establish after how many cycles the exponential phase ended and the plateau was reached. Once the optimal number of cycles was established, the remaining reactions were returned to the thermocycler for the appropriate number of cycles. The reactions were terminated by the addition of 2µl of 0.5M EDTA and combined.

5.2.2 Column chromatography

The PCR products were cleaned by extraction with one round of phenol: chloroform: isoamyl alcohol (25:24:1) and concentrated by butanol extraction (as described in sections 2.2.4 and 2.2.7). The samples were further cleaned using CHROMA-SPIN-1000 columns (Clontech, Palo Alto, CA, USA) according to the manufacturers' instructions, removing salts, solvents, nucleotides and enzymes by gel filtration chromatography.

5.2.3 *Rsa*1 digestion

The cleaned samples (375µl) were digested using 10 units of *Rsa*1 in *Rsa*1 restriction buffer (Clontech, Palo Alto, CA, USA) in a total reaction volume of 405µl for 3 hours at 37°C. The reaction was terminated by the addition of EDTA to a final concentration of 10mM.

5.2.4 Purification of digested cDNA

The digested cDNA was purified using the NucleoTrap PCR Kit (Clontech, Palo Alto, CA, USA) according to the manufacturers' instructions. The DNA binds to the NucleoTrap silica matrix in the presence of chaotropic salts, allowing impurities to be washed away before eluting the DNA in TE buffer (10mM Tris [pH 8], 1mM EDTA). Microfiltration removed any traces of the matrix suspension from the eluate, before precipitating by the addition of 0.5 volumes of 4M ammonium acetate and 2.5 volumes of 95% ethanol and incubating at room temperature for 20 mins. The cDNA was pelleted by centrifugation at 14 K rpm for 20 mins, the pellet was ethanol washed (as described in section 2.2.6) and resuspended in 6.7µl of TNE buffer (10mM Tris [pH 8], 10mM NaCl, 0.1mM EDTA). Repeating the elution of the DNA from the NucleoTrap silica matrix was found to significantly increase yield.

5.2.5 Choosing the primers for the ligation efficiency analysis

Before the tester samples (old leaf and old petal) were split into two halves and different adaptors ligated to the 5' ends of the cDNA molecules (section 5.2.6), primers needed to be chosen for the ligation efficiency analysis, which tests the efficiency with which these adaptors ligated to the cDNAs (described in section 5.2.6). Degenerate primers to amplify a 323 bp fragment of the β tubulin genes (*Pos 63*: 5'-ATGAGYGGYGTSACSTGCT-3', where Y=C/T and S=C/G; *Neg 2*: GTAGGANGAGTTCTTGTTCTG, where N=A/C/G/T; Rogers *et al.*, 1993) and primers designed to *Arabidopsis thaliana* and *Brassica napus* to amplify a 344bp fragment of the *SAG12* gene (*SAG12 F*: 5'-TTGCCGGTTTCTGTTGAYTGG-3'; *SAG12 R*: 5'-TGGTGTGCCACTGCYTTCAT-3', where Y = C/T) were trialled for this purpose. The initial PCR reactions to establish the primer set most suitable for the ligation efficiency PCR used 0.625 units of Taq polymerase (Qiagen Ltd., Crawley, UK), Qiagen buffer, 10ng of the appropriate cDNA (young petal, old petal, young leaf, old leaf), 1.5mM $MgCl_2$, 0.2mM dNTPs and 0.4mM each of the two primers (either *SAG12 F* and *SAG12 R* or *Pos 63* and *Neg 2*). Reactions were cycled in a Perkin Elmer 2700 thermocycler under the conditions 15 mins 95°C hotstart, {95°C 10 seconds, 50°C 30 seconds, 68°C 2.5 mins} for the tubulin primers, and 15 mins 95°C hotstart, {95°C 10 seconds, 65°C 30 seconds, 68°C 2.5 mins} for the *SAG12* primers. Tubes were removed from the thermocycler at 18, 24, 30, 36 and 42 cycles and the products were analysed on an ethidium bromide stained agarose gel (as described in section 2.2.10).

5.2.6 Adaptor ligation

Each of the tester (old leaf and old petal) samples were spilt in two, each half having a different adaptor ligated to the 5' end of the *Rsa*1 cut molecules- adaptor 1 and adaptor 2R (figure 5.2.1). 2μM of the appropriate adaptor was used per ligation reaction, along with 200ng tester cDNA and 400 units of T4 DNA ligase in ligation buffer (Clontech, Palo Alto, CA, USA) in a 10μl reaction volume. A small amount of each pair of ligation mixtures was combined, as an unsubtracted tester control (to be used in step 5.2.9). The ligation was allowed to proceed overnight at 16°C and the reaction was terminated by the addition of 1μl of 0.2 M EDTA/ 1mg/ml glycogen mix and heating to 72°C for 5mins.

The efficiency of the ligation reaction was determined by means of 2 similar PCR reactions run side by side. In one, a fragment spanning the adaptor/ cDNA junction of each ligated tester sample was amplified and in the other, an internal fragment of the cDNA was amplified. Comparison of the intensities of these PCR products on an ethidium bromide stained agarose gel gives an indication of the efficiency of ligation. The *SAG12* primers were used for the PCR reactions as this gene is known to be strongly expressed in old tissue, whereas tubulin levels fall in old tissue, and thus *SAG12* should be easily detectable in the tester cDNA samples. The PCR reactions used 10ng of ligated tester cDNA, 0.4μM of each primer (cross junction: *SAG12 R* and PCR primer 1 [figure 5.2.1]; internal: *SAG12 R* and *SAG12 F*), 0.2mM dNTPs in a 25μl reaction. The adaptors were extended by incubation at 75°C for 5 mins followed by 30 cycles of {94°C 10 seconds, 65°C 30 seconds, 68°C 2.5 mins}. The products were analysed on an ethidium bromide stained agarose gel (as described in section 2.2.10) to compare the intensities of the bands produced in the cross junction and the internal reactions.

5.2.7 Hybridisation

For the first hybridisation, an excess of *Rsa*1 digested driver cDNA (1.5μl) was added to 1.5μl of both the tester ligated to adaptor 1 and to the tester ligated to adaptor 2R separately in the presence of the supplied hybridization buffer, denatured at 98°C for 1.5 mins and incubated at 68°C for 8.5 hours. The second hybridisation immediately followed the first. The two first hybridisation samples were simultaneously mixed in the presence of fresh denatured driver and hybridisation buffer. To do this, a mixture of 1μl of fresh driver in buffer was prepared, then one of the hybridisation samples was drawn into a pipette tip, a small amount of air was drawn into the tip and then the fresh driver and buffer mixture, so that the pipette tip contained both samples separated by a pocket of air. The contents of the tip was transferred into the tube containing the other hybridisation sample, mixed by pipetting and incubated overnight at 68°C (without denaturing the mixture). This ensures that the two hybridisation samples and the fresh driver are mixed simultaneously. The samples were diluted with 200μl of dilution buffer, giving a total volume of 212μl, and kept at 68°C for a further 7 mins.

5.2.8 PCR amplification

The differentially expressed cDNAs (type e molecules) were selectively amplified by PCR, using 1µl of the diluted subtracted cDNAs (or unsubtracted tester control) as a template for the 25µl reaction, PCR primer 1 at a concentration of 0.4 µM (which binds to both adaptor 1 and 2R; figure 5.2.1) and 0.2 mM dNTPs, using the buffer and Advantage 2 Polymerase mix from the Advantage 2 PCR Kit (Clontech, Palo Alto, CA, USA). The adaptors were extended by incubation at 75°C for 5 mins, followed by a 94°C incubation for 25 seconds and 27 cycles of {94°C 10 seconds, 66°C 30 seconds, 72°C 1.5 mins}.

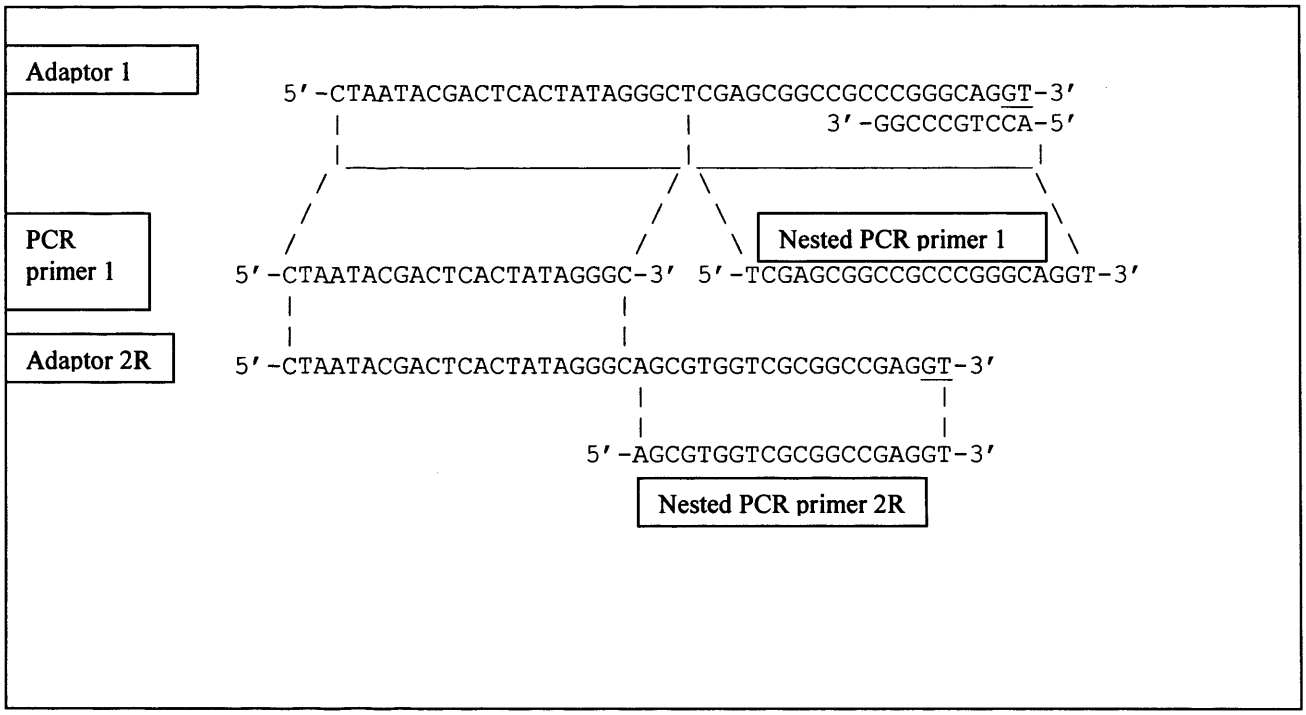


Figure 5.2.1. Sequences of the PCR –Select cDNA synthesis primer, adaptors and PCR primers. When the adaptors are ligated to *Rsa*1-digested cDNA, the half *Rsa*1 site (underlined on the 3' end of both adaptors) is restored, allowing easy removal of the adaptors before the hybridisation step of the subtraction.

A second, nested PCR was performed to further reduce background and to enrich for differentially expressed sequences, using 1µl of a 1/10 dilution of the first PCR as a template in a 25µl reaction, with nested PCR primers 1 and 2R (specific to adaptors 1 and 2R, respectively- figure 5.2.1) at a concentration of 0.4µM each, dNTPs at 0.2mM and using the buffer and Advantage 2 Polymerase mix from the Advantage 2 PCR Kit

(Clontech, Palo Alto, CA, USA), incubated for 12 cycles of {94°C 10 seconds, 68°C 30 seconds, 72°C 1.5 mins}.

5.2.9 Transformation and cloning

These differentially expressed sequences were cleaned using the QIAquick PCR purification kit (Qiagen Ltd., Crawley, UK) (as described in section 2.2.14), concentrated by precipitation with 1.5 volumes of 4M ammonium acetate and 6.5 volumes of 95% ethanol, and incubated at -20°C overnight. The cDNA was pelleted by centrifugation at 14 K rpm for 20 mins, the pellet was ethanol washed (as described in section 2.2.6) and resuspended in 10µl of sdw. A 3:1 ratio of insert: vector was used for ligation into pGEM Easy T cloning vector (Promega UK Ltd., Southampton, UK), thus 0.5µl of vector was used for each reaction with 1µl of petal SSH product and 3µl of leaf SSH product in a 10µl total reaction volume. The ligated vectors with their inserts were transformed into *E. coli* JM109 cells (Promega UK Ltd., Southampton, UK) and spread onto LB amp100 plates with 50mM IPTG and 2% X-gal for blue/ white selection (as described in sections 2.2.17 and 2.2.18). As each colony has originated from a single bacterium, each will contain a different insert in its plasmid vector. Individual white colonies were picked and grown as liquid cultures in 96 well plates LB amp100 and stored at -80°C as glycerol stocks (as described in section 2.2.18).

5.2.10 Sequencing of clones

A random selection of 16 clones were chosen for sequencing. The plasmids were isolated from the bacteria, sequenced, and analysed (as described in sections 2.2.19 and 2.2.15).

5.2.11 Isolation of partial tubulin cDNA from wallflower

Despite the fact that the tubulin primers were not suitable for the ligation efficiency control, they did work well on wallflower cDNA, and so a PCR reaction was performed to amplify a partial cDNA of wallflower tubulin. Total RNA was extracted using the Tri reagent extraction method and DNAse treated (as described in sections 2.2.3 and 2.2.11) and mixed as described above to give young petal, old petal, young leaf and old leaf RNA samples. cDNA was made as described in section 2.2.12. Both

the cDNA and the controls lacking the RT enzyme were used to set up PCR reactions to amplify the tubulin fragment (as described in section 5.2.5).

The PCR products from the tubulin leaf reactions were combined and the tubulin petal reaction products combined and cleaned using the QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK), eluting the DNA in 30µl of EB buffer (Qiagen Ltd, Crawley, UK) (as described in section 2.2.14). The cleaned PCR product was ligated into Promega P-GEM Easy T cloning vector and transformed into *E.coli* JM109 cells (as described in section 2.2.17 and 2.2.18). The bacteria were spread onto LB amp100 plates with 50mM IPTG and 2% X-gal for blue/ white selection. Four white colonies from each plate (leaf and petal) were picked and grown as liquid cultures in LB amp100 and stored at – 80°C as glycerol stocks. These eight colonies were used to set up PCR reactions to establish the size of the insert, using 0.625 units of HotStarTaq polymerase (Qiagen Ltd, Crawley, UK), Qiagen buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs and 0.4mM each of M13F and M13R primers in 100µl reactions. Reactions were cycled in a Perkin Elmer 2700 thermocycler under the conditions: 15 mins 95°C hotstart, {95°C 1 minute, 58°C 1 minute, 72°C 2 mins} and a final extension at 72°C for 5 mins. The plasmid was extracted from one of each of the petal and leaf clones using the QIAquick miniprep kit (Qiagen Ltd, Crawley, UK), according to the manufacturer's instructions, with the DNA eluted in 30µl of warm EB buffer and the insert was sequenced (as described in sections 2.2.19 and 2.2.15).

Clones from both the leaf and petal SSH libraries were differentially screened by arraying the clones and hybridising them with cy 3 and cy 5 labelled probes of both tester and driver cDNA. Clones recognised by the tester probe but not the driver probe were confirmed as differentially expressed (chapter 6).

5.3 Results

A series of controls were performed during the SSH procedure, as recommended by the manufacturers, to ensure that each of the steps had worked correctly.

5.3.1 SMART cDNA synthesis

The LD PCR step of the SMART cDNA synthesis was performed, taking out aliquots every 3 cycles between 15 and 24 cycles, to find the optimum number – to ensure that the double-stranded cDNA remains in the exponential stage of amplification (figure 5.3.1.1). Overcycled cDNA is a poor template for subtraction, whereas undercycling results in a poor yield. It was recommended to use one cycle less than needed in order to reach the plateau. 17 cycles proved to be optimal for each of the samples with the exception of the old leaves, where 19 cycles was optimal (figure 5.3.1.1).

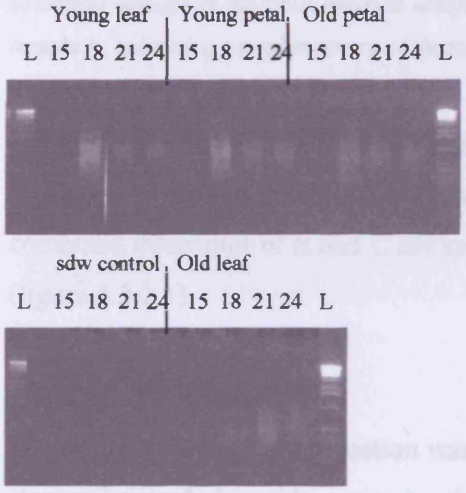


Figure 5.3.1.1. Optimisation of cycle number for LD PCR (second strand SMART cDNA synthesis). The plateau is reached at 18 cycles for the young petal, old petal and young leaf samples, and thus 17 cycles proving optimal, and at 21 cycles for the old leaf samples, and so 19 cycles were optimal.

5.3.2 Column chromatography

To ensure maximum recovery of DNA after purification by column chromatography, a sample of unpurified cDNA (sample A) was electrophoresed alongside two purified samples: early (sample B) and late (sample C) column eluate, on an ethidium bromide stained agarose gel. A sample of very early eluate (sample 15) was also run, to ensure that no DNA eluted from the column prematurely (figure 5.3.2.1).

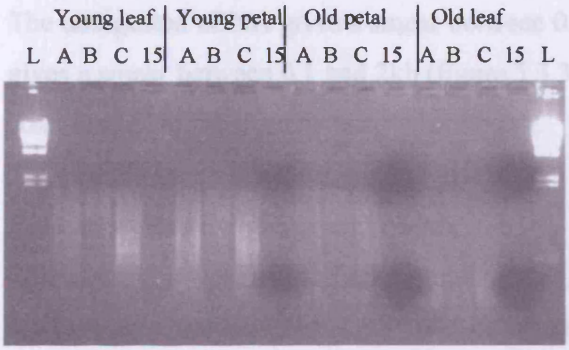


Figure 5.3.2.1 DNA sample before column chromatography (A), very early eluate from column (15), early eluate from column (B), late eluate from column (C). DNA was eluted in samples B and C. The combined strength of the DNA bands in samples B and C compares favourably to the band strength in sample A, indicating a good recovery of cleaned DNA from the column.

DNA was present in both fractions B and C of the eluate, so these fractions were combined to continue with the procedure. Nothing was present in fraction 15. The combined intensities of B and C are similar to the intensity of A, indicating little loss (figure 5.3.2.1).

5.3.3 *Rsa*1 digestion

To confirm that the *Rsa*1 digestion was successful, a sample of uncut cDNA was electrophoresed alongside a sample of digested cDNA on an ethidium bromide stained agarose gel (figure 5.3.3.1).

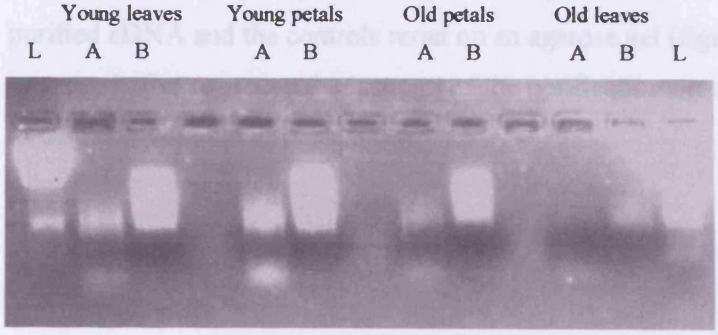


Figure 5.3.3.1 Comparison of cDNA before (B) and after (A) *Rsa*1 digestion. The sizes of the bands are similar to the expected sizes quoted in the manufacturer's handbook and show the digested cDNA to be smaller in size than the undigested, as would be expected.

The undigested cDNA gives a smear between 0.5 and 3kb and the digested cDNA gives a smear between 0.1 and 2kb (figure 5.3.3.1). This agrees with the expected sizes quoted in the manufacturer's handbook.

5.3.4 Purification of digested cDNA

To analyse the yield of purified digested cDNA, equivalent amounts of the following samples were run alongside each other on an ethidium bromide stained agarose gel: *Rsa*1 digested cDNA before purification, purified diluted cDNA before ethanol precipitation, and purified cDNA after ethanol precipitation (figure 5.3.4.1).

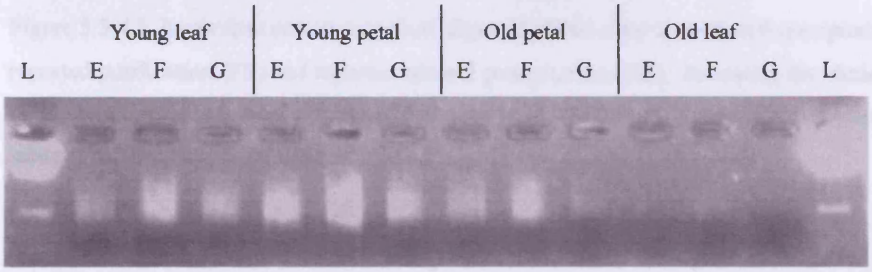


Figure 5.3.4.1 Equivalent amounts of *Rsa*1 digested cDNA before purification (E), after purification (F) and after ethanol precipitation (G). No cDNA is lost after purification, but yield after ethanol precipitation is poor.

DNA yield after ethanol precipitation was poor. The previous step in the procedure was repeated. The pellet of NucleoTrap purification matrix and was reeluted, and the microfiltration and precipitation steps were repeated. This was added to the original purified cDNA and the controls reran on an agarose gel (figure 5.3.4.2). Yield greatly improved after repeating the last part of the purification procedure and was sufficient to continue with the SSH protocol.

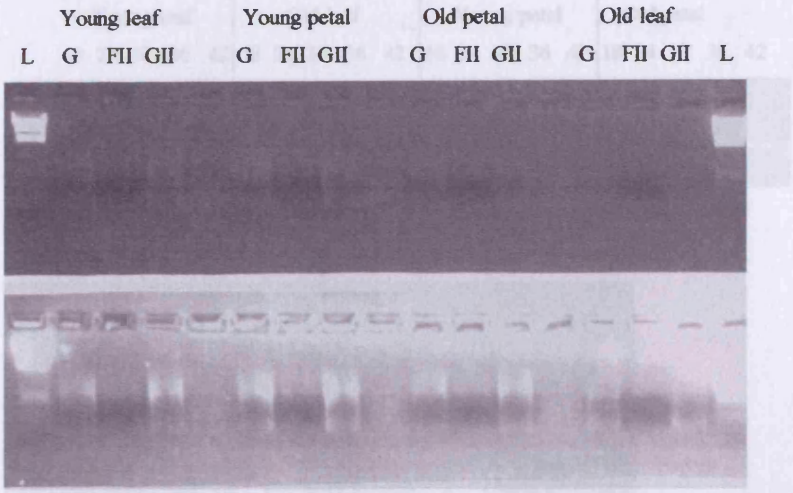


Figure 5.3.4.2 Equivalent amounts of *Rsa*1 digested cDNA after first ethanol precipitation (G), repeated purification (FII) and repeated ethanol precipitation (GII). Repeating the elution and precipitation steps increased cDNA yield dramatically. Both images represent the same gel, but were taken at different exposure times.

5.3.5 Choosing primers for the ligation efficiency analysis

To analyse the efficiency of the ligation, a PCR reaction was run to verify that at least 25% of the cDNAs had adaptors on both ends. Both tubulin and *SAG12* primers were trialled for this purpose. Comparison of the results of the trial tubulin and *SAG12* PCRs show that *SAG12* expression is much stronger in old tissues than tubulin expression, making these the most suitable primers to use for the ligation efficiency analysis (figure 5.3.5.1).

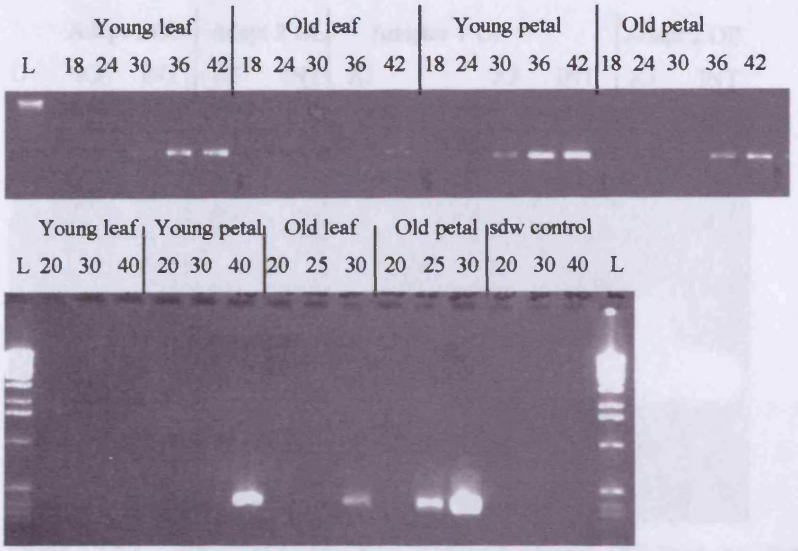


Figure 5.3.5.1 Comparison of tubulin (top) expression and *SAG12* (bottom) expression in old leaves and petals at different cycle numbers. *SAG12* is expressed strongly in both old leaves and old petals, whereas tubulin is expressed only weakly in old tissues.

5.3.6 Adaptor ligation

Two similar PCR reactions were run, one amplifying a fragment spanning the adaptor/cDNA junction of the testers ligated to one of the adaptors, and the other amplifying a fragment within the tester cDNA (resulting in a slightly smaller band), and the intensities of the bands produced by both reactions when run on an ethidium bromide stained agarose gel were compared (figure 5.3.6.1). *SAG12* primers were chosen for the internal PCR, as this gene is strongly expressed in old tissues, and so should be abundant in the tester cDNA. For the cross junction PCR, one of the *SAG12* primers (R) was used together with a primer designed to bind to the adaptor.

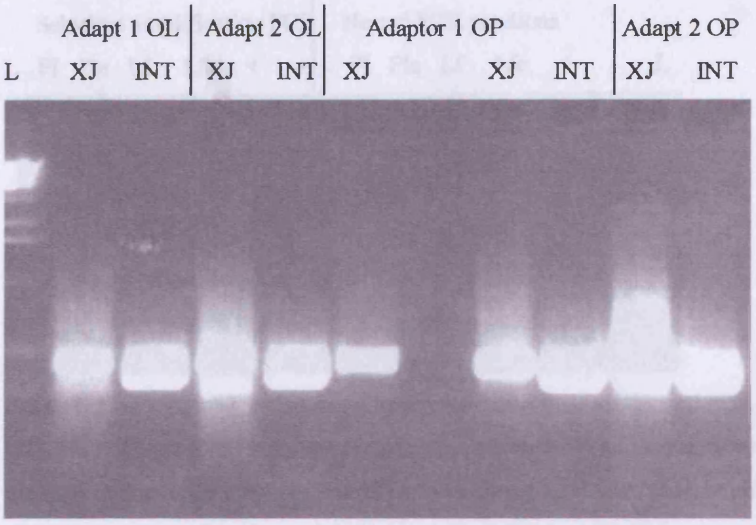


Figure 5.3.6.1 Ligation efficiency PCR. The cross-junction (XJ) bands are slightly larger in size and of similar intensity to the internal (int) bands for both petal tester samples, indicating good ligation efficiency. The int bands are stronger than the XJ bands in the leaf tester samples, showing those ligation reactions to be less efficient.

In both petal tester samples, the intensity of the internal band and the cross junction band were similar, indicating good ligation efficiency. In the leaf tester samples, the internal bands were stronger than the cross junction band in both cases, indicating that these ligations were not as successful as the petal ones. However, the cross junction band was in excess of 25% of the intensity of the internal band, which was sufficient to continue the procedure.

5.3.7 Hybridisation

There were no control steps for the two hybridisation reactions

5.3.8 PCR amplification

The final control step was to analyse the products of the selective amplification PCR and nested PCR reactions, which amplified the differentially expressed cDNAs. Aliquots of each sample PCR and the subsequent nested PCR and of each unsubtracted tester control PCR and associated nested PCR were run alongside each other on an ethidium bromide stained agarose gel (figure 5.3.8.1).

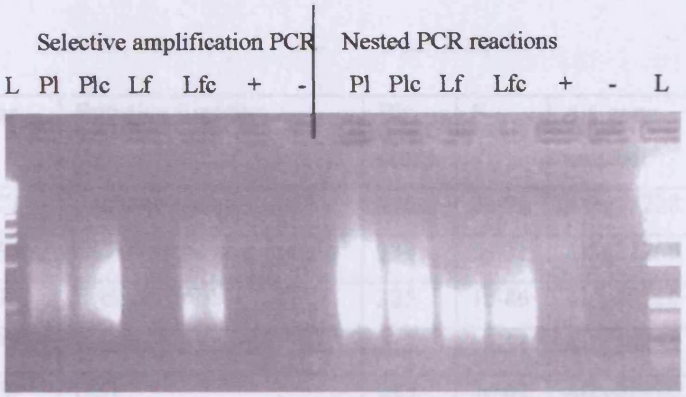


Figure 5.3.8.1 Samples of the selective amplification PCR and nested PCR amplifications for petal (Pl), leaf (Lf), petal and leaf controls (Plc/Lfc), positive (+) and negative (-) controls. The thickness of the band is due to the amplification of many different cDNAs of differing sizes.

The bands of the nested PCRs were much stronger than those of the corresponding selective amplification PCR, indicating that both PCRs worked well. After the selective amplification PCR, the bands of the unsubtracted tester controls (c) were considerably brighter than those of the subtracted samples, as described in the manufacturer's handbook, and as might be expected. However, after the nested PCR, the difference in brightness became less marked (figure 5.3.8.1). The PCRs worked well, indicating that the subtraction was successful and thus that the differentially expressed cDNAs are suitable for cleaning, ligation into a plasmid vector and transformation into *E. coli*.

5.3.9 Transformation and cloning

After plating the transformed bacteria onto LB plates with blue white selection, all of the white colonies were picked and grown as liquid cultures in 96 well plates. 1018 colonies were obtained from the leaf subtraction and 614 from the petal subtraction, giving a total of 1632 colonies and therefore 1632 putatively differentially expressed sequences.

5.3.10 Sequencing of clones

The results of searching the BLAST database with the sequences of the 16 randomly chosen clones are summarised in table 5.3.10.1.

Clone	Putative function	Bit score	E	*A.t.* gene number	Molecular weight	DNA homology	Protein homology
PL C1	Pectinesterase	276	2e-78	At2g45220	55976.8	90.8	86
LP H9	CCR4-associated protein	138	6e-31	At5g22250	32168.7	-	-
P1 D11	Pectinesterase	325	1e-86	At2g45220	55976.8	92.8	86
P1 F4	Peptide transporter	217	1e-54	At1g32450	67761.4	93.4	100
P1 F8	GST	243	3e-62	At2g02930	24120.7	91.8	83
P1 F10	GST	79	1e-17	At1g02920	23598.2	72.1	89
P2 B1	Lipid transfer protein	96.4	1e-31	At3g22600	17305.6	93.4	-
P5 B10	Heat shock protein	177	4e-45	At5g12020	17623.4	89.6	98
P4 D6	*Sen1*	155	6e-36	At4g35770	20078.8	83.9	84
P6 C7	Cytochrome P450	159	5e-37	At3g26200	57033.4	-	-
L1 B4	Inositol polyphosphate 5-phosphatase	149	8e-40	At1g47510	37250.8	90.2	98
L1 B5	Bifunctional nuclease	36.8	3	At1g11190	34884.8	-	-
L1 F6	Ribosomal protein S14	200	3e-42	-	-	-	-
L1 H3	γ glutamyltransferase	59.7	1e-12	At4g29210	69148.6	80.4	-
L2 D8	No match found	-	-	-	-	-	-
L4 F4	No match found	-	-	-	-	-	-

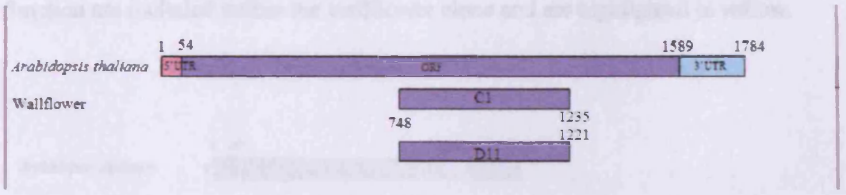
Table 5.3.10.1 Table summarising the results of searching the BLAST database with the sequenced clones. The plate reference of the clone is given, along with a putative function assigned based on the *Arabidopsis thaliana* gene with which the clone had highest homology. The bit score relates to the number of bases that were matched, accounting for gaps in the sequence that may affect the likelihood of a match (NCBI, 2003*a*). The E value is the Expect, and is a statistical analysis of the likelihood of the sequence being matched purely by chance. The lower this value is, the more statistically valid the match (NCBI, 2003*b*). The *A.t.* (*Arabidopsis*) gene number is an unique identifier for each *Arabidopsis* gene. The number following the prefix At indicates which chromosome the gene is located on in *Arabidopsis*. The molecular weights are those of the predicted protein for the *Arabidopsis* gene, obtained from their respective Target P web page (Emanuelsson and Rapacki, 2003). The % similarity of the wallflower gene to *Arabidopsis thaliana* at the DNA level (DNA homology) was calculated by megalign (DNASTAR, Madison, WI) for the sequenced clone from wallflower aligned against only the corresponding section of the *Arabidopsis* gene. The % similarity of the wallflower gene to *Arabidopsis thaliana* at the protein level (protein homology) was calculated by BLAST for the translation of the sequenced clone from wallflower aligned against only the corresponding section of the *Arabidopsis* protein.

Of the 16 genes sequenced, 14 returned a match on the BLAST database. Four of the 14, despite being a good enough match to identify a homologous gene (as searched by

tBLASTx), did not align well, and so the percentage homology at the DNA or protein level could not be calculated. A further 2 of the remaining 10 aligned to untranslated regions of their homologous genes, and so only DNA homology could be calculated. Six of the clones were more than 90% homologous at the DNA level to the corresponding *Arabidopsis* gene over the section sequenced, a further three were over 80% homologous and another one over 70% homologous. Similarly, a high degree of homology was also shown at the protein level, with all clones showing over 80% and three showing over 90% homology to the respective *Arabidopsis* protein. Each of the wallflower clones has aligned to a different *Arabidopsis* gene, with the exception of clones *PL C1* and *P1 D11*, both of which have aligned to the same pectinesterase gene (At2g45220).

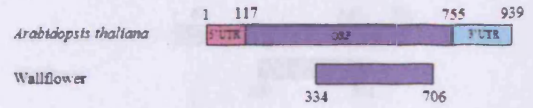
Each of the wallflower sequences was aligned to both the DNA and protein sequences of the corresponding *Arabidopsis* gene (figures 5.3.10.1 to 5.3.10.9) to establish which part of the gene the wallflower clone represented (graphic part of each figure) and the degree of protein-level similarity (text part of each figure). The numbers on the graphic indicates the nucleotide position on the *Arabidopsis* gene. Four of the wallflower clones: two pectinesterase clones (*PL C1* and *P1 D11*) aligning to the same area of the same gene (figure 5.3.10.1) and the two clones with homology to two GST genes (*P1 F8* and *P1 F10*; figures 5.3.10.2 and 5.3.10.3, respectively) included key amino acids of functional significance. This strongly suggests that these genes are functional homologues of the *Arabidopsis* genes, performing similar functions in wallflower. Clone *P5 B10* showed 98% similarity to a putative class two 17.6 kilo dalton heat shock protein, termed *At* HSP 17.6-II, over a large portion of the ORF, which includes the 100 amino acids at the 3' end (figure 5.3.10.4), which are known to be important for HSP function, again indicating that the wallflower protein probably performs a similar function to the *Arabidopsis* protein. The homology of clone *P1 F4* to the peptide transporter (figure 5.3.10.5) did not cover known functional regions of the protein, however, the homology was very high (100% similarity), strongly suggesting functional homology. Clone *P4 D6* shows 84% amino acid homology to the *Arabidopsis Sen1* gene over a large proportion of the ORF (figure 5.3.10.6), indicating a high likelihood of functional homology, although as the precise function of the *Sen1* gene remains to be elucidated, little is known about which amino acids have functional significance, and thus whether or not these are

included within the wallflower clone. Clone *L1 B4* aligns to a small proportion of the ORF of an *Arabidopsis* gene with homology to an inositol polyphosphate-5-phosphatase gene (figure 5.3.10.7). Again, little is known about the amino acids of functional significance in this gene, but the degree of homology at the amino acid level is high (98%), indicating a high probability of the wallflower and *Arabidopsis* clones having similar functions. Clones *P2 B1* and *L1 H3* show homology to the 3' UTR of an *Arabidopsis* lipid transfer protein and γ-glutamyl transferase, respectively (figures 5.3.10.8 and 5.3.10.9). Although DNA level homology is high, at 91.4% for *P2 B1* and 80.4% for *L1 H3*, the fact that both clones align to the 3' UTR makes it difficult to speculate on the functional homology between these two clones and their respective *Arabidopsis* homologues.



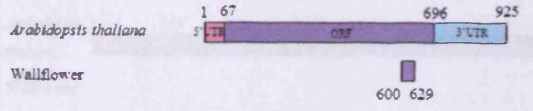

```
WF: 39   FVIYVKQGVYSENLEIRKKNVMLRGDGKGRXXXXXXXXXXXXXXXFNSATVAAVGDGFIA 218
         FVIYVKQGVYSENLEIRKKNVMLRGDG G+                FNSATVAAVGDGFIA
At: 750  FVIYVKQGVYSENLEIRKKNVMLRGDGIGKTIITGSKSVGGGTTTFNSATVAAVGDGFIA 929

WF: 219  RGITFRNTAGANNGQAVALRSGSDLSVFYQCSFEGYQDTLYVHSNRQFYRDCDVYGTVDF 398
         RGITFRNTAGA+N QAVALRSGSDLSVFYQCSFE YQDTLYVHSNRQFYRDCDVYGTVDF
At: 930  RGITFRNTAGASNEQAVALRSGSDLSVFYQCSFEAYQDTLYVHSNRQFYRDCDVYGTVDF 1109

WF: 399  IFGNAAAVLQNCNIFAR 449
         IFGNAAAVLQNCNIFAR
At: 1110 IFGNAAAVLQNCNIFAR 1160
```

Figure 5.3.10.1 Alignment of *PL C1* and *P1 D11* to the *Arabidopsis* pectinesterase gene. Both wallflower clones align to the same part of the *Arabidopsis* open reading frame (ORF), shown in lilac. The signature motif for pectinesterase enzymes is included within the wallflower clone, as is shown highlighted in yellow.



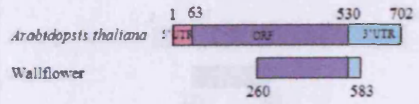

```
WF: 28  EYIAHRYENQGTNLLPADSKNVAQYATMAIGLEVESHQFDPVASKLAWEQIFKLIYGLTT 207
        +YIAHRYENQGTNLLPADSKN+AQYA M+IG++VE+HQFDPVASKLAWEQ+FK  YGL T
At: 333 QYIAHRYENQGTNLLPADSKNIAQYAIMSIGIQVEAHQFDPVASKLAWEQVFKFNYGLNT 512


WF: 208 DQXXXXXXXXXXXXXXDVYDARLEEFKYLAGDTFTLTDLHHIPVIQYLLGXPTKKLFTEX 387
        DQ              DVY+ARL+EFKYLAG+TFTLTDLHHIPVIQYLLG PTKKLFTE
At: 513 DQAVVAEEEAKLAKVLDVYEARLKEFKYLAGETFTLTDLHHIPVIQYLLGTPTKKLFTER 692


WF: 388 PHVS 399
        P V+
At: 693 PRVN 704
```

Figure 5.3.10.2 Alignment of *P1 F8* to the *Arabidopsis* GST gene. The wallflower clone aligns to a large proportion of the *Arabidopsis* ORF (shown in lilac). Important amino acid residues for GST function are included within the wallflower clone and are highlighted in yellow.




```
WF: 30  GTPTKKLFDARPHVSAWVADITSRPSSQKVL*VNQSV 140
        GTPTKKLFD RPHVSAWVADITSRPS++KVL*V   +
At: 601 GTPTKKLFDERPHVSAWVADITSRPSAKKVL*VTHKL 711
```

Figure 5.3.10.3 Alignment of *P1 F10* to the *Arabidopsis* GST gene. The wallflower clone aligns to a small proportion of the *Arabidopsis* ORF (shown in lilac). Important amino acid residues for GST function are included within the wallflower clone and are highlighted in yellow.

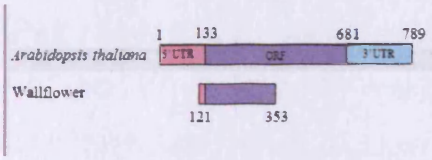



```
WF: 78   YAFVVDMPGIKGDEIKVQIENENVLVVSGGRKRESKENEGVKYVRMERRMGKFMRKFQLP 257
         YAFVVDMPGIKGDEIKVQ+EN+NVLVVSG R+RE+KENEGVKYVRMERRMGKFMRKFQLP
At: 260  YAFVVDMPGIKGDEIKVQVENDNVLVVSGERQRENKENEGVKYVRMERRMGKFMRKFQLP 439

WF: 258  ENADLEKISAVCNDGVLRVTV 320
         ENADL+KISAVC+DGVL+VTV
At: 440  ENADLDKISAVCHDGVLKVTV 502
```

Figure 5.3.10.4 Alignment of *P5 B10* to the *Arabidopsis* HSP gene. The wallflower clone aligns to a region spanning the ORF and 3'UTR of the *Arabidopsis* gene (shown in lilac and blue, respectively). The 100 amino acids at the 3' end of the HSP protein are important for its function, which comprises most of the length of these very small proteins. The entirety translatable section of the wallflower clone is included within this range, and thus all of the translatable protein sequenced from wallflower is important for HSP function.

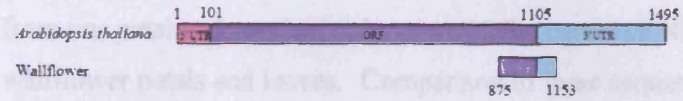

```
WF: 32   YVGQLEFFNAQTPDGLKSFGSALCMMSMSMGNFVSSLLVTMVVKISTEDHMPGWIPRNLN 211
         YVGQLEFFNAQTPDGLKSFGSALCMMSMSMGNFVSSLLVTMVVKISTEDHMPGWIPRNLN
At: 1625 YVGQLEFFNAQTPDGLKSFGSALCMMSMSMGNFVSSLLVTMVVKISTEDHMPGWIPRNLN 1804

WF: 212  KGHLDRFYFLLAALTSIDLVIYIACAKWY 298
         KGHLDRFYFLLAALTSIDLV+YIACAKWY
At: 1805 KGHLDRFYFLLAALTSIDLVVYIACAKWY 1891
```

Figure 5.3.10.5 Alignment of *P1 F4* to the *Arabidopsis* peptide transporter gene. The wallflower clone aligns to the 3' end of the *Arabidopsis* ORF, shown in lilac.




```
WF: 18  SRPRYAGEEKMETTAFNTSTRIGSWSSTISAPLQTCGSFNWQLPTRRRVIVANLQNSNFR 197
        S+  +  E KMETTAFNT++RIG+WSS IS PLQTCGSF  QLPTRR VIVA+L+NSNFR
At: 103 SKRSFKEEVKMETTAFNTTSRIGNWSSAISPPLQTCGSFKCQLPTRRGVIVADLRNSNFR 282

WF: 198 WRKAATTNRANVAAEAVRIPTSVPARAA 281
        WRKA TT+R NVAAEAV+IPTSVP R A
At: 283 WRKATTTSRGNVAAEAVKIPTSVPVRVA 366
```

Figure 5.3.10.6 Alignment of *P4 D6* to the *Arabidopsis Sen1* gene. The wallflower clone aligns to a region spanning the 5'UTR and the ORF of the *Arabidopsis* gene (shown in pink and lilac, respectively).




```
WF: 96   DTSHKVRIPAWTDRILFKIQDTDNIQATLHSYDSIDQVYGSDHKPVKADLCLKWVNN*ID 275
         DTSHK+R+PAWTDRILFKIQDTDNIQATLHSYDSIDQVYGSDHKPVKADLCLKWVN+*I+
At: 932  DTSHKIRVPAWTDRILFKIQDTDNIQATLHSYDSIDQVYGSDHKPVKADLCLKWVNS*IN 1111
```

Figure 5.3.10.7 Alignment of *L1 B4* to the *Arabidopsis* homologue of an inositol polyphosphate 5-phosphatase gene. The wallflower clone aligns to a region spanning the ORF and the 3'UTR of the *Arabidopsis* gene (shown in lilac and blue, respectively).

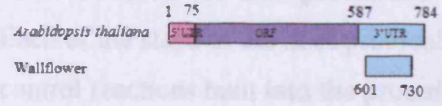


Figure 5.3.10.8 Alignment of *P2 B1* to the *Arabidopsis* lipid transfer protein gene. The wallflower clone aligns to the 3' untranslated region (UTR) of the *Arabidopsis* gene (shown in blue), thus as the wallflower clone does not encode a part of the translated protein, no protein alignment could be made.

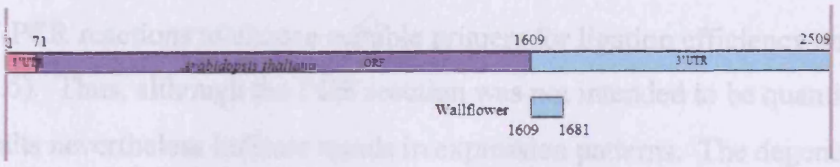


Figure 5.3.10.9 Alignment of *L1 H3* to the *Arabidopsis* γ-glutamyl transferase gene. The wallflower clone aligns to the 3' UTR of the *Arabidopsis* gene (shown in blue), thus as the wallflower clone does not encode a part of the translated protein, no protein alignment could be made.

5.3.11 Isolation of partial tubulin cDNA from wallflower

A trial PCR using the tubulin primers on the cDNA and the control reactions lacking reverse trasnscriptase gave bands in the cDNA reactions and none in the control reactions – demonstrating the complete removal of genomic DNA in the DNAse treatment step. As each of the eight white colonies picked proved to contain inserts of the same size after PCR amplification, plasmids were only isolated and sequenced from one petal and one leaf colony. A 324bp partial cDNA was obtained from both wallflower petals and leaves. Comparison of these sequences proved them to be identical, with the exception of three base pair differences: base 6 was a C in the petal sequence and T in the leaf, base 98 was a T in the petal and C in the leaf and base 201 was a G in the petal and an A in the leaf. When entered into BLAST, the wallflower sequences pulled out a variety of β tubulin genes from other plant species.

5.4 Discussion

5.4.1 Controls for SSH procedure

Each of the steps of the SSH protocol appear to have worked well and all of the control reactions built into the procedure have given the expected results, based on the guidelines given in the manufacturer's handbook, thus indicating that both subtractions worked effectively. As controls, two partial wallflower cDNAs were amplified - *SAG12* and β tubulin. The *SAG12* primers designed to *Arabidopsis* and *Brassica napus SAG12* genes worked well on wallflower cDNA, amplifying a 344bp fragment of the *SAG12* gene, indicating that the gene is conserved across all three species. The abundance of the *SAG12* PCR product in young and old wallflower tissues reflected the findings of chapter 4 on the pattern of expression of *SAG12* in wallflower leaves and petals. Northern analysis showed *SAG12* expression to increase in both old wallflower leaves and wallflower petals (chapter 4), as did these

RT-PCR reactions to choose suitable primers for ligation efficiency analysis (section 5.3.5). Thus, although the PCR reaction was not intended to be quantitative, the results nevertheless indicate trends in expression patterns. The degenerate β tubulin primers amplify a 323bp fragment and have worked on all plant species tested to date, and also worked on wallflower cDNA, amplifying a 324bp fragment. β tubulin is a multigene family which, in *Arabidopsis*, contains 9 members (Snustad *et al.*, 1992), with members expressed in all plant tissues at varying levels (Dixon *et al.*, 1994; Hussey *et al.*, 1988). It is likely that the primers amplify all of the family members, as the primers are degenerate and not specific to a particular member of the β tubulin family. As there are some minor differences between the leaf and petal sequences, a different family member may have been pulled out of each. However, these differences are very small, since the primers amplify a conserved region of the gene and it is probable that this region has very similar sequence in all wallflower family members. Tubulin is expressed strongly in young petals and young leaves and expressed at a lower level in old petals and leaves, with a similar degree of downregulation in both tissues. This is similar to results found in *Arabidopsis* cell culture, where β tubulin expression decreased almost 2-fold in senescing cultures (Swidzinski *et al.*, 2002). Although this PCR reaction was not intended to be quantitative, the similarity of the result of the *SAG12* PCR to the *SAG12* expression patterns established from northern analysis and the accordance of the β tubulin result in wallflowers with known expression patterns in *Arabidopsis* give good reason to believe that β tubulin expression decreases to a similar extent with increasing age in both petals and leaves of wallflowers.

5.4.2 Sequencing of clones

The random sequencing of some clones and their analysis identified some genes that are expressed during leaf senescence and late petal development in wallflowers, and putative functions have been assigned to these, based on homology to *Arabidopsis* genes. The high degree of homology between the wallflower clones and their *Arabidopsis* gene counterparts along with the alignments confirm that the homologues appear to be meaningful. Using the SSH method has allowed identification of clones aligning to all areas of the protein, thus showing this method to be better than differential display, where typically only clones aligning to the 3' end of the gene are

identified. The possible functions of these genes and their role in senescence and late development will be discussed with reference to wallflowers and whether or not the events in leaves and petals bear any similarity to each other.

5.4.2.1 Pectinesterases

The sequence of clones *PL C1* and *P1 D11* closely matches a putative pectinesterase gene in *Arabidopsis*. Both clones originated from the subtracted wallflower petal cDNA, indicating that this gene is upregulated during late petal development. Pectinesterases de-esterify the pectin in the cell wall and are known to be associated with abscission and senescence (Nari *et al.*, 1991). Pectin methyl esterase is one of the major hydrolases involved in the post-harvest softening of tomatoes (Barka *et al.*, 2000; Sethu *et al.*, 1996). As well as being found in higher plants, they are also present in phytopathogenic fungi and bacteria (Collmer and Keen, 1986). In wallflowers, a likely role for this enzyme would be to de-esterify the cell walls, and thus it may be involved in wilting.

It is interesting that the two pectinesterase clones sequenced appear to represent the same PCR product. It is possible that this is as the same bacterial colony was picked twice, but unlikely, as the two clones were obtained from different 96-well plates, which also makes contamination highly unlikely as an explanation. The most convincing explanation is that either the same PCR product was cloned twice into separate bacteria, or that the two clones represent different pectinesterase mRNAs, but appear the same as they are from a highly conserved region. This is a plausible theory, as both wallflower clones contain the signature motif for pectinesterases (Richard *et al.*, 1996), indicating that the cloned region may be highly conserved.

5.4.2.2 CCR4-associated proteins

The sequence of clone *LP H9* matches a CCR4-associated protein from *Arabidopsis*, and originated from the subtracted wallflower leaf cDNA. This gene is involved with the transcriptional control of mRNA, affecting the expression of genes, usually by increasing transcription (Denis and Malvar, 1990). It contains five leucine-rich binding domains (Braun *et al.*, 1991) and binds to other proteins forming a CCR4 complex, resulting in an increase in its functional ability (Liu *et al.*, 1997; Malvar *et al.*, 1992). It is thought that the CCR4 complex is conserved in eukaryotes, with

evidence for its presence in yeast, *Arabidopsis*, human, *C. elegans* and mouse (Lee *et al.*, 1999). A gene has been found in mice that is similar to an *Arabidopsis* CCR4-associated protein, which interacts with CCR4 to activate transcription (Draper *et al.*, 1995). It would seem likely that this gene may play a similar role in wallflowers, and its increased expression during senescence may indicate a regulatory role for this gene in increasing the expression of other senescence associated genes.

5.4.2.3 Peptide transporters

The petal clone *P1 F4* closely matched a peptide transporter gene in *Arabidopsis*, with the role of catalysing the transport of oligopeptides across the cell membrane. Peptide transport is associated with protein hydrolysis and recycling in senescing leaves (Higgins and Payne, 1981) and the expression of this gene during late petal development may indicate similar events in the two organs. As recycling is the defining characteristic of senescence, this may indicate that wallflower petals also recycle the nutrients that they contain, and thus, senesce.

5.4.2.4 Glutathione-S-transferases

The two petal derived clones *P1 F8* and *P1 F10* both matched closely to *Arabidopsis* GST genes. GSTs comprise a large and diverse gene family, with 48 members present in the genome of *Arabidopsis*, divided into 5 classes (Dixon *et al.*, 2002). Levels of GST expression are known to increase during leaf senescence (Guo *et al.*, 2004; Jepson *et al.*, 1994; Zhou and Goldsbrough, 1993), and are known to be regulated by ethylene in *Arabidopsis* (Zhou and Goldsbrough, 1993). They are also upregulated in response to many biotic and abiotic stresses (Marrs, 1996). As befits such a diverse family of genes, their roles and functions are also varied, but share the feature of using the tripeptide glutathione as a cosubstrate or coenzyme (Dixon *et al.*, 2002). GSTs are involved in secondary metabolism, tyrosine metabolism, as well as in herbicide, oxygen and radical detoxification (Cobbet and Goldsbrough, 2002; Dixon *et al.*, 2002; section 1.5.5.1), where they may be involved with maintaining cell viability during senescence. GSTs are induced in response to cytokinin hormones (Gonneau *et al.*, 1998; Marrs, 1996) which retard senescence, which therefore mediated in part through the protective effects of GSTs. They have also been implicated in UV-inducible cell signalling and apoptosis regulation (Dixon *et al.*, 2002), and glutathione, which is necessary for GST activity, is a known inducer of

plant defence genes (Dron *et al*, 1988; Wingate *et al.*, 1988), which may therefore indicate a role in senescence regulation.

The two wallflower clones showing homology to GST genes each showed homology to a different *Arabidopsis* gene, indicating that the two clones are independent. There are 6 amino acids in GST genes that are conserved between all animal and plant GSTs (Droog *et al.*, 1993), two of which were present in both wallflower GST clones, and are also present in the carnation gene pSR8, which shows homology to GSTs and is upregulated during late petal development and in response to ethylene in carnation petals (Meyer *et al.*, 1991).

5.4.2.5 Lipid transfer protein

The *P2 B1* clone from subtracted wallflower petal cDNA closely matched a putative lipid transfer protein (LTP) gene from *Arabidopsis*. LTPs were initially thought to be involved with the transfer of fatty acids and phospholipids between membranes within the cell, but the discovery that they are extra-cellular proteins led to the need to revise this function (Kader, 1997). A wide range of extracellular roles have been suggested for LTPs, such as in pathogen-defence reactions or the adaptations of plants to environmental changes (Kader, 1997) and homology has been found between the promoter of an LTP and the promoters of stress induced genes and the LTP promoter has been shown to respond to certain stresses (Yubero-Serrano *et al.*, 2003). The *in vivo* roles of these proteins remains unclear, but it is possible, from their different patterns of expression, that different LTP genes may have different functions (Yubero-Serrano *et al.*, 2003). LTP expression is highly localised, for example being localised to the flowers in carrot (Sterk *et al.*, 1991). Thus it is likely to play a similar role in the petals of wallflowers as it does in carrot flowers, and it is possible that the product of this gene may be involved in wilting. It is also possible that they may be involved in lipid transfer in the extracellular environment, transferring lipids from senescing cells and recycling them to other parts of the plant.

5.4.2.6 *Sen1*

The petal derived clone *P4 D6* showed homology to the *Arabidopsis* senescence associated gene *Sen1*. *Sen1* is involved with cellular ageing, but its function is, at present, a mystery (Oh *et al.*, 1996). Work by Chung *et al.* (1997) shows this gene to

be negatively regulated by sugar, with sugar starvation causing it to be upregulated, supporting the theory that sugar may be involved with the regulation of senescence (see section 1.4.2.4). The expression of this gene in late petal development in wallflowers as well as in senescing *Arabidopsis* leaves hints towards similar processes being at work in both organs, although it is difficult to speculate on the extent of this similarity without knowing the function of the gene.

5.4.2.7 Heat shock proteins

The *P5 B10* clone, from the subtracted petal cDNA showed homology to class II heat shock protein (HSP) in *Arabidopsis*. HSPs are involved with cell rescue, defence, ageing and death, but their function remains elusive. They are low molecular weight proteins and likely to be found in the cytoplasm (Vierling, 1991). HSPs are produced in flowers under normal field conditions (Hernandez and Vierling, 1993) and low molecular weight HSPs have been found to increase in abundance in the presence of abscisic acid, which has been linked to senescence in leaves (Smart, 1994). As was the case with *Sen1*, the presence of these proteins in old petals as well as in senescing leaves indicates a similarity between the two processes, but as the function is not known then it is difficult to determine whether the two processes are intrinsically or merely superficially similar.

The wallflower HSP clone shows homology to a large proportion of the ORF of the *Arabidopsis* HSP gene, partially on account of the protein being so small. The last approximately 100 amino acid residues of HSP proteins (which accounts for about two thirds of the length of the protein) are conserved, and are important in HSP function (Kim *et al.*, 1998). The entire translatable section of the wallflower clone's sequence falls into this range, and thus is likely to be important in HSP function.

5.4.2.8 Cytochrome P450

The *P6 C7* clone from petals was homologous to a cytochrome P450-like protein from *Arabidopsis*, which are a large superfamily of genes involved in the oxidative degradation of various compounds, mainly toxins and mutagens (Chapple, 1998). A similar gene is upregulated in the late development of daylily petals (Panavas *et al.*, 1999) and cytochrome P450s are part of the HR to bacterial infection in *Arabidopsis* (Godiard, 1998). They are also involved in many plant biochemical pathways and in

the biosynthesis of many products, including hormones, lipids, secondary metabolites, and plant growth regulators, such as gibberellins (Chapple, 1998; Schuler, 1996). It is difficult to speculate on the actual role of this protein superfamily during senescence and late petal development, as the possibilities are so varied, and thus the expression of this gene during late petal development only serves to demonstrate a similarity between late developmental processes in leaves and petals, as well as the HR.

5.4.2.9 Inositol polyphosphate 5-phosphatase

The *L1 B4* clone from the leaf subtracted cDNA matches an *Arabidopsis* inositol polyphosphate 5- phosphatase (IPP) gene. Enzymes of the IPP family specifically hydrolyse inositol 1,4,5 trisphosphate (IP_3), a secondary messenger molecule, to inositol 1, 4-diphosphate (IP_2). An increase in levels of IP_3 is involved in triggering the release of calcium from intramolecular stores, in turn advancing the progression of senescence (section 1.4.3.1). Thus, hydrolysis of the IP_3 molecule by IPP could lead to a decrease in calcium levels in the cell, retarding the progress of senescence. In animals, IPP inhibits protein kinase B phosphorylation, leading to apoptotic cell death.

The At1g47510 gene, which was found by BLAST to be the closest match to the wallflower *L1 B4* clone, encodes one of the smallest members of the IPP family in any organism and has a slightly different substrate specificity to other plant IPPs: hydrolyzing 5-phosphates from a group of phosphoinositide substrates, including phosphatidylinositol 4,5 bisphosphate (PIP_2), phosphatidylinositol 3,5 bisphosphate and phosphatidylinositol 3,4,5 trisphosphate, and not active on IP_3 or inositol 1,3,4,5 tetraphosphate (Ercetin and Gillaspy, 2004). PIP_2 is the preferred substrate, which has a role in cytoskeletal rearrangements, potential involvement in vesicle trafficking and ion transport and is known to act as a docking site for proteins (Toker, 1998). Thus, loss of PIP_2 by hydrolysis would lead to a loss of such docking sites and could influence many downstream processes. PIP_2 is also a precursor of IP_3 (Ercetin and Gillaspy, 2004), and thus breakdown of PIP_2 would lead to reduced IP_3 production, obstructing calcium-based regulation of senescence (section 1.4.3.1). The IPP encoded by At1g47510 is regulated by ABA, JA and auxin, which have all been implicated in senescence signalling (sections 1.4.2.3.3 and 1.4.2.3.4), although usually as promoters of senescence. ABA is also known to stimulate PLC activity, resulting

in new IP_3 synthesis (Meijer and Munnik, 2003), as well as stimulating production of other IPP family members, which hydrolyse IP_3 and thus terminate ABA signalling (Burnette *et al.*, 2003). JA is eventually produced in response to the action of IP_3 (section 1.4.3.1), which would thus activate this IPP gene.

As only a small part of the wallflower gene sequence was available to query the BLAST database, it is possible that this gene may encode any of the IPP family members in wallflowers. However, whichever family member the gene encodes, it functions as a negative regulator of senescence. The fact that this gene has been identified from a SSH library, indicating that it is upregulated during senescence, coupled with its putative action in preventing calcium release, thus delaying senescence, initially seem at odds. However, this gene is activated at the end of a signalling cascade promoting senescence, it must be considered that it may be a negative regulator controlling the rate of senescence, preventing the signalling cascade from becoming self-perpetuating (see section 1.4.3.1). Additionally, the IP_3 which releases calcium comes from the hydrolysis of PIP_2, whereas the action of this IPP enzyme prevents *de novo* synthesis of IP_3. Thus, it does not directly negatively interfere with the G-protein-calcium signalling cascade, and indeed prevents newly synthesised IP_3 from positively interfering with such signalling. Therefore this gene may be important for the regulation of leaf senescence in wallflowers, and it would be interesting to discover whether it is also upregulated during the late development of petals. There is also the consideration that the SSH method only enriches for genes expressed at higher levels in old tissues compared to young, and the identification of this gene by this method is therefore not a guarantee of its upregulation during late development.

5.4.2.10 Bifunctional nuclease

The leaf derived *L1 B5* clone showed homology to a bifunctional nuclease from *Arabidopsis*. Nuclease levels are known to increase during leaf senescence (section 1.5.4.3), breaking down the phosphate- and nitrogen-rich nucleic acid molecules so that the nutrients can be recycled. The bifunctional nuclease (*BFN1*) found in senescing *Arabidopsis* leaves and stems has both RNAse and DNAse activities and its expression characteristics suggest a role in nucleic acid degradation to facilitate nucleotide and phosphate recovery during senescence (Perez-Amador *et al.*, 2000).

5.4.2.11 Ribosomal proteins

The *L1 F6* clone from leaves shows homology to an *Arabidopsis* gene that encodes the ribosomal protein S14, which encodes one of the proteins making up the 30S subunit of the ribosome. The upregulation of this *S14* mRNA during wallflower leaf senescence is in contrast to findings in lupin cotyledons, where it is only detectable in young tissue and is upregulated by cytokinins, which negatively regulate senescence (Cherepneva *et al.*, 1998). However, *S14* is also upregulated in the HR in tobacco leaves (Karrer *et al.*, 1998). Ribosomes are complex organelles with the central role of translating the mRNA into proteins. Thus, as senescence progresses and protein synthesis generally declines, fewer ribosomes are needed to fulfil this task. In keeping with this, levels of ribosome inactivating proteins increase with senescence (Stirpe *et al*, 1996). However, a certain number need to be retained in order to translate the genes which are upregulated during senescence. Senescence in leaves also sees a decrease in the polymer state of ribosomes, caused by a decrease in magnesium, which can be delayed by the addition of magnesium, in turn delaying senescence (Kiss, 1995). Thus, ribosomal proteins and their breakdown may have a role to play during the senescence of leaves, and it would be interesting to learn more of what precisely that role is, and whether they are similarly involved in late petal development.

5.4.2.12 γ glutamyl transferase

The leaf *L1 H3* clone shows homology to the γ glutamyl transferase gene from *Arabidopsis*. Transglutaminases are related to growth, differentiation (Grandi *et al.*, 1992; Serafini-Fracassini *et al.*, 1989), stress (Dondini *et al.*, 2001), leaf senescence (Ori *et al.*, 1999) and late petal development (Serafini-Fracassini *et al.*, 2002) and may be involved in glutathione catabolism. The best known role of transglutaminases is in stabilising proteins (Lorand and Conrad, 1984), which may be an important function during senescence, when proteolysis is such a prominent event. It is possible that transglutaminases have multiple roles in plant PCD, as is the case in apoptosis in mammalian cells (Melino and Piacentini, 1998). Specific γ glutamyl transferases are involved in metal detoxification in higher plants (Zenk, 1996).

Thus, all of the genes identified are known to be upregulated during late development and therefore are likely to play a role in late developmental events, even if in some cases it is not clear exactly what that role may be. A certain degree of overlap has been noted between leaves and petals, with many of the genes identified from the subtracted petal cDNA being known to increase in expression in senescing leaves. However, the lack of information on the function of many of these genes means that the significance of these genes being found in both petals and leaves is unknown. If the genes encode products that are involved in the senescence process in definitive ways, such as being involved in the recycling of nutrients, then their presence in both tissues would indicate that late developmental processes in leaves and petals are fundamentally similar. However, if the genes encode proteins that merely indicate degradative events, without there necessarily being any recycling, or stress response proteins, then although this still shows that there are common features between leaf senescence and late petal development, the fundamental processes at work may still be different.

6 Microarrays

6.1 Introduction

6.1.1 How microarrays work

Traditionally, molecular biologists have worked on a 'one gene in one experiment' basis, limiting throughput and making it difficult to obtain an overall picture of gene function. Microarrays are a powerful tool that can be used to measure the differential expression of thousands of genes simultaneously, bridging the gap between sequence information and functional genomics. Microarray systems comprise three key parts:

- The array, which contains nucleic acid sequences, or targets, immobilised on a solid support, usually a glass microscope slide
- One or more labelled mRNA-derived samples, or probes, that are hybridised to the array
- A detection system that quantitates the hybridisation signal

6.1.1.1 The array. There are two general types of microarrays, DNA fragment-based microarrays and oligonucleotide-based microarrays. Oligonucleotide-based microarrays can be made at higher densities than DNA fragment arrays (250,000 oligonucleotides per cm^2), particularly if produced by photolithography methods, and are more consistent from one array to another (Lemieux *et al.*, 1998; Eisen and Brown, 1999). The oligonucleotides are synthesised *in situ* based on gene sequences from databases, and so construction is dependent on the availability of accurate sequence data, making this type of array unsuitable for most plant species, including *Erysimum linifolium*, for which large amounts of sequence data are not available. The hybridising sequence in this type of microarray is very short (20-25 nt) and so the technique is sensitive to single nucleotide mismatches (Kehoe *et al*,. 1999). Thus, although sequence data is available for *Arabidopsis*, which is very closely related to wallflowers, the technique may be unsuitable for cross-species hybridisation.

DNA fragment based microarrays are made by robotically depositing specific fragments of DNA (500-5,000 nt) on microscope slides at indexed locations. Currently, up to 10,000 spots can be arrayed per $3.24cm^2$ (Kehoe *et al.*, 1999). At this

density, all 20,000- 25,000 *Arabidopsis* genes can be arrayed on one microscope slide. These fragments can be from a variety of sources, and may be anonymous cDNA clones, EST clones, anonymous genomic clones or DNA amplified from open reading frames (Welford *et al.*, 1998; Penn *et al.*, 2000). However, the number of clones required to ensure a high probability of a low-abundance mRNA being represented at least once in the cDNA library used to create an array is very large. To combat this problem in this study, wallflower transcripts identified by the SSH work were arrayed, reducing the number of clones needed to ensure representation of low-abundance mRNAs (see chapter 5). Known senescence-related genes from *Arabidopsis* were also spotted.

6.1.1.2 The probes. The probes need to be labelled in order to be detected. Radioactive labelling is one option, but has the shortcomings of being less sensitive, only being able to hybridise the array with one probe at a time and, of course, the dangers to the investigator. Fluorescent labelling is thus becoming the method of choice for increased safety and the ability to hybridise an array with multiple samples, each labelled with a different fluor. CyDye fluors are commonly used, as they are relatively photostable. Cy3 and Cy5 are particularly stable, partially explaining the popularity of the use of this particular pair of CyDyes. They are also bright dyes that give strong fluorescent signals and the good spectral separation of Cy3 and Cy5 means that each can be excited at different wavelengths and their emissions can be detected separately with minimal overlap by the use of two different lasers, typically a 532nm and 633nm laser for Cy3 and Cy5, respectively. The fluorescence of Cy3 and Cy5 is also minimally affected by factors such as pH, the presence of DMSO and the temperatures and conditions normally encountered in molecular biology applications. The fluorescently labelled cDNA probes can be made in two different ways, either by direct or indirect labelling. With direct labelling, the RNA sample is reverse transcribed to create the probe using three unmodified dNTPs and a fourth CyDye labelled dNTP. However, the addition of a bulky side chain to one of the dNTPs adversely affects the ability of the reverse transcriptase enzyme to incorporate this dNTP into the cDNA, thus limiting probe synthesis. In addition, as the Cy5 molecule is larger than the Cy3 molecule, the two probes within the pair do not label with equal efficiency. The indirect, or post-labelling, method, mRNA is converted into first-strand cDNA that contains amino-allyl-dUTP by incorporating an amino-allyl-

modified nucleotide, and the mRNA template degraded. The amine groups on the cDNA are reacted with a CyDye-NHS (N-hydroxyl succinimidyl) ester, resulting in the generation of fluorescently labelled cDNA. As the amino-allyl dNTP is smaller than the CyDye labelled dNTP, it is incorporated more efficiently into the cDNA, resulting in longer cDNA fragments and a higher yield. It is also incorporated equally into the two samples that will make up a pair of probes.

6.1.1.3 Hybridisation. The microarray is probed by hybridisation with the fluorescently labelled mRNA-derived probes. The excess unbound probe is washed off and the remaining bound probe is excited by light. Pair-wise comparisons of samples can be made, by using two fluorescent tags with different excitation and emission optima to label two different probes. The probes can be mixed and allowed to hybridise to the same microarray, allowing direct comparisons to be made, as the ratio of fluorescent emission at the two wavelengths reflects the ratio of abundance of that sequence in the two probes. Microarrays allow the impact of a specific treatment, environmental factor or developmental stage to be assessed on all aspects of plant biology, as even subtle changes in gene expression can be detected. Thus, in wallflowers, gene expression in old and young tissues could be compared in this way to establish whether the same genes are involved in the late developmental processes of the two organs, petals and leaves. A ratio of two or greater is generally required between the fluorescence intensity for the two probes for it to be considered to be significant differential expression (Girke *et al.*, 2000; de Risi *et al.*, 1997; Schena *et al.*, 1996). However, in studying PCD in *Arabidopsis* using this technique, Swidzinski *et al.* (2002) argued that a ratio of 1.5 between the two probes is suitable, particularly in the study of PCD upregulated genes, on account of the lower abundance of other cellular transcripts present in older tissue with the total RNA content of the cells decreasing up to 10-fold (Quirino *et al.*, 2000; Buchanan-Wollaston, 1994). This lower ratio has also been used to represent significant changes in differential expression in other microarray analyses (Desikan *et al.*, 2001; Kawasaki *et al.*, 2001; Perez-Amador *et al.*, 2001). The intensity of the fluorescent signal emitted from each spot on the microarray is related to the abundance of the corresponding sequence in the probe. Thus, the fluorescence intensities of the two differently labelled probes hybridised to the same spot can be directly compared. However, the microarray does not give information about absolute gene expression

levels in the samples, as the intensity of the fluorescent signal is not only proportional to the number of hybridised fragments, but also to the length of those fragments and the number of fluorescent labels that each fragment carries. These, in turn, are dependent on the length and the nucleotide sequence of the fragment. The unique secondary structures, melting temperatures and reassociation kinetics of the different probe species (Southern *et al.*, 1999) also affect relative hybridisation of the probe to different spots. Thus, the relative intensity of the fluorescence between one spot and another gives no information on the relative expression levels of the two genes represented by those spots. mRNA species can be detected at levels of 1 in 100,000 (Girke *et al.*, 2000; Ruan *et al.*, 1998) to 1 in 500,000 (Schena *et al.*, 1996), equating to about 1 copy per cell (Ruan *et al.*, 1998) at the lower end of the range, up to a few thousand copies of transcript per cell at the upper limit of detection (Ruan *et al.*, 1998). The broad dynamic range of the signal output means that both strong and weak signals can be monitored quantitatively on the same microarray (Wodicka *et al.*, 1997).

6.1.1.4 Detection and analysis. Microarray scanners typically contain two different lasers that emit light at wavelengths that are suitable for exciting the fluorescent dyes used as labels. A confocal microscope attached to a detector system records the emitted light from each of the microarray spots, allowing high-resolution detection of the hybridisation signals. Due to the large amount of data produced, computerised data processing by means of specialist software is necessary. The primary data is extracted from the scanned microarray slide images, normalised to remove the influence of experimental variation and manipulated so that biologically meaningful conclusions can be drawn.

6.1.2 Alternative technologies

Dot blots of clones arrayed on membrane filters can be screened in a similar way to microarrays. Such arrays are known as macroarrays and can hold approximately 6000 clones of denatured double stranded DNA on a 12 X 18 cm membrane (Kuhn, 2001) or five times this density when robotically printed (Baldwin *et al.*, 1999). These are normally probed with radioactively labelled probes, and thus, only one probe can be used at a time. Although it also possible to use fluorescently labelled probes in a similar way to for microarrays (Chen *et al.*, 1998), the autofluorescence of the

membrane limits the sensitivity (Baldwin *et al.*, 1999). Microarrays thus have the advantage of being 50 times more sensitive (Kuhn, 2001; Baldwin *et al.*, 1999), have a broader dynamic range (Kehoe *et al.*, 1999), allow simultaneous analysis of two probes and allow genome scale analysis of gene expression patterns and hence were chosen in preference to macroarrays for this study.

Northern blots or other profiling techniques give detailed and reliable information on expression patterns, and are not subject to the same degree of experimental variation as microarrays, but it would be impossible to study such a large number of genes in this way. However, these techniques need to be used on genes of interest identified by the array to confirm the results obtained by arraying and to obtain further detail of the expression patterns of the genes. Those exhibiting interesting expression patterns can be sequenced and further analysed. Additional studies would also be necessary to ascertain whether the altered transcript levels are due to changes in synthesis or turnover. All of these methods, including microarrays, only identify genes whose expression is induced or repressed at the transcriptional level. Other gene products may also be of interest with regulation occurring at the level of phosphorylation or dephosphorylation of the protein. Proteomic approaches would need to be used to identify changes in polypepties.

As well as hybridisation-based techniques, for which no sequence information is needed, there are also sequencing-intensive methodologies for quantitative high-throughput display, such as Serial Analysis of Gene Expression (SAGE) and introduced Amplified Fragment Length Polymorphisms (iAFLPs). SAGE (Velculescu *et al.*, 1995) involves producing an unique sequence tag (of approx. 9 bp) from each species present in a cDNA population. The tags are linked together, the concatenates inserted into a plasmid vector, cloned and sequenced. In the sequence, the tags are separated by the recognition sequence of the anchoring enzyme; hence, multiple tags can be sequenced within a single clone. A transcription profile is obtained by determining the total frequency of each tag in a SAGE clone library, reflecting the abundance of the corresponding mRNA in the tissue. Due to technical difficulties and inherent drawbacks, this technique is less frequently used than microarrays for transcript analysis (Kuhn, 2001). It also requires extensive sequence information, which is not available for wallflowers, making this technique unsuitable

for this study. iAFLP measures the concentrations of known transcripts in numerous different probes (Kawamoto *et al.*, 1999). cDNA from each probe is restriction cut and ligated to one of up to six polymorphic adaptors, which have short insertions of various lengths in a common adaptor sequence. The differentially adapted cDNAs are then pooled and amplified using a gene specific primer and a fluorescently labelled adapter primer. The amplicons are separated on an automatic sequencer by length heterogeneity introduced by the polymorphic adaptors and transcript abundance determined by comparing peak areas on the electropherogram. The main limitation of this technique is that only known transcripts can be quantified, as gene specific primers are required, again making this technique unsuitable for the study of wallflowers at present.

6.1.3 MGED standards

A problem with microarray technology is the absence of a standard format for storing the large amount of data that one experiment can yield, and the sizable additional information required to interpret the data, such as the origin of the biological material, experimental design, array composition and design, hybridisation conditions and so on. This leads to a lack of adequate information being available on published data, and results in people finding it difficult to use the large amount of data that has been published, to compare their own results to published findings and to replicate published experiments in new systems (de Francesco, 2002; Nature editorial, 2002, Spellman *et al.*, 2002). There is also the problem that gene expression data is highly context dependent (Brazma *et al.*, 2000), and as much information as possible needs to be known, particularly about the biological origins of the samples. The members of the Microarray Gene Expression Data Society (MGED) have been working with the scientific community towards the development of international publication standards for DNA microarray data and have written an open letter to scientific journals proposing standards for publication (Ball *et al.*, 2002) to make microarray data more useful and accessible. The MGED-devised MIAME (Minimum Information About a Microarray Experiment) standard outlines what information needs to be made available in order to facilitate data sharing (Brazma *et al.*, 2001).

However, MIAME does not specify the format in which the data should be provided, only its content. If MIAME is to be useful, a standard format for the transmission of

the data is required, a need which has recently been responded to with the MAGE-ML (Microarray Gene Expression Mark-up Language) framework for moving and storing microarray data sets that incorporates the principles set down in MIAME. This is an XML (extensible markup language)-based system to facilitate the annotation of experiments. MIAME currently specifies a large amount of information as 'minimal', and will survive based on whether it is reasonable to provide this level of annotation, rather than whether it is unreasonable not to. Thus tools that allow straightforward and rapid ways of providing this information are necessary (Spellman *et al.*, 2002).

Once compiled and formatted, there are three possibilities for publishing microarray data, firstly, for authors to make the data available on their own websites, second, for journals to take this responsibility, and thirdly to submit data to a central public repository (Ermolaeva *et al*, 1998). The advantages of the third over the other two are that all of the data will be available in one centralised location, with a single structure and retrieval system and that all of the data could be queried simultaneously. The success of public sequence databases is an example of what could be achieved for microarray data. Two databases are emerging as the main public repositories: GEO (www.ncbi.nlm.nih.gov/geo/) and ArrayExpress (www.ebi.ac.uk/arrayexpress), and the MGED group are suggesting that submission of data to one of these should be made a requirement for publication.

In conclusion, microarrays are the most suitable methodology for the large scale study of wallflowers at present, but care must be taken to ensure that all of the data resulting from the array and the associated information needed for its interpretation is stored in an accessible format.

6.2 Materials and methods

Details of the optimisation procedures for the array experiment leading to the use of this methodology are given in appendix 4.

6.2.1 Producing the array

The bacterial cultures of the subtracted, selectively amplified clones produced by the SSH procedure (see chapter 5) and bacterial cultures of gene clones known to be

upregulated during leaf senescence from *Arabidopsis thaliana* (obtained from V. Buchanan-Wollaston, HRI Wellesbourne) were used as a source of DNA for the spots to be printed on the microarray. Details of numbers of clones printed and the source of the clones is given in table 6.2.1.1, with the identities of the *Arabidopsis* genes given in appendix 3, and details of the control spots printed are given in table 6.2.1.2. This gives a total of 6912 spots representing 1737 different clones to be spotted on the microarray.

Test DNA source	Number of clones	Number of replicates/slide
Wallflower leaf	1018	3
Wallflower petal	614	3
Arabidopsis leaf (see appendix 3)	91	3

Table 6.2.1.1 Table showing the number of clones printed, number of replicates printed of each clone and the source of the clones.

Controls	Number of replicates/slide	Purpose of control
SAG12	32 16(n)+16 ½conc.(h)	Positive control: known expression pattern, expressed highly in old tissue (see northern blots, chapter 4). Wallflower derived clone.
Tubulin	32 16(n)+16 ½conc.(h)	Positive control: known expression pattern, low expression in old tissue (see RT-PCR, chapter 5). Wallflower derived clone.
LP H9	32 16(n)+16 ½conc.(h)	Positive control: identified from old leaf tissue (see SSH, chapter 5). Wallflower derived clone.
P1 F4	32 16(n)+16 ½conc.(h)	Positive control: identified from old petal tissue (see SSH, chapter 5). Wallflower derived clone.
No DNA	579	Negative control. Identifying DNA carryover during printing.
Human TFR	3	Negative control. Determining hybridisation specificity.
Lambda	3	Negative control. Determining hybridisation specificity.
ALIENs (Stratagene, LaJolla,USA)	256 (4 ALIEN clones, 16reps X 4conc.s)	Negative control. Artificial sequences with no significant homology to any known nucleic acids and with low secondary structure. Determining hybridisation specificity.
Marker	192	Autofluorescent marker spot (DNA labelled with Cy3 dye- see section 6.1.1.2) for spotfinding process of analysis (see section 6.2.4) and positive control for effective binding of target DNA to slide surface.
Blank	576	Negative control. Three blank spots surround each marker, aids spotfinding process. Also identifies DNA carryover during printing.

Table 6.2.1.2 Table showing the control spots printed, number or replicates of each control and the purpose of the control.

The insert within the plasmid in each bacterial clone was amplified by colony PCR (see section 2.2.20) for spotting on the array. For plates where colony PCR failed to work for a significant number of the clones on that plate after several attempts, the lysis of bacterial cells method (see section 2.2.21) was tried. For plates where both methods failed to work for a significant number of clones after several attempts, the plasmids needed to be isolated before amplification. For these, 100µl of the transformed bacteria containing the clones were used to inoculate a 2ml LB-amp100 culture which was grown overnight at 37°C with shaking at 250 rpm. The plasmids containing the DNA clones of interest were isolated from the bacteria using the Whatman 96-well Plasmid Miniprep Kit (Whatman, Maidstone, UK) according to the manufacturers' Vacuum Filtration protocol. The procedure is based on the alkaline lysis of the bacterial cells followed by the clearing of the lysate through a Lysate Clarification Plate. The plasmid DNA is then captured by a DNA Binding Plate, washed, and eluted in 100µl of elution buffer. The clone contained within the plasmid was amplified by PCR, using 0.625 units of Qiagen HotStarTaq polymerase and Qiagen buffer (Qiagen Ltd., Crawley, UK) with 1.25mM $MgCl_2$, 2µl of plasmid DNA, 0.2mM dNTPs, and 10mM each of M13F and M13R primers, using the M13 forward and reverse primer sites on the regions of the vector flanking the insert. Reactions were given a 15 minute 95°C hotstart then cycled in a Perkin Elmer 2700 thermocycler for 35 cycles of {95°C 1 minute, 58°C 1 minute, 72°C 2 mins}. All PCR products (of colony PCRs and isolated plasmid PCRs) were cleaned using the Whatman 96-well PCR Cleanup Kit (Whatman, Maidstone, UK) to remove salts, unincorporated nucleotides, primers and the enzyme. The manufacturers' Vacuum Filtration protocol was followed, binding the PCR product to the DNA Binding Plate, washing, and eluting in 25µl of nuclease-free water. Once amplified and cleaned, 5µl of each PCR product was visualised on an ethidium bromide stained agarose gel (as described in section 2.2.10) and the intensities of the PCR bands viewed under UV compared to the intensity of 2µl of hyperladder II (Bioline, London, UK) to verify whether the reaction had produced sufficiently concentrated DNA for spotting (20-40ng/µl).

2.5µl of each PCR reaction was added to 2.5µl of DMSO in 384-well plates using a Perkin Elmer multiprobe liquid handling robot, then spotted as a 4 X 12 metagrid of 12 X 12 subgrids onto UltraGAPS II (Gamma Amino Propyl Silane) Coated slides

(Corning,Corning, NY) using 150μm solid pins on a Genomic Solutions Flexys workstation and air-dried for 12 hours, baked at 80°C for 2 hours and UV cross-linked with a Stratagene autocrosslink cross-linker to fix the DNA onto the slide. Slides were stored with dessicant in the dark at room temperature until needed.

Staining in a 1 in 10,000 solution of sybr green (Invitrogen, Paisley UK) in filter sterilised TAE (sections 2.1 and 2.2.1) also served as a useful control to confirm that the target DNA had been successfully printed on the slide and had bound effectively. This stains the DNA, showing that it is present, without impairing its ability to bind to the probe once hybridised. The sybr green stain is washed off the slide prior to hybridisation to ensure that signal from the stain does not interfere with the signal from the probe during scanning.

6.2.2 Making the probe

Total RNA was extracted from leaves and petals of each developmental stage, using the Tri reagent extraction method with column purification (see sections 2.2.3 and 2.2.8). Equal amounts of RNA from petal stages –2, -1 and 0 were mixed to give young petal RNA and RNA from petal stages 3, 4 and 5 were combined to give old petal RNA. Leaf stage 3 was used as young leaf RNA and RNA from leaf stages 5 and 6 were mixed and used as old leaf RNA.

6.2.2.1 MessageAmp aRNA. Due to the difficulties in obtaining large amounts of high-quality RNA from wallflower leaves and particularly petals, and after several unsuccessful attempts at making probes without message amplification (see appendix 4 for details), the MessageAmp aRNA kit (Ambion, Cambridge, UK) was used to amplify the mRNA, according to the manufactures' protocol. This technique boosts the sensitivity for rare mRNAs while maintaining the original relative ratio for each message in the population. Indeed, the sensitivity has been reported to be sufficient to detect transcripts present at less than 0.1 (on average) copies per cell in yeast (Wodicka *et al.*, 1997). In addition, this technique evens out the potential problem raised by Buchanan-Wollaston (1994): that total mRNA yields per cell (as a proportion of the total RNA) may not be equal between the young and old tissues. It is based on the RNA amplification protocol developed in the Eberwine laboratory (van Gelder *et al.*, 1990). The mRNA undergoes reverse transcription using an

oligo(dT) primer containing a T7 promoter, followed by *in vitro* transcription of the resulting DNA with T7 RNA Polymerase, generating hundreds to thousands of antisense RNA (aRNA) molecules. This can be used to make the Cy3 and Cy5 labelled probes by reverse transcription. This method does not significantly distort the relative abundance of individual mRNA sequences within an RNA population (Baugh *et al.*, 2001; Pabon *et al.*, 2001; Poirier and Erlander, 1998) as RNA polymerase activity is generally not affected by either the concentration of individual templates in a complex mixture or by the sequences of the templates being transcribed. Although a few templates may be transcribed more or less efficiently than others, the amplification bias is typically equivalent in all samples, and so it is possible to compare expression profiles of different amplified RNA samples.

All short incubation steps, PCR reactions and cDNA synthesis incubations were carried out in a Perkin Elmer 2700 thermocycler, and incubations longer than 2 hours were carried out in an air incubator. Incubations at sub-ambient temperatures were performed in a cooled waterbath or a refrigerator (4°C). To synthesise the first-strand cDNA, 5µg of each total RNA sample was incubated with 1µl of T7 Oligo(dT) primer in a total volume of 12µl for 10 mins at 70°C. 8µl of RT master mix (2µl 10X First Strand Buffer, 1µl Ribonuclease Inhibitor, 4µl dNTP mix, 1µl RT) was added to each reaction, mixed thoroughly by pipetting and incubated at 42°C for 2 hours. To synthesise the second strand cDNA synthesis, 63µl of nuclease-free water, 10µl of 10X Second Strand Buffer, 4µl of dNTP Mix, 2µl of DNA Polymerase and 1µl of RNase H were added to the first strand cDNA, mixed by pipetting and incubated at 16°C for 2 hours. The double-stranded cDNA was purified using the cDNA Filter Cartridges provided with the kit according to the manufacturers' instructions. The purified cDNA was eluted in 16µl of warm (50°C) nuclease-free water. For the *in vitro* transcription step, 24µl of *in vitro* transcription master mix (4µl T7 ATP solution (75mM), 4µl T7 CTP solution (75mM), 4µl T7 GTP solution (75mM), 4µl T7 UTP solution (75mM), 4µl T7 10X Reaction Buffer, 4µl T7 Enzyme Mix) was added to each purified cDNA sample, mixed by pipetting and incubated for 14 hours at 37°C. The aRNA was purified using the aRNA Filter Cartridges provided with the kit according to the manufacturers' instructions. The purified aRNA was eluted in 16µl of warm (50°C) nuclease-free water and quantified using a Nanodrop ND-1000 (Labtech International, Ringmer, E. Sussex, UK).

6.2.2.2 cDNA post-labelling with CyDyes. The CyScribe Post-Labelling Kit (Amersham Biosciences, Little Chlafont, UK) was used to amplify the aRNA, according to the manufacturers' instructions. Duplicate reactions were set up for each type of RNA (YP, OP, YL, OL), each one containing 1µg of aRNA with 1µl of Anchored oligo(dT) primers and 1µl of random nonamer primers in a total reaction volume of 11µl. After mixing, these were incubated at 70°C for 5 mins then cooled at room temperature for 10 mins to permit template-primer annealing. A master mix of the following reagents was prepared, and 9µl added to each reaction on ice: 4µl of 5X CyScribe buffer, 2µl of 0.1M DTT, 1µl of Nucleotide mix, 1µl of amino-allyl-dUTP nucleotide and 1µl of CyScribe RT, and incubated at 42°C for 1.5 hours. The RNA template was degraded by alkaline hydrolysis treatment by the addition of 2µl of 2.5M NaOH, incubated at 37°C for 15 mins and neutralised by the addition of 10µl of 2M HEPES free acid. The aminoallyl-modified cDNA was purified to remove any unincorporated nucleotides and compounds containing amino groups in order to achieve optimum conditions for CyDye coupling by overnight ethanol precipitation and washing (as described in sections 2.2.5 and 2.2.6). The pellet was resuspended in 40µl of filter sterilised (section 2.2.1) 0.1M sodium bicarbonate buffer (pH 9.0). The purified aminoallyl cDNA was added directly to one aliquot of CyDye NHS ester and the ester resuspended by pipetting. One of each duplicate reaction was added to an aliquot of Cy3 NHS ester and the other to an aliquot of Cy5 NHS ester. Thus, each probe pair was made in duplicate with the only difference between the two duplicates being that in the first, the young tissue was labelled with Cy3 and the old with Cy5, whereas in the second, the dyes were swapped, so that the old tissue was labelled with Cy3 and the young with Cy5. The reactions were incubated at room temperature in the dark for 90 mins for the CyDye NHS esters to react with the aminoallyl groups incorporated in the cDNA before being stopped by the addition of 15µl of 4M hydroxylamine with a further 15 minute incubation in the dark to inactivate any unreacted CyDye NHS ester molecules. The fluorescent cDNA probe was purified from the unreacted CyDye in order to maximise hybridisation signal and minimise non-specific background using the CyScribe GFX Purification Kit (Amersham Biosciences, Little Chalfont, UK) provided with the CyScribe Post-Labelling Kit. Each probe pair to be used together was mixed (Cy3 YP & Cy5 OP, Cy5 YP & Cy3 OP, Cy3 YL & Cy5 OL and Cy5 YL & Cy3 OL) and lyophilized using an Edwards Freeze Dryer Modulyo Pirani 501. These were resuspended in 50µl of hybridisation

buffer containing 25% formamide, 5X SSC, 0.1% SDS, 0.5mg/ml poly(dA), 0.5mg/ml yeast tRNA.

6.2.3 Prehybridisation and Hybridisation

Slides were prehybridised for 45 mins in a 5X SSC, 0.1%SDS, 1%BSA solution preheated to 42°C to wash away any unblocked target and to block any sites on the slide surface that are capable of binding the probe non-specifically, washed 5 times in milliQ water and twice in isopropanol and air dried. The slides were hybridised immediately. The probe was heated to 95°C for 5 mins and applied to the microarray slide surface. A second microarray slide was carefully lowered over the probe, ensuring good coverage of the printed area of both slides by the probe and that there were no air bubbles. The slides were hybridised back-to-back overnight in a humid chamber at 42°C.

The slides were separated by immersion in 2X SSC, 0.1% SDS at 42°C and washed in the same solution for 5 mins. The slides were further washed in a solution of 0.1X SSC, 0.1% SDS for 10 mins at room temperature then in four changes 0.1X SSC for 1 minute each at room temperature. The slides were rinsed in isopropanol and dried by centrifugation for 1 minute at 2krpm in a MSE Mistral 2000 centrifuge.

6.2.4 Detection and analysis

The slides were scanned using an Affymetrix 428 array scanner with the supplied software (Affymetrix, Santa Clara, CA, USA). The slide was scanned at 532nm (Cy3) and 633nm (Cy5) using a gain setting that allows identification of less intense spots, without compromising the quantification of the brighter spots. The gain is the laser power (in volts) of the photomultiplier tube, and thus increasing the gain increases the laser power that the slide is scanned at, resulting in increased excitation of the CyDye molecules and a brighter image. Although scanned at slightly different gains, these differences were equalised at the image analysis step (section 6.2.4.1) when the background signal as well as the spot signal was measured. The background was then subtracted from the signal at the data analysis step (section 6.2.4.1). At a higher gain setting, the vale for spot intensity is increased, but the value for background is also increased proportionally. The images were analysed using the microarray image analysis software package Imagene (version 5; BioDiscovery, Marina Del Rey, CA)

and the resulting data processed using the microarray data analysis software package GeneSpring (version 6; Silicon Genetics, Redwood City, CA).

6.2.4.1 Image analysis. Using Imagene, a template was fitted over the image of the slide to detect the spots and assign their identities (known identity in the case of the *Arabidopsis* clones, putative identity based on gene homology for the sequenced wallflower SSH clones and a plate reference for the remaining wallflower SSH clones), a process known as 'spotfinding'. The relative expression level of each spot was quantified in both channels (Cy3 and Cy5) by converting the fluorescent intensities of all the pixels in the image, including those within (signal) and those outside (background) the DNA spots, into numerical values. Imagene's automatic segmentation was used to differentiate between signal and background values for each spot independently. In order to establish how trustworthy the expression data from each spot is, the spot quality flagging schemes on Imagene were set to help remove suspicious spots from consideration. The empty spots flag identifies low-expressed or missing spots. The sensitivity threshold of this flag was set so that the spots identified by Imagene as being missing matched those that could not be identified visually on the microarray image. A threshold value of 1 (on a scale of 0 to 4) was thus chosen, to which the ratio

R = (signal mean – background mean)/ background standard deviation)

was compared. If the calculated ratio for the spots was lower than the threshold value, then the spot was flagged as empty. The negative spots flag identifies spots with a signal mean lower than the background mean. As negative spots did not occur on this array, this quality flag was disabled. The poor spots flag detects low-quality spots, using 7 different criteria to describe poor spots, searching for evidence of contamination, deviation from expected position and irregular shape and size. The default parameters were used, apart from the shape regularity setting, which measures the closeness of the spot's border to circular by inscribing the signal area of a spot into a circle, computing the number of non-signal pixels within the circle and dividing this by the circle's area. The ratio is subtracted from 1 and called "shape regularity". This was set to 0.4. The median signal intensity of each pixel across each spot and the median background intensity were calculated in both channels, and these data were saved along with the gene identity and quality flag information as a tab-delimited text file for export into GeneSpring. The median value is used rather than the mean as this

is more resistant to variation caused, for example, by fluorescent speckles. The spots flagged by Imagene as being empty were removed from the analysis before exporting the data to GeneSpring.

6.2.4.2 Data analysis. The tab-delimited text files created by Imagene were imported into GeneSpring, with the 'signal' data referring to the spot intensity in the probe derived from old tissue and the 'control' referring to that in the probe derived from young tissue. GeneSpring subtracts the background intensity from the spot intensity for both channels, giving the background-corrected spot intensity, and divides this value for the signal with that for the control, giving a ratio for the expression of that gene in old tissue relative to young tissue. Although spot intensity is dependent on gene expression, it is also dependent on a number of other factors, such as the length of the probe molecule that hybridises to a spot. Thus a spot which appears very bright on the array may be very highly expressed, or may be a very long probe transcript which has incorporated a lot of the CyDye. An example of this is the metallothionein genes on the *Alstroemeria* arrays of Breeze *et al.* (2004), where the arrays showed little change in expression of the metallothioneins, yet northern analysis showed strong upregulation of their expression. A possible reason given for this discrepancy was that the high representation of different metallothionein genes on the *Alstroemeria* array could have resulted in reduced intensity of hybridisation to each individual spot on the array, but that the sequence similarity between the metallothionein genes would result in cross-hybridisation on the northern blots and thus a high expression level (Breeze *et al.*, 2004). Thus, spot intensity cannot be taken as a direct measure of gene expression, and spot intensities are not comparable between spots due to the differences is the lengths and label incorporation of the probe transcripts. However, the intensity of one CyDye compared to the other for the same spot is directly comparable, as the probe transcripts are the same length, and the only difference between the transcripts is the CyDye with which they've been labelled. Thus, the ratios for relative gene expression are comparable between spots.

The steps involved in analysing the data are:

- Normalisation, to equalise for experimental differences
- Set up linear and log data interpretations for analysis

- Quality control, removing unreliable spots from the analysis:
    - Spots with poor replication (log interpretation; standard deviation filter)
    - Spots that do not change in expression between young and old tissues (linear interpretation; expression level filter)
- T-test to establish which genes show significant changes in expression with age (log interpretation)
- Produce lists of genes significantly and reproducibly showing changes in expression with increasing age for petals and for leaves
- Establish degree of overlap between petal and leaf gene lists using Venn diagrams

Normalisation is performed in order to compare ratio data between microarray slides and to account for differences in Cy3 and Cy5 signals, perhaps due to the amounts of mRNA used in the two labelling reactions, the efficiency of the cDNA synthesis reactions from the RNA, the efficiency of incorporation of the two Cy dyes and the efficiency of the detection system in detecting the two dyes. Linear normalisation assumes a single normalisation factor is required across the whole signal range and can either be obtained using the signal ratio from a gene expressed at the same level in both the signal and control channels (such as housekeeping genes), using spiked exogenous sequences that hybridise to homologous control spots on the array, or using total signal (the sum of all the Cy3 signals compared to the sum of all the Cy5 signals). However, during senescence, the expression level of most genes, including housekeeping genes, falls, whereas the expression of other genes increases. No genes are known to remain expressed at a constant level throughout development (Swidzinski *et al.*, 2002), and thus no single gene is suitable to be used for normalisation. RNA spike controls disregard any purity and integrity problems of the actual RNA samples. Using total signal assumes that few of the spots change in expression between the two RNA samples under investigation, or that the changes are balanced (van de Peppel *et al.*, 2003). This would not be the case for this array, as the genes spotted are a subtracted library produced by SSH, enriched for genes expressed specifically or preferentially in old tissue, and thus the old tissue channel is expected to hybridise much better to the slide than the young tissue channel, making the slides unsuitable for normalisation using total signal. There is also the concern that the normalisation factor is not constant over the whole signal range in most cases (Tseng

et al., 2001), with the Cy3/C5 ratio being higher at low Cy5 signal levels compared to high Cy5 signal levels. Therefore, no normalisation was used. Although not ideal, it is argued that this is unlikely to prove a problem in this instance, because the chance of there being a quantitative difference between the Cy3 and Cy5 channels has been minimised in several ways:

1. The same amount of RNA has been used in all labelling reactions
2. The chance of differences due to errors in quantification (due to differential efficiency of each amplification reaction, or due to pipetting or instrument accuracy, for example) reduced by pooling each stage of RNA to be used to make up the young and old tissue probes before amplification and quantification.
3. The post labelling method used to make the probe minimised the possibility of the two dyes being incorporated unevenly in the two labelling reactions (see section 6.1.1.2)
4. The replication of the probe making procedure with the dye-swap would show any differences between the Cy3 and Cy5 labelling reactions or between the detection of Cy3 and Cy5 by the scanner.

The distribution of the ratios was looked at for all of the spots on each chip. Both a log interpretation and a linear interpretation were set up for the data. Thus, the data can either be analysed directly in the linear interpretation, or the logged data can be analysed in the log interpretation. The log interpretation is the most important of the two, as any statistical tests carried out assume that the data is normally distributed, which is only true of the logged data. In setting up both interpretations, the Cross-Gene error model (an alternative measure of variability to sample replication) option was deactivated, the view replicates parameter set to 'continuous' (showing relative gene expression of the replicate slides as a line graph rather than a histogram, giving a visual description of the level of reproducibility of the gene expression ratio for a given clone between replicate slides) and the file name parameter set to 'do not display' (thus not taking file name into consideration).

The cross gene error model estimates measurement and sample-to-sample variation in experiments with low numbers of replicate samples by assuming that the amount of variability is a function of the control strength within all the measurements for a single experimental condition. Using this assumption, the number of measurements

used to estimate the global error is equal to the total number of clones on the array. If active, this model would be used, amongst other things, as the basis for the standard deviation, representing the variability of individual population members, and for the t-test p-value, representing the statistical test of differential expression for a specific condition. When inactive, the replicate data for a particular clone or condition is used as a basis for these statistical measurements. The cross-gene error model is also dependent upon the expression levels on each slide being centred around 1, which is achieved by normalisation across the slide in dividing each intensity value by the median of all the intensity values on the slide. As the degree of replication was good in both the petal and leaf experiments with 12 replicate spots per clone (and more in the case of many controls), and as the expression levels on the slides are not centred around 1 (see figure 6.3.3.2.1) due to more clones being upregulated with increasing age than are downregulated (which is on account of the clones being derived from an SSH library), the cross gene error model was deemed inappropriate for data analysis in this instance.

The QC (quality control) genes step aims to filter out genes with unreliable measurements, such as bad spots or poor replicates, as well as those which do not show significant differential expression, prior to analysis. Firstly, the standard deviation filter removed the genes with poor reproducibility between replicates (using the log interpretation) by measuring the spread of the replicate data for each gene around its median value and filtering out those with a standard deviation greater than 1.4. The gene list kept was the genes with reproducible data, which equates to 90% of the area under the normal distribution curve. Secondly, the expression level filter removed the genes that did not alter significantly in their expression between the signal and the control (using the linear interpretation). A minimum value of 0.667 and maximum value of 1.333 were set, identifying those that change by 30% between the signal and the control. This ratio of 1.333 is slightly lower than the ratio of 1.5 used by Swidzinski *et al.* (2002), allowing a buffer for those clones that just fall short of the 1.5-fold mark. Those clones within the buffer zone can be filtered out later if necessary. The gene list saved was the genes that did not change significantly. Thus, in order to produce a list of those genes that show both reliable and significant changes in expression level, the list of genes that did not change in expression must be subtracted from the this list of genes showing reproducible results. To this end, a

Venn diagram was constructed with the standard deviation filter gene list in one circle and the expression level filter gene list in another circle. The reproducibly significantly changing genes were those in the non-overlapping part of the standard deviation filter circle. This is the QC'd gene list used for all further analysis.

A t-test was then performed in the log interpretation to establish which genes showed a ratio significantly different from 1 at the 95% confidence level. Multiple testing correction (MTC) is a statistical method that tries to limit the number of samples that pass the t-test by chance alone relative to the proportion of identified clones, thus eliminating false positives. The Benjamini and Hochberg false discovery rate (BH FDR) was chosen as the most suitable method of MTC, as it is sufficiently stringent in eliminating false positives without excessively increasing the number of false negatives.

From here, lists of genes that are significantly and reproducibly upregulated and downregulated with increasing age in both leaves and petals were produced. The overlap between these lists could be established by the use of Venn diagrams to establish whether any genes are shared between leaves and petals as they get older, and thus whether any of the late developmental processes are shared between the two organs.

6.3 Results

6.3.1 Producing the array

The PCR products from the colony PCR or plasmids isolated from the bacterial clones gave strong bands when run on an ethidium bromide stained agarose gel. Comparison of the intensities of the PCR products to the intensity of hyperladder II bands confirmed that they were free from contaminating oligos and within the recommended concentration range for printing (figure 6.3.1.1).

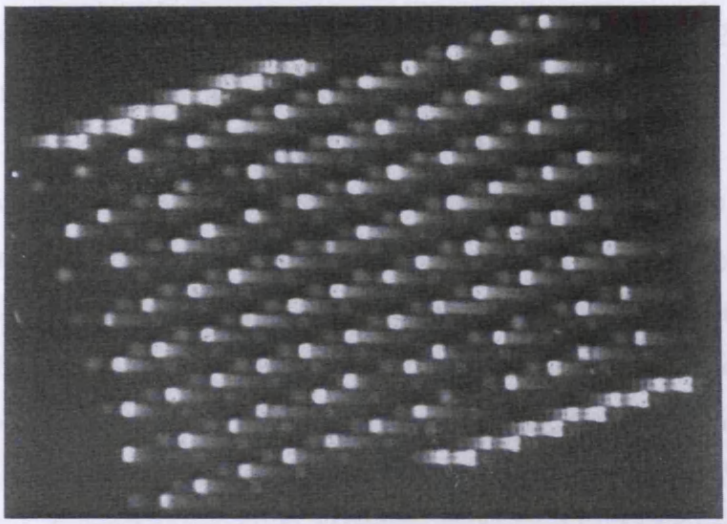


Figure 6.3.1.1 Photograph of the PCR products derived from one of the 96-well plates of purified plasmids isolated from bacterial clones from the SSH procedure. Comparison of the band intensities with the intensities of the hyperladder II (Bioline, London, UK) bands confirms that the PCR products have concentrations within the range 20-40 ng/µl, necessary for slide printing.

DNA was printed as described in section 6.2.1 and sybr green staining confirmed the presence of spots on the slide, showing that the DNA had been printed and fixed to the slide successfully (data not shown).

6.3.2 Making the probe

6.3.2.1 MessageAmp aRNA. Amplification of the wallflower mRNA proved remarkably successful, significantly increasing the amount of messenger RNA present. From a starting amount of 5µg of total RNA, table 6.3.2.1.1 shows the amount of aRNA produced for each of the four samples.

Sample	Young petal	Old petal	Young leaf	Old leaf
Quantity aRNA	24µg	25.6µg	12.8µg	19.2µg

Table 6.3.2.1.1 The quantity of aRNA produced from 5µg of total RNA for each of the four tissue types using the MessageAmp aRNA kit (Ambion, Cambridge, UK).

The overall amount of RNA increased between 2.5- and 5-fold, with this increase completely due to an increase in the amount of message present, which initially only accounted for about 1% of the total RNA. Thus, if there is 2.5 to 5 times the amount of RNA present, which is derived from the amplification of 1% of the starting

population, then the level of amplification gives between 250 and 500 times the starting amount of message.

6.3.2.2 cDNA post labelling with CyDyes. It is possible to quantify the degree of incorporation of dye into each probe by spectrophotometry, or by running an aliquot of the probe on an agarose gel. Spectrophotometry gives an indication of amount of dye present in the probe sample, but not of how much is actually incorporated in the probe molecules. Gel analysis shows the dye incorporated into the probe as a smear, and the unincorporated dye as a separate, smaller smear. Neither of these methods was deemed necessary to establish probe quality, and from visual inspection, each of the probes appeared to have good incorporation of CyDye.

6.3.3 Detection and analysis

6.3.3.1 Image analysis.

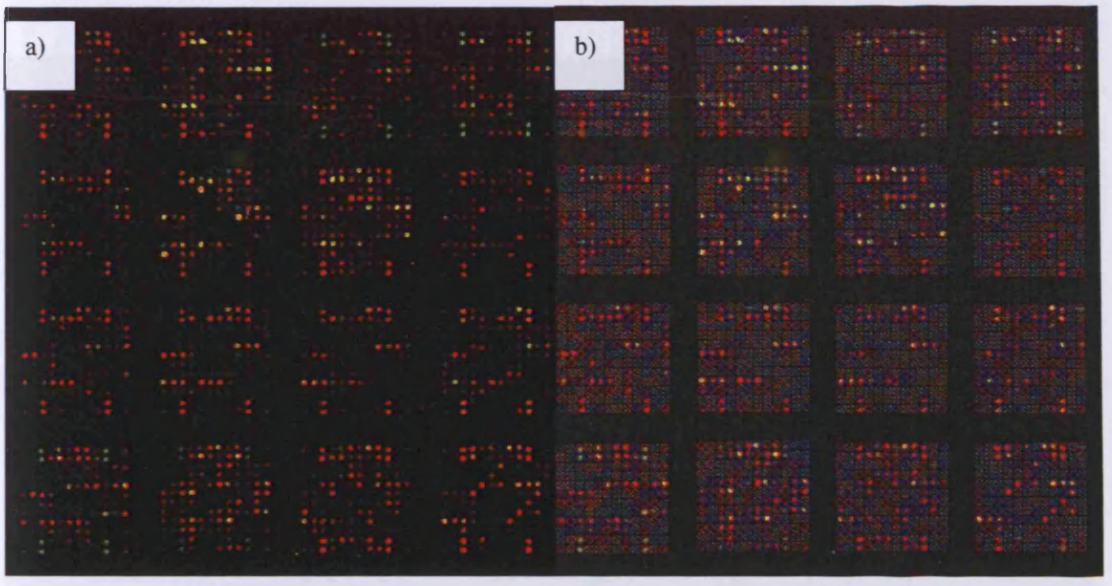


Figure 6.3.3.1.1 a) Imagene false colour composite screenshot of a section of one of the petal microarray slides. The prominence of the Cy5 dye (red) is due to the fact that that the old petal RNA was labelled with this dye and the majority of spots on the array were derived from the genes expressed in old tissue. Spots hybridising to the Cy3 probe (green) indicate clones that are expressed in young petals and the spots hybridising to both probes (yellow) show clones that are expressed in both young and old tissues. b) Imagene screenshot of the same area of the same slide with the spotfinding template overlaid. The 'signal' (within the spot) and 'background' (area surrounding the spots) are measured for each spot in both channels. Empty spots (X) and poor spots (+) have been flagged by Imagene.

The two images obtained for each slide from the scanner (Cy3 image and Cy5 image) were overlaid and analysed using Imagene (figure 6.3.3.1.1). The marker spots and the spots flagged by Imagene as being 'empty' (including the negative controls) were removed from the analysis. Of the remaining spots (1018 clones derived from wallflower leaf, 614 from wallflower petals, 91 clones from *Arabidopsis* leaves and the 4 duplicated positive controls of clones of known sequence), an average of 40% of spots on the leaf slides and 46% on the petal slides were flagged by Imagene as being empty. In some cases, all 3 replicates on all four slides were empty, whereas in other instances only one or two of the 12 replicates of a spot were flagged as empty.

6.3.3.2 Data analysis. The ratios of spot intensity for old tissue probe to young tissue probe were plotted on a log scale for each of the four petal slides and for each of the four leaf slides, to see whether the distribution is normal and to see the range of ratios of expression (figure 6.3.3.2.1).

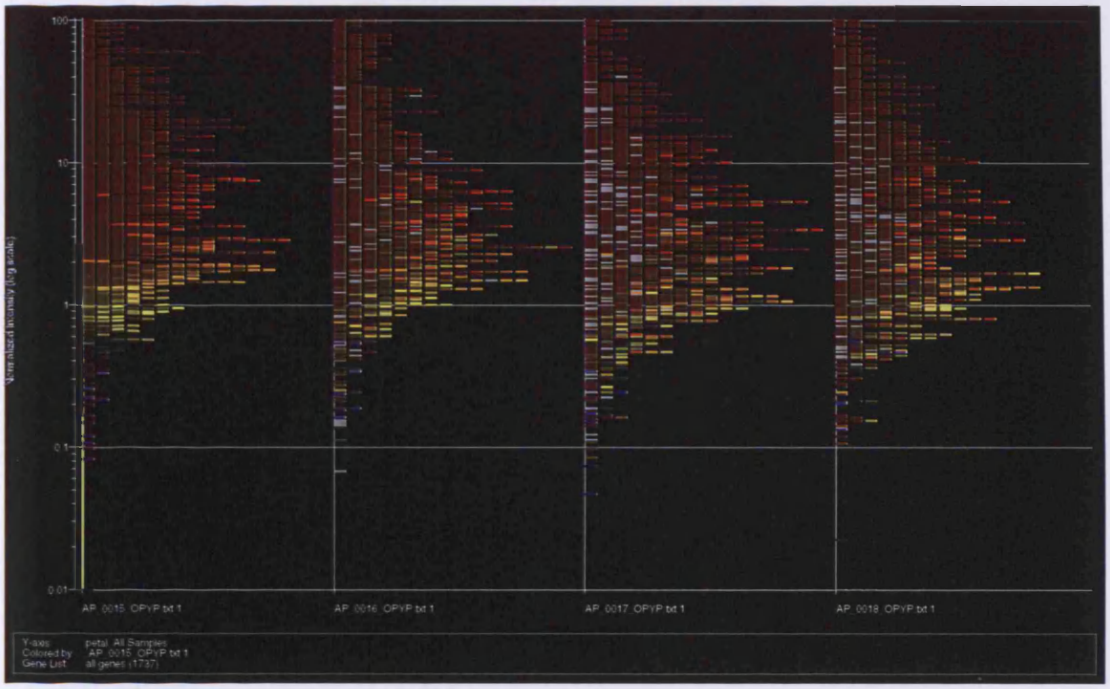


Figure 6.3.3.2.1 Log plot of the distribution of the average expression ratios across the replicate spots for each of the 1727 clones on the four petal slides. Red indicates genes upregulated with increasing age, blue, genes downregulated with age and yellow, genes whose expression didn't change between the old and young tissue samples. Most of the genes are red, which is expected as the clones spotted were derived from an SSH library of senescence enhanced genes.

The distribution was close to normal for each of the slides, although the Anderson-Darling normality test indicates that the logged ratio data are not normally distributed. Most of the clones spotted were upregulated during senescence, which is to be expected from clones derived from an SSH library subtracted in one direction, leading to a skewed distribution. Most were upregulated about 3-fold between young and old petals, and many upregulated up to 100-fold. This is unimportant from the point of view of the statistics that will be used on the data, as the distribution of the log data for the replication for each spot is normal, which is essential for statistical validity.

GeneSpring also gave a correlation coefficient for each slide against each of the others within an experiment. The correlation coefficients for the slides in the petal experiment are given in table 6.3.3.2.1 and those for the slides in the leaf experiment in table 6.3.3.2.2.

Petal slides	**15**	**16**	**17**
16	0.929		
17	0.891	0.879	
18	0.881	0.874	0.919

Table 6.3.3.2.1 Correlation coefficients between the four slides within the petal experiment

Leaf slides	**11**	**12**	**13**
12	0.872		
13	0.766	0.768	
14	0.726	0.818	0.838

Table 6.3.3.2.2 Correlation coefficients between the four slides within the leaf experiment

Next, the expression ratios were looked at across the four slides, to establish the reproducibility of the results across the four slides (figure 6.3.3.2.2). The relatively straight and horizontal lines across each of the four slides (seen on figure 6.3.3.2.2), indicate that the average ratios of the three replicates for each clone were fairly consistent between the four slides.

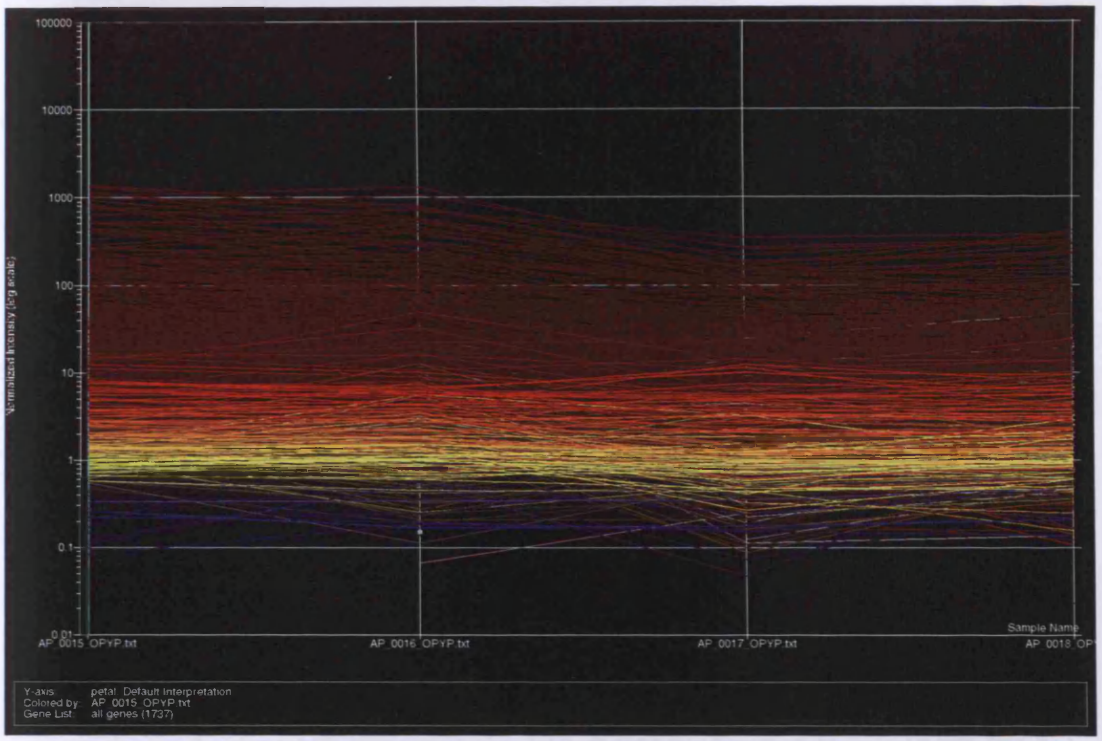


Figure 6.3.3.2.2 Log plot of the average expression ratios across the replicate spots for each clone across the four petal slides. Relatively straight and horizontal lines across the four slides show good replication across the four slides with each of the four sets of three replications of each clone returning a similar ratio on each of the slides.

The clones that did not give reproducible results across the replicates (those with a large standard deviation) were removed from the analysis as being statistically unreliable. The clones that did not change significantly in expression between the young and old tissues were not looked at immediately; as they do not indicate what processes may be upregulated or downregulated during late development in leaves and in petals, and thus give little information on shared processes. However, the information was retained, as they may prove interesting should a gene that is upregulated in one organ remain constant in its expression in the other organ. In order to look at the clones with significant and reproducible changes in gene expression; a Venn diagram (figure 6.3.3.2.3) was constructed showing the clones with reproducible data and those that did not change significantly over time.

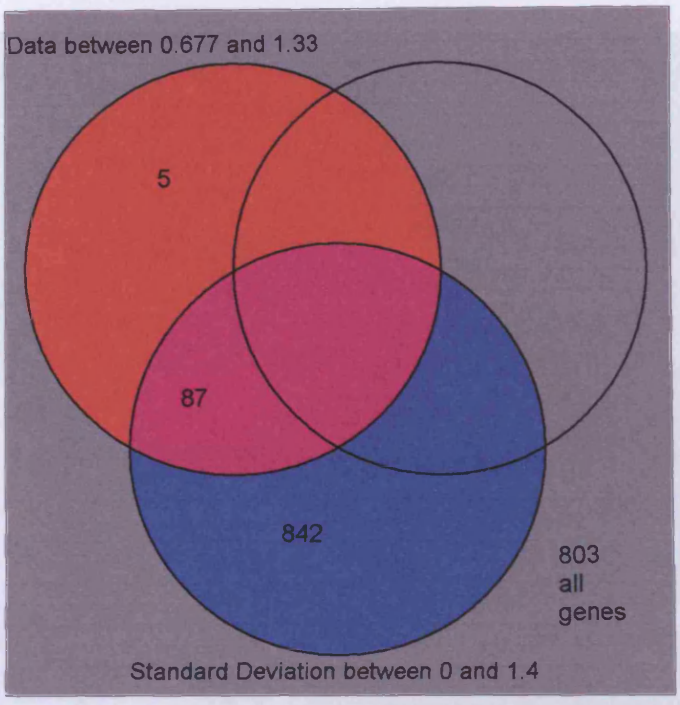


Figure 6.3.3.2.3 Venn diagram showing the clones that to not change significantly over time (red circle) and the clones with reproducible data between the replicates (blue circle). The pink overlap shows the clones that reproducibly remain constant between the two tissue ages. The clones of interest are the 842 clones that change in expression reproducibly between the two ages of tissue, represented by the non-overlapping section of the blue circle.

842 clones were identified as changing significantly and reproducibly in expression between young and old petals. A t-test was performed on these clones to establish which showed a ratio of old/young which was significantly different from 1 at the 95% confidence level, with the BH FDR multiple testing correction applied to eliminate false positives that pass the t-test by chance alone (reproducibility across slides shown in figure 6.3.3.2.4), leaving 515 clones.

The same process was carried out for the data from the slides probed with young and old leaf RNA (eventually leaving 415 genes reproducibly and significantly changing in expression), and from here, lists were produced to establish the identities of the clones that are upregulated and downregulated with increasing age in leaves and in petals. Venn diagrams were constructed to establish the degree of overlap between the leaf and petal lists, and further lists were made to establish the identities of the shared genes.

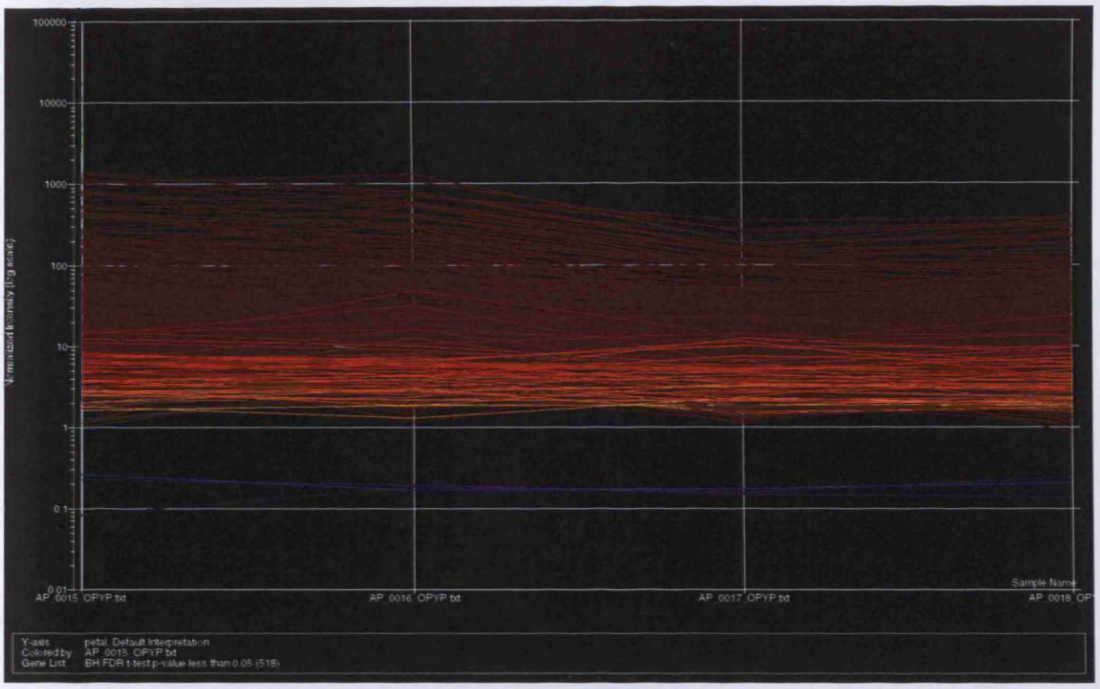


Figure 6.3.3.2.4 Log plot of the average expression ratios across the replicate spots for each clone across the four petal slides for the significantly and reproducibly changing clones, significantly different from 1 at the 95% confidence level after application of the BH FDR to eliminate false positives.

6.3.4 Results of analysis

Details of the data produced from the image and data analyses, including identity of clones excluded from the analysis, signal and control intensities, expression ratios of all genes included in the analysis and their t-test P-values, are given in appendix 5. Taking into account all of the replicates printed across each of the four slides for each clone (3 replicates X 4 slide = 12 replicates for all test clones, see table 6.2.1.2 for details of control replication) for each experiment (petal and leaf), the proportions of clones reproducibly detected, and showing up or down regulation with age at a significant level were compared (see figure 6.3.4.1).

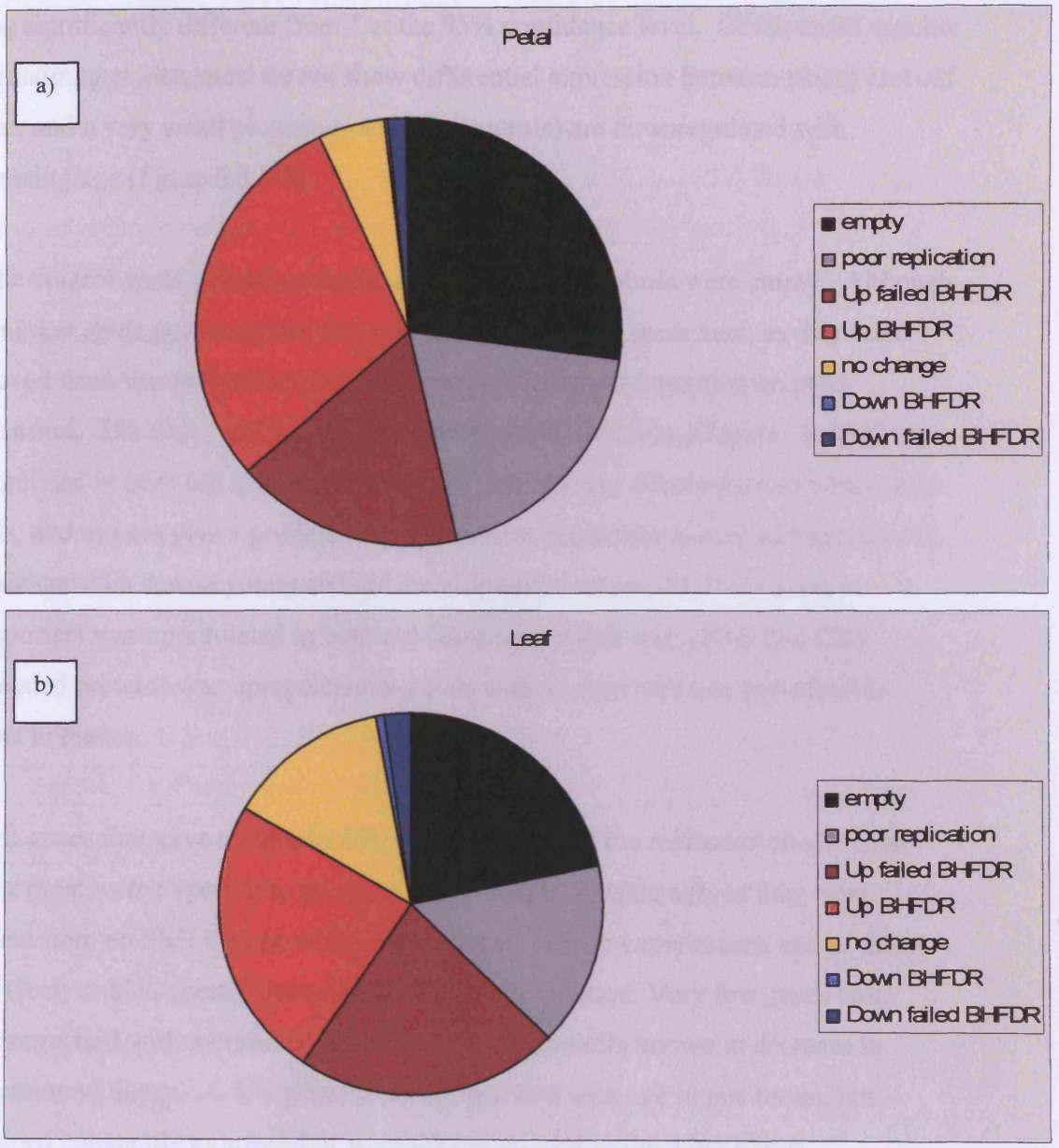


Figure 6.3.4.1 Pie charts showing the proportion of the 1737 clones spotted on the array that gave empty spots for all replicates (black), that showed poor reproducibility between replicates (grey), that did not change in expression with age (yellow) and were upregulated (red) or downregulated (blue) with increasing age in the tissue under investigation with (bright red or blue) and without (dark red or blue) significant difference from 1 at the 95% confidence level after application of the BH FDR to eliminate false positives in a) the petal experiment and b) the leaf experiment.

In both experiments, nearly half the clones were eliminated from the analysis for either being empty or for poor replication. Nearly half the clones were upregulated with increasing age in the tissue under investigation, with about half of these ratios

being significantly different from 1 at the 95% confidence level. Of the small number of remaining clones, most do not show differential expression between young and old tissue, and a very small proportion (mostly controls) are downregulated with increasing age (figure 6.3.4.1).

Of the control spots printed on the array, the negative controls were empty. Although the marker spots gave a signal, they are counted as empty spots here, as they were removed from the analysis on account of not giving any information on gene expression. The four positive controls gave positive results in all cases. *SAG12* was upregulated in both old leaves and old petals, tubulin was downregulated with age in petals, and did not give significant or reproducible results for leaves, but appeared to remain constant across young and old developmental stages, *P1 F4* (a peptide transporter) was upregulated in both old leaves and petals and *LP H9* (a CCR4 associated protein) was upregulated in petals with no significant or reproducible results in leaves.

Of the genes that gave a reproducible ratio across all of the replicates on all of the slides, most were expected to be upregulated with increasing age, as they were derived from an SSH library, which was the case in both experiments, and of these, 52% (leaf) to 63% (petal) showed significant upregulation. Very few genes were downregulated with increasing age, mostly being controls known to decrease in expression with age. A few genes were upregulated with age in one tissue, but remained constantly expressed or were downregulated in the other tissue.

Table 6.3.4.1 lists all of the genes of known or putative identity printed on the microarray, ordered alphabetically according to their pattern of expression. Looking at the clones showing significant and reproducible expression, 415 clones were upregulated in senescing leaf tissue, 515 in old petal tissue and 298 of these clones were upregulated in both tissues. Of the 298 shared clones, the ones of known identity represented clones sequenced from the SSH library and senescence associated genes from *Arabidopsis*, and are shown in section 1 of Table 6.3.4.1. Of these, many were upregulated to a similar degree in both leaves and petals, however, others were upregulated to a much greater degree in old petals than old leaves whereas none showed significantly greater upregulation in leaves compared to petals.

No clones were identified that were upregulated in leaves and downregulated in petals, but 12 clones were upregulated in petals and downregulated in leaves. The ones of known or putative identity are summarised in section 2 of table 6.3.4.1, and all represent GSTs. Again, degree of change in expression levels was much greater in petals than in leaves. The genes of known identity upregulated in petal and not showing significant or reproducible expression patterns in leaves are summarised in section 3, and those of the 20 clones upregulated in leaves but not showing significant or reproducible expression in petals in section 4 of table 6.3.4.1. Similarly, genes upregulated in petals and reproducibly showing no change in expression in leaves are listed in section 5 and those upregulated in leaves and reproducibly showing no change in petals are recorded in section 6 of table 6.3.4.1.

Of the 15 clones downregulated in senescing leaves and the three downregulated in old petals, only one was downregulated in both, which did not have a known or putative identity, and hence is not listed in table 6.3.4.1. The remaining two clones downregulated in petals, were not expressed significantly or reproducibly in leaves and both represented tubulin controls (section 7 of table 6.3.4.1). Of the remaining 14 clones downregulated in leaves, most gave reproducible expression in petals, and most of those of known or putative identities were upregulated in petals, and thus have already been summarised in section 2 of table 6.3.2.1. One clone, *B113*-representing the ribulose bisphosphate carboxylase small subunit from *Arabidopsis*, was downregulated in leaves but did not give significant or reproducible results in petals (section 8 of table 6.3.4.1).

87 petal clones and 239 leaf clones reproducibly showed no change in expression between young and old tissue, as defined in this study (shown on figure 6.3.4.1 as 'no change'), with 51 of these clones shared between both lists (section 9 of table 6.3.4.1 catalogues those of known or putative identity). Section 10 of table 6.3.4.1 records the genes reproducibly showing no change in expression in petals, with no reproducible or significant results in leaves and section 11 of the same table conversely lists the genes reproducibly showing no change in expression in leaves, with no reproducible or significant results in petals. Finally section 12 of the table

holds the remaining clones of known or putative identity, whose gene expression patterns were not reproducible or significant in either organ.

Clone ID	Indentity	*At* gene number	Petal ratio	petal t-test P-value	Leaf ratio	leaf t-test P-value	origin of clone
1. Upregulated in both leaves and petals							
L1 B4	Inositol polyphosphate 5-phosphatase	At1g47510	7.9790	0.0039	4.6664	0.0091	Wallflower SSH library
L1 B5	Bifunctional nuclease	At1g11190	29.0486	0.0090	7.6234	0.0057	Wallflower SSH library
P1 F4	Peptide transporter	At1g32450	67.7231	0.0000	9.3505	0.0007	Wallflower SSH library
P1 F4h	Peptide transporter	At1g32450	141.4174	0.0004	8.6033	0.0000	Wallflower SSH library
P1 F4n	Peptide transporter	At1g32450	144.6308	0.0001	9.6387	0.0075	Wallflower SSH library
P2 B1	Lipid transfer protein	At3g22600	12.2710	0.0000	13.3044	0.0000	Wallflower SSH library
AtWRKY4	WRKY factor	At1g13960	2.1798	0.0041	2.0229	0.0002	*Arabidopsis SAG*
CCH	Copper homeostasis factor	At3g56240	4.0291	0.0000	1.5134	0.0204	*Arabidopsis SAG*
HIN1	Harpin induced	At2g35980	5.7201	0.1884	5.1748	0.0001	*Arabidopsis SAG*
LSC141	Xylosidase?	At5g49360	17.0924	0.0000	2.8515	0.0001	*Arabidopsis SAG*
LSC301	12-OPDR	At1g76680	2.1243	0.0080	1.4864	0.0215	*Arabidopsis SAG*
LSC337	Histone H1-3	At2g18050	20.2440	0.0010	5.6873	0.0002	*Arabidopsis SAG*
LSC648	hydrolase/lipase	At5g13800	5.2353	0.0000	2.6030	0.0068	*Arabidopsis SAG*
PLC1	Phospholipase C	At4g38530	2.4951	0.0018	1.6804	0.0032	*Arabidopsis SAG*
RD21a	Cysteine protease	At1g47128	3.9395	0.0000	2.4001	0.0093	*Arabidopsis SAG*
RNS1	RNAase	At2g02990	7.5360	0.0000	3.7700	0.0003	*Arabidopsis SAG*
SAG12	cysteine protease	At5g45890	66.1236	0.0004	34.3507	0.0000	*Arabidopsis SAG*
SAG12h	cysteine protease	At5g45890	593.2599	0.0002	35.2898	0.1762	*Arabidopsis SAG*
SAG12n	cysteine protease	At5g45890	498.0351	0.0003	268.9586	0.0026	*Arabidopsis SAG*
SAG13	ADH	At2g29350	2.8373	0.0002	1.8036	0.0011	*Arabidopsis SAG*
SRG3	beta- glucosidase	At3g02040	2.3034	0.0234	2.1795	0.0043	*Arabidopsis SAG*
2. Upregulated in petals, downregulated in leaves							
P1 F10	GST	At1g02920	5.6331	0.0003	0.3411	0.0164	Wallflower SSH library

P1 F8	GST	At2g02930	19.5872	0.0000	0.3648	0.0205	Wallflower SSH library
GST1	GST Atpm24.1	At4g02520	9.4091	0.0002	0.2842	0.0104	*Arabidopsis SAG*
3. Upregulated in petals only							
HP C1	Pectinesterase	At2g45220	17.2821	0.0076	3.1764	0.0414	Wallflower SSH library
L1 H3	γ glutamyltransferase	At4g29210	4.0394	0.0000	1.3144	0.1497	Wallflower SSH library
AtWRKY53	WRKY factor	At4g23810	1.5526	0.4979	0.7727	0.3127	*Arabidopsis SAG*
Cyp83A1	cytochrome P450	At4g13770	4.4702	0.1495	2.2338	0.0423	*Arabidopsis SAG*
EAT1	ACC oxidase	At1g05010	5.8336	0.0046	1.7971	0.1908	*Arabidopsis SAG*
Erd1	Clp protease	At5g51070	7.5026	0.1522	2.5963	0.1488	*Arabidopsis SAG*
Lox1	lipoxygenase	At1g17420	2.6440	0.1541	1.3120	0.3337	*Arabidopsis SAG*
LSC213	POP dikinase	At4g15530/ At4g15520	4.8795	0.0078	1.3964	0.2407	*Arabidopsis SAG*
LSC226	cytochrome P450	At4g31500	3.4269	0.0111	0.6059	0.1100	*Arabidopsis SAG*
LSC331	flavonol glucosyl transfera	At4g01070	13.4463	0.0000	1.3578	0.3324	*Arabidopsis SAG*
LSC332	Unknown	At2g17710	6.9256	0.0019	1.4508	0.3488	*Arabidopsis SAG*
LSC460	GS	At5g37600	2.6581	0.0003	1.2971	0.1359	*Arabidopsis SAG*
LSC54	metallothionein	At1g07600	31.5423	0.0002	1.7135	0.1260	*Arabidopsis SAG*
SAG15	*ERD 1*	At5g51070	3.7384	0.0002	1.4300	0.0767	*Arabidopsis SAG*
Sen4	xyloglucan transferase	At4g30270	4.5494	0.0001	1.9396	0.0691	*Arabidopsis SAG*
4. Upregulated in leaves only							
SAG27	Nitrilase 2	At3g44300	1.4835	0.5027	2.5029	0.0217	*Arabidopsis SAG*
5. Upregulated in petals, reproducible no change in leaves							
ACO2	ACC oxidase	At1g62380	3.0318	0.0000	1.1248	0.5513	*Arabidopsis SAG*
HP H9	CCR4-associated protein	At5g22250	3.0415	0.0001	1.0796	0.6896	Wallflower SSH library
HP H9h	CCR4-associated protein	At5g22250	4.2626	0.0000	0.8709	0.2151	Wallflower SSH library
APHPH9n	CCR4-associated protein	At5g22250	3.8654	0.0000	0.3388	0.3314	Wallflower SSH library
CAT1	catalase	At1g20630	3.5689	0.0000	1.1288	0.5289	*Arabidopsis SAG*
LSC222	chitinase	At2g43570	2.3294	0.0055	1.0615	0.7857	*Arabidopsis SAG*

LSC223	unknown	At2g38640	4.8038	0.0000	1.2961	0.1709	*Arabidopsis SAG*
LSC327	unknown	At1g27020	3.7363	0.0452	0.8416	0.5587	*Arabidopsis SAG*
LSC334	NADPH quin oxidoreduct	At4g21580	1.9919	0.0607	1.1611	0.4973	*Arabidopsis SAG*
LSC336	unknown	BAC F4N2	3.9546	0.0370	0.7007	0.5899	*Arabidopsis SAG*
LSC7	cysteine protease	At5g60360	6.1992	0.0038	0.9144	0.7146	*Arabidopsis SAG*
LSC760	aspartic protease	At1g11910	3.9404	0.0118	1.0208	0.9370	*Arabidopsis SAG*
LSC803	glutathione peroxidase	At4g11600	1.7942	0.0297	0.9108	0.6627	*Arabidopsis SAG*
LSC812	Carnitine racemase	At1g65520	2.4910	0.0020	0.8894	0.6786	*Arabidopsis SAG*
LSC815	SRP	At4g30600	2.9194	0.0093	0.8815	0.5805	*Arabidopsis SAG*
PLD(1)	Phospholipase D	At3g15730	2.4974	0.0009	1.3135	0.1513	*Arabidopsis SAG*
PLD(3)	Phospholipase D	At4g35790	2.6974	0.0136	1.0205	0.9563	*Arabidopsis SAG*
SAG21	LEA pro	At4g02380	6.8071	0.0000	1.1567	0.4850	*Arabidopsis SAG*
6. Upregulated in leaves, reproducible no change in petals							
LSC30	ferritin	At5g01600	1.2002	0.6920	3.6868	0.0003	*Arabidopsis SAG*
7. Downregulated in petals							
TUBh	b tubulin	-	0.1735	0.1487	1.6771	0.5882	*Arabidopsis SAG*
TUBn	b tubulin	-	0.1999	0.1055	1.2018	0.7337	*Arabidopsis SAG*
8. Downregulated in leaves							
B113	RBCS	At1g67090	0.5563	0.8675	0.2802	0.0194	*Arabidopsis SAG*
9. Reproducible no change in both petals and leaves							
AtWRKY15	WRKY factor	At2g23320	1.0192	0.9708	1.0906	0.7170	*Arabidopsis SAG*
CAT3	catalase	At1g20620	1.2373	0.5478	0.8362	0.4384	*Arabidopsis SAG*
ERD16	Ubiquitin extension CEP52	At3g52590	1.1174	0.9673	0.6167	0.4858	*Arabidopsis SAG*
Sen5	unknown	At3g15450	0.7072	0.5993	1.1072	0.6487	*Arabidopsis SAG*
10. Reproducible no change in petals only							
LSC320	unknown	At1g27330	0.7682	0.6515	0.5712	0.0808	*Arabidopsis SAG*
SAG18	Unknown	At1g71190	1.1900	0.7178	1.4388	0.0454	*Arabidopsis SAG*

11. Reproducible no change in leaves only							
138	Alpha tubulin	At4g14950	0.2873	0.6125	0.8688	0.6482	*Arabidopsis* SAG
139	Actin	At1g49240	0.4197	0.6841	0.8142	0.4825	*Arabidopsis SAG*
L1 F6	Ribosomal protein S14	-	2.0004	0.4394	0.8419	0.6040	Wallflower SSH library
At103-A	Glutathione S transferase	At2g29450	0.3732	0.3981	0.8799	0.5961	*Arabidopsis SAG*
Bax1	Cell death factor	At5g47120	1.7730	0.1810	0.8098	0.4078	*Arabidopsis SAG*
CAT2	catalase	At4g35090	1.3834	0.8319	0.7853	0.6583	*Arabidopsis SAG*
CST	Citrate synthase	At2g44350	1.6617	0.2492	0.8491	0.6564	*Arabidopsis SAG*
DHOR	Dihydro-orotate reductase	At3g17810	1.6758	0.2020	1.1397	0.6424	*Arabidopsis SAG*
ERD6	Sugar transporter ATZF1	At1g08930	3.3152	0.2833	1.0626	0.8918	*Arabidopsis SAG*
Lox2	lipoxygenase	At1g55020	1.3059	0.8013	0.4712	0.1908	*Arabidopsis SAG*
Lox3	lipoxygenase	At3g45140	0.2241	0.7443	0.8358	0.5804	*Arabidopsis SAG*
LSC215	xyloglucan fucosyl trans ?	At2g15390	1.5461	0.1835	1.0406	0.8689	*Arabidopsis SAG*
LSC323	Isopropylmalate dehase	At5g03290	1.4857	0.2243	1.0822	0.6837	*Arabidopsis SAG*
LSC813	unknown	At3g17800	1.4719	0.2379	0.9825	0.9324	*Arabidopsis SAG*
nClpP3	Cys protease	At1g66670	2.1324	0.3250	0.9919	0.9816	*Arabidopsis SAG*
PR21	Zeaxanthin epoxidase	At5g67030	0.3227	0.5789	0.9177	0.8648	*Arabidopsis SAG*
RNS2	RNase	At2g39780	1.6249	0.6028	1.2028	0.6853	*Arabidopsis SAG*
SAG14	Blue Cu binding	At5g20230	6.8509	0.0193	0.7656	0.4015	*Arabidopsis SAG*
UBIQ	Polyubiquitin UBQ4	At5g03240	1.6340	0.1372	1.2618	0.2578	*Arabidopsis SAG*
12. Not preset in any lists							
ACC2	ACC synthase	At4g26200			0.0100	no reps.	*Arabidopsis SAG*
P1 D11	Pectinesterase	At2g45220	5.7979	0.0000	2.2799	0.0102	Wallflower SSH library
P4 D6	*Sen1*	At4g35770	4.3792	0.5304	1.5661	0.1693	Wallflower SSH library
P5 B10	Heat shock protein	At5g12020	10.4446	0.0028	2.4196	0.0317	Wallflower SSH library
P6 C7	Cytochrome P450	At3g26200	2.0936	0.7406	1.2616	0.6099	Wallflower SSH library
AtWRKY17	WRKY factor	At2g24570	1.7058	0.6101	2.2842	0.1349	*Arabidopsis SAG*
AtWRKY6	WRKY factor	At1g62300	8.7602	0.0738			*Arabidopsis SAG*

B72	Cytochrome P450	At2g45570	3.6334	0.0001	1.6282	0.0327	*Arabidopsis SAG*
CAB	CHLOROPHYLL A/B	At2g34420	0.5204	0.6897	0.3731	0.0636	*Arabidopsis SAG*
Cyp71B3	cytochrome P450	At3g26220					*Arabidopsis SAG*
Cyp71B4	cytochrome P450	At3g26280	7.5106	0.0520	1.9057	0.1307	*Arabidopsis SAG*
Cyp91A2	cytochrome P450	At4g37430			3.7483	0.1613	*Arabidopsis SAG*
LSC322	Aquaporin MIPC	At2g39010	0.2596	0.8030	0.2886	0.1074	*Arabidopsis SAG*
LSC326	Gamma VPE	At4g32940			9.2353	no reps.	*Arabidopsis SAG*
LSC418	Cu amine oxidase	At4g12280	2.3664	0.5598	2.2644	0.5070	*Arabidopsis SAG*
LSC550	cytochrome P450	At1g64950	5.2662	0.2216	3.2195	0.0381	*Arabidopsis SAG*
LSC639	ABC transporter	At4g04770	1.8046	0.1544	1.8144	0.0510	*Arabidopsis SAG*
LSC790	cysteine protease	At4g39090	2.9720	0.0000	1.4830	0.0410	*Arabidopsis SAG*
LSC813A	Acid phosphatase	At3g17790	2.2609	0.5440	1.5855	0.4918	*Arabidopsis SAG*
LSC814	DNA J homolog	At5g06910	1.3426	0.5296	1.3473	0.3332	*Arabidopsis SAG*
LSC88	Wali 7 like/stress response	At5g19140					*Arabidopsis SAG*
LSC94	PR1a	At2g14610	4.2051	0.0001	0.5099	0.0491	*Arabidopsis SAG*
nClpP5	Cys protease	At1g49970	1.5200	no reps.	0.1797	0.3069	*Arabidopsis SAG*
PDF1..2	defensin	At5g44420	2.9419	0.7432	1.4781	0.5707	*Arabidopsis SAG*
PED1	Thiolase	At2g33150	3.2607	0.2188	1.7005	0.2867	*Arabidopsis SAG*
STP4	sugar transporter	At3g19930	2.7650	0.1962	1.2044	0.7507	*Arabidopsis SAG*

Table 6.3.4.1 Table listing all of the genes of known or putative identity spotted on the array, with *Arabidopsis* gene number, ratio for both the petal and leaf experiment, P-values for the t-test for both the petal and leaf experiment and the origin of clone.

6.4 Discussion

6.4.1 Producing the array

The comparison of the numbers of spots flagged by Imagene as being empty between the plates of clones that were amplified by colony PCR and the plates of clones from which the plasmid was isolated before PCR amplification showed that there was no difference in the success rate of the spots regardless of the complexity and expense of the method of amplification of the clones.

6.4.2 Making the probe

6.4.2.1 MessageAmp aRNA. Message amplification was found to be essential to make high quality probes from wallflower RNA. Without amplifying the mRNA, 20μg of total RNA was needed for each Cy dye labelling reaction. However, using this amplification technique, the 5μg of total RNA from each age and type of tissue needed for the MessageAmp aRNA reaction provided enough aRNA to make all of the probes used in this experiment, and only $^1/_6$ to $^1/_{12}$ of the total amount of aRNA made by the reactions was used. This represents a considerable saving of time in extracting RNA from the wallflower tissue, which is a time consuming protocol, especially as several extractions would be needed from each of the developmental stages of leaves and especially petals in order to obtain the amount of RNA needed to make the probes without amplification. The quality of the probes produced using the MessageAmp aRNA kit was considerably higher than that of the probes made directly from total RNA, as the amplified probes hybridised successfully to the array, whereas the unamplified probes hybridised poorly, and did not produce any meaningful or measurable results (appendix 4). This could be due to the large amount of rRNA present in total RNA inhibiting the labelling reaction by preventing the active components of the reaction from interacting with each other. Also, the reaction using the aRNA could be primed with both anchored oligo (dT) primers and the random nonamer primers, whereas reactions using total RNA could only be primed with the former. It is likely that more probe molecules are produced when both priming methods are produced compared to only one, and the probes produced equally represent the 3', middle and 5' portions of the mRNAs rather than being biased towards the 3' ends of the mRNAs, as would be the case when only using oligo (dT) priming, providing further explanation of why the probes produced from aRNA are of

better quality than those produced from total RNA. The level of amplification observed in this experiment was between 250 and 500 times the original amount of RNA present. However, 2000-fold amplification of the starting amount of RNA has been achieved in a single round of amplification using a similar method (Eberwine *et al.*, 1992; van Gelder *et al.*, 1990).

6.4.2.2 cDNA post labelling with CyDyes. The petal probe and the leaf probe labelled with the Cy3 and Cy5 dyes in one orientation gave similar results to the petal and leaf probes which were reverse labelled, with regard to the background corrected ratios of gene expression in young compared to old tissue. This indicates that the two dyes were incorporated into the two halves of each probe approximately equally, as should be the case when using post-labelling. For future reference, to experimentally confirm whether this is the case, more controls would need to be included in the experiments. If mRNA corresponding to one of the ALIEN control spots had been spiked in equal amounts into both the Cy3 and Cy5 RNA making up the probe, this control spot should give a ratio of 1 between the two channels if both halves of the probe labelled equally, giving an accurate measure of the efficiency of the two labelling reactions.

6.4.3 Detection and analysis

6.4.3.1 Image analysis. Due to the positions of the marker spots and the blank spots, the slide was visually identical in both orientations: the right orientation for fitting the template and the 'upside-down' orientation for fitting the template. Should the experiment be repeated, image analysis could be made quicker and easier by printing the marker spots asymmetrically, so that the correct orientation could be identified visually.

6.4.3.2 Data analysis. Had the control described in section 6.4.2.2 been included, normalisation of the slide would have been possible prior to data analysis. However, there would still be the concerns that the measurement of the amount of spike added would be subject to the same errors as the measurement of the RNA samples themselves, and that such a spike might not show the same purity and integrity problems of the actual RNA samples – thus potentially giving a false normalisation, however, it could help identify problems with chemical contaminants. There is also

the problem of the normalisation factor not being constant across the whole signal range (Tseng *et al.*, 2001), but this can be overcome by using several similar normalisation factors calculated from different spots, with each different spike present at a different concentration in the two RNA samples, producing a standard curve of normalisation factors spanning the whole signal range. Similarly, different amounts of the same spike could be added to each of the two samples within a probe so that the dynamic range of the expression ratios could be established by means of a standard curve. Normalisation would be best achieved using the signal ratio from genes expressed at the same level in both signal and control channels, or using total signal, rather than external spikes. Although this experiment has identified genes that do not change in their expression between the young and old leaf and petal stages defined in this study, further characterisation of the expression patterns of these genes in other developmental stages would be necessary to establish whether their expression does remain unchanged throughout development before they could be used as controls in this way, and several different genes would need to be identified to normalise across the whole signal range. Total signal is inappropriate in this instance, as described in section 6.2.4.2. Thus, as these two preferred options for normalisation remain impossible at present, normalisation using external spikes is the only remaining option, and should be used in future experiments at several concentrations across the signal range and across the dynamic range of expression ratios.

The minimum and maximum values set for the expression level filter of 0.667 and 1.333 respectively identified genes that changed in expression by 30% between the signal and the control. This ratio is slightly lower than the ratio of 1.5 (50%) used by Swidzinski *et al.* (2002), allowing a buffer for those genes that just fall short of the 1.5-fold mark. Those genes within this buffer zone could be removed at a later stage if necessary and if not filtered out by the t-test, but this approach retained as much information as possible, which was particularly important as the slides have not been normalised.

The level of replication of spots per clone on the slides was good, and four slides were probed per experiment, giving replication between slides as well as between spots. Two slides were hybridised to the same probe, comparing differences due to printing between the two slides and measuring the effect of the orientation of the slides during

hybridisation. No effect was seen on the efficiency of hybridisation with regard to which was the 'bottom' slide and the 'top' slide. The other two slides in the experiment were hybridised with a probe labelled in the reverse orientation to the first, removing the effect of any bias introduced by the labelling reaction. The correlation coefficients for the four slides in each experiment indicates that the replication between the slides within an experiment was good (see tables 6.3.3.2.1 and 6.3.3.2.2), particularly so in the petal experiment. The correlation coefficients for the pairs of slides hybridised with the same probe are somewhat higher than those for two slides hybridised with different probes within the experiment, as would be expected, but the difference is not great. The two probes used for each experiment were made with RNA pooled from up to three different developmental stages, with the RNA from each developmental stage extracted separately, and in the case of young leaves, where only one developmental stage was used, RNA from several different extractions was pooled. This replicates and averages the effect of different batches of extractions and different collections of plant material. The plant material used for different RNA extractions for the probes was collected separately, but within the same season, so as not to introduce an additional variable into the experiment. Although wallflower morphology during development was compared seasonally, with no differences seen, it is still possible that there may be differences in gene expression. A separate experiment would be needed to ascertain whether or not this is the case.

6.4.4 Results of analysis

The negative controls on the array gave no signal above background. Thus, the blank spots and 'no DNA' spots showed that there was no DNA carryover during the printing process, and the DNA of non-plant origin showed that hybridisation was specific. The marker spots indicated that the DNA had, indeed, bound successfully to the slide surface and were useful in spotfinding. The four positive controls gave the expected results in all cases. *SAG12* was upregulated in both old leaves and old petals, as was shown to be the case using northern blots (chapter 4). Tubulin was downregulated with age in petals, as was expected from the preliminary results obtained from the RT-PCR (chapter 5), and did not give significant or reproducible results for leaves, but preliminary RT-PCR results show it to be expressed in both young and old leaves (chapter 5). The microarray also demonstrated α tubulin (a control gene from *Arabidopsis*) to reproducibly show no change in expression in leaf

tissue (with no reproducible results in petals). *P1 F4* (a peptide transporter) was upregulated in both old leaves and petals. As this SSH clone was derived from the petal subtraction, it would be expected to be upregulated in old petals, but nothing further is known about its expression pattern in leaves. *LP H9* (a CCR4-associated protein) was upregulated in petals with no significant or reproducible results in leaves. This is interesting, as this clone was derived from the leaf subtraction, and so from this, would be expected to be upregulated in leaves. Nothing further is known about the expression pattern of this gene in petals. An useful control to add to the array for future reference would be some polyA spots. These would act as a control against the polyA tails of the mRNAs being reverse transcribed within the probe and hybridising non-specifically to the polyA tails that may be present on the mRNAs that have been cloned and spotted. Other useful controls that could be included in future array experiments have been described in sections 6.4.2.2 and 6.4.3.2.

Of the empty clones that did not represent negative controls, possible reasons for their lack of hybridisation include failure to pick a bacterial colony successfully, poor quality PCR product, or that the gene represented by that clone may not be expressed in the tissue under investigation. The poor replication of some clones may have been due to differences between spot replicates (such as poor or inconsistent binding of some replicates spotted onto the slide), local differences on the slide surface during hybridisation (such as small air bubbles) or differences between slides.

The genes that were found to be significantly and reproducibly expressed in both leaves and petals represent transcripts which are shared between the two organs. The processes that these genes are responsible for are therefore active in both organs, indicating similarities in late developmental processes. Thus, a lot of information can be gleaned from the lists of shared gene expression, especially if key genes involved in leaf senescence are also upregulated with age in petals. However, the same does not apply to the lists of genes that are 'exclusively' expressed in one of the two organs. There will, of course, be some genes that are expressed in one organ but not the other, even if late developmental processes are found to be equivalent, as the functions and therefore some of the contents of the two organs are distinct. For example, leaves must breakdown and detoxify chlorophyll as they senesce, whereas

this is not a problem for petals. The ribulose bisphosphate carboxylase small subunit gene was found to be downregulated in senescing leaves, as would be expected as photosynthesis declines, but did not give significant or reproducible expression data in petals, which can be explained as these are not photosynthetic organs. Similarly, the petals contain bright purple pigments that the leaves do not have, and so are likely to express genes concerned with their formation and breakdown that will not be expressed in leaves. However, it is also possible that a gene recognised by the microarray as being significantly and reproducibly upregulated in only one organ is, in actual fact, also upregulated in the other organ, but was not detected by the microarray due to poor replication or as it is only upregulated to a small degree and the microarray is not sensitive enough to detect the change in expression, particularly without slide normalisation. Indeed, many of the genes identified as being upregulated significantly and reproducibly only in petals were known SAGs from *Arabidopsis* leaves with known roles in leaf senescence, and as such are likely to be involved in wallflower leaf senescence. Thus, the fact that known *Arabidopsis* leaf SAGs are showing upregulation in late wallflower petal development is further indication of shared processes between the two organs. There were comparatively few genes that were identified by the microarray as uniquely upregulated in leaves compared to in petals. These considerations should be borne in mind when considering the lists of genes produced by the analysis, so as not to read too much into the fact that the expression of certain genes may appear not to be shared between the two processes. Further characterisation of the expression of such genes in both tissues would be necessary before any reliable conclusions can be drawn on gene expression that is unique to one of the organs.

In the list of genes whose expression is upregulated with age in both leaves and petals of wallflowers and the list of genes with upregulated expression only in old wallflower petals but known to be involved in leaf senescence in other species, a number of different classes of genes were represented and are summarised, with examples, in table 6.4.4.1.

Functional class	Example genes	Thesis section
Macromolecule breakdown		1.5.4
Proteins	Cysteine protease	1.5.4.2
	Aspartic protease	
	Clp protease	
	SRP (signal recognition particle)	
	Peptide transporter	(5.4)
Nucleic acids	Bifunctional nuclease	1.5.4.3 (5.4)
	RNAse	
Lipids	Hydrolase	1.5.4.4
	Lipase	
	Lipoxygenase	
	Lipid transfer protein	(5.4)
	Phospholipase D	
	NADPH quinone oxidoreductase	
	Carnitine racemase	
Carbohydrate	β- glucosidase	1.5.4.5 (5.4)
Cell walls	Pectinesterase	1.5.3.6 (5.4)
	Xylosidase	
	Xyloglucan transferase	
Signalling and control		
Ethylene synthesis	ACC oxidase	1.4.2.3.1
Jasmonic acid synthesis	12-OPDR (oxophytodienoate reductase)	1.4.2.3.3
	Lipoxygenase	
Calcium signalling	Inositol polyphosphate 5-phosphatase	(5.4)
	Phospholipase C	1.4.3.1
Phosphorylation	POP dikinase	1.4.3.2 ; 1.5.4.2
Transcriptional control	WRKY transcription factors	1.4.5
	CCR4-associated protein	(5.4)
Other		
Stress response	Harpin induced	1.5.5.2
	γ glutamyl transferase	(5.4)
	Chitinase	
	ERD1 (early response to dehydration)	
	Histone H1-3	
	Lipid transfer protein	
	Alcohol dehydrogenase	
Antioxidants	Catalase	1.5.5.1
	Glutathione peroxidase	

Metal-binding proteins	Copper homeostasis factor	1.5.5.3
	Metallothionein	
	Blue copper-binding protein	
Unknown function	Cytochrome P450	1.5.5.3 (5.4)
	LEA (late embryogenesis abundant) proteins	

Table 6.4.4.1 Table summarising the different functional classes of the genes active in both leaf and petal late development. Examples of genes found by the microarray to be upregulated in both leaf senescence and late petal development or only in late petal development but derived from senescing *Arabidopsis* leaves are given, with reference to the section of thesis containing further information on that gene or class of genes.

Many genes with a known involvement in leaf senescence have appeared in lists of old petal upregulated genes. Genes involved in the breakdown of all kinds of macromolecules are upregulated in old petals, as well as genes involved with remobilisation, such as a peptide transporter and lipid transfer protein, indicating that senescence processes may be active in wallflower petals, as there is evidence of recycling the contents of the organ. Cysteine and aspartic proteases have been reported as being senescence-enhanced in many systems (Gepstein *et al.*, 2003; Lohman *et al.*, 1994; Smart, 1994; Drake *et al.*, 1996; Buchanan-Wollaston and Ainsworth, 1997; Weaver *et al.*, 1998; Kinoshita *et al.*, 1999; section 1.5.4.2). Clp proteases are ATP-dependent serine proteases, often localised to the chloroplast (Adam *et al.*, 2001; Miller *et al.*, 1999; Weaver *et al.*, 1998; Nakashima *et al.*, 1997; Lohman *et al.*, 1994; Kiyosue *et al.*, 1993), where 90% of the remobilised nitrogen comes from (Smart, 1994). They have also been found in the non-photosynthetic plastids of flower petals (Peltier *et al.*, 2004) as well as in mitochondria (Peltier *et al.*, 2004; Adam *et al.*, 2001), and increased expression is associated with leaf senescence in *Arabidopsis* (Nakabayashi *et al.*, 1999; 1997). The Clp C subunit is the ATPase subunit of a chloroplast located protease (van Doorn *et al.*, 2003). However, despite *ERD1* (which encodes the Clp C subunit) mRNA levels increasing with senescence, levels of the protein fall strongly with increasing age (Weaver *et al.*, 1999). The SRP is an ubiquitous system for the targeting of membrane and secreted proteins (Groves *et al.*, 2001) and its expression during senescence may be involved with the targeting of some SAGs, such as the enzymes for macromolecule breakdown, to specific areas of the cell. SRPs are known to be involved in chloroplast biosynthesis in the targeting

of LHCP to the thylakoid membranes (Klimyuk *et al.*, 1999; Schuenemann *et al*,. 1998), and thus may similarly be involved in chloroplast transdifferentiation.

Increase in nuclease expression is, again, a commonly documented event during senescence (Gepstien *et al.*, 2003; Buchanan- Wollaston, 1997; Green, 1994; Taylor *et* al., 1993; Blank and McKeon, 1991; 1989; section 1.5.4.3).

Lipids can be hydrolysed by a variety of enzymes, including hydrolase, lipase, liopxygenase and phospholipase D (Gepstein *et al.*, 2003; He and Gan, 2002; Hong *et al.*, 2000; Thompson *et al.*, 1998; Fan *et al.*, 1999; 1997). The products of LOX action can also be converted into precursors of JA (Vick and Zimmerman, 1984), and thus may have a regulatory role in senescence (He *et al.*, 2002) and the products of phospholipase D action may also play a role in lipid signalling (Wang, 2004). Genes which may be involved in the breakdown of toxic products of macromolecule breakdown, such as the NADPH quinone oxidoreductase, certain types of which break down one of the major toxic products generated by lipid peroxidases (Mano *et al.*, 2002), show the importance of retaining cell viability throughout late developmental processes. Carnitine racemase functions in carnitine metabolism in *E.coli* (Eichler *et al.*, 1994) and L-carnitine plays a major role in eukaryotes in transporting fatty acids into the mitochondira (Kunau *et al.*, 1995). Fatty acids are known to be respired during senescence (sections 1.5.3.3 and 1.5.4.4), and although there is evidence that they are also converted to sugars by the glyoxylic acid cycle in the glyoxysome (figure 1.5.4.2.1), this does not preclude the idea that they may also be transported directly into the mitochondria. It is also likely that lipid metabolism is not just a symptom of senescence, but also a factor in regulating its progression (He and Gan, 2002).

The β-glucosidase enzyme hydrolyses the terminal non-reducing β-D-glucose residues with the release of β-D-glucose (Zorb *et al.*, 2004), with sugars being important for translocation, and also sugar levels have been implicated in the control of senescence (Rolland *et al.*, 2002; Yoshida *et al.*, 2002*c*; Xiao *et al.*, 2000; Quirino *et al.*, 2000; Dai *et al.*, 1999; Bowling *et al.*, 1997; Jang *et al.*, 1997). Enzymes involved in cell wall breakdown have also been previously reported to show senescence-enhanced

expression (Gepstein *et al.*, 2003; Fujiki *et al.*, 2001; Yoshida *et al.*, 2001; Obergon *et al.*, 2001; Park *et al.*, 1998*a*).

Many genes employed in the signalling and control of leaf senescence, at all levels from external signalling to inter- and intra-cellular signalling, are also upregulated during late petal development. This indicates that there may be shared mechanisms for controlling the two processes, which stands to reason if the two processes themselves are similar. Genes involved in the synthesis of both ethylene and jasmonic acid are upregulated during late development, and both hormones are known to be involved in regulating senescence (sections 1.4.2.3.1 and 1.4.2.3.3). Genes involved with calcium signalling (section 1.4.3.1) are also upregulated, which is an important signalling mechanism in all kinds of programmed cell death in plants (Jones, 2001; Cheng *et al.*, 1983), and so the fact that this signalling mechanism is shared does not help answer the question of whether or not wallflower petals senesce. Similarly, phosphorylation is a common regulatory mechanism, which is not unique to senescence and although involved in senescence, the transcriptional control genes are not unique to the process either. Therefore, although indicating that control mechanisms are shared between leaf senescence and late petal development, the presence of these genes does not provide evidence for senescence in petals.

Stress response genes, as well as being upregulated during leaf senescence, are also expressed in old petals, again showing similarities between the two processes. This is concordant with the results of a microarray study of *Iris* tepals, where the expression of many stress related genes is upregulated during late development (van Doorn *et al.*, 2003). It is possible that these genes may have a causative role to play in the process, or that they are upregulated as a pre-emptive measure to protect the old tissues in their compromised state, thus allowing them to remain viable for the maximum time possible and allow the maximum possible recovery of nutrients. These include a harpin induced gene, induced in response to the harpins produced by bacterial pathogens (Desikan *et al.*, 2001; Lindgren, 1997; He *et al.*, 1994; Baker *et al.*, 1993), and is also upregulated during leaf senescence in *Aradidopsis* (Gepstein *et al.*, 2003; Yoshida *et al.*, 2001), chitinase, which breaks down the chitin in the cell walls of fungal pathogens, *ERD1* which is expressed in response to dehydration and histone H1-3 which is also induced by drought (Deleu *et al.*, 1999; Ascenzi and Gantt, 1997).

Recent studies indicate that lipid transfer proteins may not have the role that they were initially thought to have and were named after, but may be involved in plant resistance to biotic and abiotic stresses (Blein *et al.*, 2002; section 5.4.2.5). As well as being upregulated during senescence (Lohman *et al.*, 1994), alcohol dehydrogenase (ADH) gene expression is induced under anaerobic conditions (Imhori *et al.*, 2004; Perata and Alpi, 1993), as well as in pollen (Bucher et al., 1995) and in ripening fruit (Chervin *et al.*, 1999; Sarni-Manchado *et al.*, 1997; Longhurst *et al.*, 1994). Similarly, expression of antioxidant genes, such as catalase and glutathione peroxidase, which is also upregulated in response to oxidative stress (Navabpour *et al.*, 2003), are upregulated during natural late developmental processes, possibly to protect cell viability at a time when macromolecule breakdown results in increased ROS activity (section 1.5.5.1).

There are also leaf SAGs of unknown function, or whose roles in senescence are unknown that are also upregulated in late petal development. These include the metal binding proteins like metallothioneins, which also increase in expression during leaf senescence of *Arabidopsis* (Gepstein *et al.*, 2003; Cobbet and Goldsbrough, 2002; Miller *et al.*, 1999; Lohman *et al.*, 1994), *Brassica napus* (Buchanan-Wollason and Ainsworth, 1997; Buchanan-Wollaston, 1994) and rice (Gibbings *et* al., 2003; Hsieh *et al.*, 1995), as well as in old rose (Channeliere *et al.*, 2002), daffodil (Hunter *et al.*, 2002) and *Alstroemeria* (Breeze *et al.*, 2004) petals. Metallothioneins, and a variety of other proteins such as CCH (copper chaperone) and BCB (blue copper-binding protein) are involved with metal ion binding, with metallothioneins binding a range of divalent cations, (Buchanan- Wollaston, 1997) and the other two binding copper ions (Miller *et al.*, 1999; Himelblau *et al.*, 1998; Lohman *et al.*, 1994; van Gysel *et al.*, 1993) and BCB also implicated in defence against aluminium toxicity (Ezaki *et al.*, 2000). It is possible that they may sequester the metal ions released as proteins are broken down, such as the copper released from pigment complexes and electron carriers, either for the detoxification of the metals or alternatively, they may even be involved in storage and transport, so that the metals can be recycled (Buchanan-Wollaston, 1997). Indeed, levels of many metals (Mo, Cr, Fe, Cu and Zn) were reduced by over 40% in senescent leaves compared to green leaves (Himmelblau and Amasino, 2001). There is also evidence to suggest that metallothioneins may protect DNA from oxidative damage by free-radicals in mammalian systems (Chubatsu and

Meneghini, 1993). So it is possible that they may have a similar role in the oxidative process of leaf senescence, as chlorophyll and membrane breakdown produces free-radicals (Buchanan- Wollaston, 1997) and there is also the consideration that free metal ions could cause oxidative damage (Himelblau and Amasino, 2000). Again, this would help the cell remain viable for longer, allowing maximum recovery of nutrients. Three different cytochrome P450 genes are expressed exclusively in senescent *Brassica napus* leaves (Buchanan-Wollaston, 1997) and upregulated during the late development of daylily petals (Panavas *et al.*, 1999). Cytochrome P450s are a diverse family of proteins, important in many plant biochemical pathways, the detoxification of many compounds and in the biosynthesis of many products, including hormones, lipids, secondary metabolites, such as flavonoids, phytoalexins and lignin, and plant growth regulators, such as gibberellins (Chapple, 1998; Buchanan-Wollaston, 1997; Schuler, 1996). The role of these enzymes in senescence is not yet understood. The LEA protein family are thought to be involved in protecting the developing seed from dehydration stress, and have also been found in adult plants, often in response to stress or hormones (Miller *et al.*, 1999; Gosti *et al.*, 1995; Naot *et al.*, 1995; Speulman and Salamini, 1995; Galau *et al.*, 1993; Yamamoto *et al.*, 1992), and their expression in senescence could therefore be a stress response, protecting the organ during late development. However, their expression pattern in *Arabidopsis* shows a peak at or shortly before visible senescence, followed by a decline in expression (Weaver *et al.* 1998), which concords with the class 4 expression pattern of regulatory genes described by Smart (1994) (section 1.5.2, table 1.5.2.1), implicating this gene in the regulation of senescence.

As well as there being many genes that are similarly regulated in the two organs, there were also some that had opposing expression patterns in leaf development compared to petal development. No genes were identified that were significantly and reproducibly upregulated in leaves and downregulated in petals, but there were genes that were upregulated in petals and downregulated in leaves. Of the genes with this expression pattern that had a known or putative identity, all represented GSTs. It is therefore possible that these enzymes have a role to play in late petal development, but are downregulated during leaf senescence. However, expression of GST genes is known to be upregulated with age in maize leaves (Smart *et al.*, 1995; Jepson *et al.*, 1994) and is upregulated with age and in an ethylene dependent manner in

Arabidopsis (Zhou and Goldsbrough, 1993) and one of the GST genes found to be upregulated in wallflower petals and downregulated in leaves (*GST1*) was a SAG from *Arabidopsis* leaves. Thus, it would seem more likely that GSTs are also upregulated in wallflower leaves during senescence, but they may either have been upregulated earlier than the stages defined here as old, be upregulated at a later stage than those defined here as old, or they may be upregulated in one of the stages defined here as old, but may have very low expression in the other stages, resulting in an appearance of down-regulation overall. This may indicate that the leaf 'old' tissue and the 'old' petal tissue are not equivalent stages, which is unlikely, due to the small number of genes with opposing expression patterns in the two tissues compared to the large number with similar expression patterns, or that the timing of expression of the same genes in different tissues can be variable. Yet another possibility is, as the GST gene family is so large and as there are several classes of GSTs, that one class is expressed as described by the microarray, but that other classes are upregulated in senescing leaves. As the GST gene family is large and varied (Dixon *et al.*, 2002), the variety roles that these proteins play in the cell is also diverse, including an involvement in secondary metabolism, tyrosine metabolism, as well as in herbicide, oxygen and radical detoxification (Cobbet and Goldsbrough, 2002; Dixon *et al.*, 2002; section 1.5.5.1). GSTs are induced in response to cytokinin hormones (Gonneau *et al.*, 1998; Marrs, 1996), and so the protective effects of this hormone may be mediated through the action of GSTs. They have also been implicated in the regulation of apoptosis (Dixon *et al.*, 2002). Thus, GSTs may have a regulatory role in senescence and may be involved in maintaining cell viability during late development, allowing programmed processes, such as senescence, to take place. It is also possible that should the expression pattern of these GSTs be confirmed by other methods of expression analysis, such as northern blots or RT-PCR, that these other methods may show that they are upregulated in both tissues. Discrepancies between the results of microarrays and other expression profiling methods have been reported by other investigators, such as Breeze *et al.* (2004), who found metallothioneins not to change in expression with age from the microarray study, but to be strongly upregulated with age according to northern blots.

With regard to the genes that do not change in expression between young and old tissues, as defined in this study, it is interesting that 51 genes reproducibly did not

change in expression in both leaves and petals, particularly as the clones were either derived from the leaf or petal SSH library or represented known *Arabidopsis* SAGs. Similar results were seen in *Alstroemeria* where metallothionein genes identified from old petal tissue by SSH showed no change in expression with age on the microarray, yet showed strong upregulation on northern blots (Breeze *et al.*, 2004). However, the SSH technique only enriched for genes with increased expression in old tissue compared to young tissue, and confirmation of upregulation is needed by other means. There is also the consideration that the SSH procedure is designed to equalise for different degrees of upregulation, and thus to identify genes which are upregulated at a very low level, as well as those that are highly upregulated. Thus, it is possible that the ratios set to define differential expression for the microarray (a 30% change in expression between young and old tissues) may exclude some of the genes upregulated to a low degree in old tissues. However, setting a lower ratio for this experiment, without slide normalisation, would have been inappropriate. Many of the genes that did not show any reproducible increase in expression in leaves that have known or putative identities are upregulated during *Arabidopsis* leaf senescence and have known roles in the process. In such cases there is also the consideration that these genes may be expressed at similar levels in the developmental stages defined in this study as 'young' and 'old', but further details of their expression pattern are, as yet, unknown. Their expression may increase in mature tissues, and fall again in old tissues, or their expression may increase only in very old tissues. It is also possible that their expression increases in one of the developmental stages included within the old tissue and not the others, but that this increase is not seen, as the effect of averaging several different developmental stages masks it. There is also the possibility of cross-hybridisation between different members of gene families. If some members of that gene family are decreasing in expression with age and some members are upregulated in late development, the results seen on the array would be that there is no overall change in expression. Further characterisation of the expression patterns of these genes would be necessary to establish whether they are constantly expressed throughout development, or if one of the other possibilities is the case.

7 RT-PCR

7.1 Introduction

In order to verify the results of the microarray experiment, and to discover more about the expression patterns of some of the genes showing enhanced expression during late development in petals, they were subjected to analysis by semi-quantitative RT-PCR. This technique was chosen over northern analysis, which has given successful results with wallflower petal and leaf RNA (chapter 5), because less RNA is required for RT-PCR than for northern blotting, which is an important factor in this study, as wallflower RNA is difficult and time-consuming to extract.

This technique involves running similar PCR reactions on the same starting amount of cDNA from each developmental stage for a limited number of cycles and comparing the intensities of the resulting PCR products, as visualised under UV light on an ethidium bromide stained agarose gel. As the same amount of cDNA was used in each PCR reaction, the intensity of the band is therefore proportional to the amount of message present, providing a measure of gene expression. Limiting the cycle number ensures that the reaction remains in exponential phase, and thus differences in band intensity result from and are directly proportional to actual differences in the number of transcripts in the initial mRNA population. If too low a cycle number is used, the exponential phase will not have been reached, and overcycling will result in stationary phase being reached, and in each case, a change in band intensity of the PCR product will not result from a change in the mRNA template. The intensities of the bands within an experiment are comparable directly to each other, making it possible to show whether genes are upregulated or downregulated in older tissues relative to younger ones. However, this method only gives relative, and not absolute, quantification of expression levels, as the number of cycles chosen are estimated and may not be fully accurate, making the technique only semi-quantitative.

The genes chosen for this investigation were *SAG12*, tubulin, *LP H9* (CCR4-related protein, chapter 5) and *P1 F4* (peptide transporter, chapter 5). All four genes chosen for RT-PCR were used as positive controls on the microarray experiment (chapter 6), and the reasons for their choice as microarray controls (section 6.2.1) were also good

reasons for selecting them for RT-PCR analysis. In addition, the microarray data for these four clones is particularly reliable, because, as positive controls, they were replicated on the array many more times than the other test clones (tables 6.2.1.1 and 6.2.1.2 give details of replication of test clones and controls, respectively). *SAG12* was chosen as a positive control for the RT-PCR experiment, as its expression pattern in the leaves and petals of wallflowers are known in detail from northern analysis (chapter 4), it shows significant and reproducible up-regulation in both organs on the microarray (chapter 6), as would be anticipated from the results of the northern analysis, and as RT-PCR reactions using wallflower cDNA from both petals and leaves with the *SAG12* primers had worked to test the ligation efficiency of the SSH (chapter 5). Similarly, the tubulin primers were also known to work with both leaf and petal cDNA from wallflowers, as a preliminary RT-PCR using them had worked for the ligation efficiency analysis of the SSH (chapter 5), although these primers were not eventually chosen to test for ligation efficiency. The tubulin clone gave a significant and reproducible result in the petal microarray experiment (chapter 6) showing the gene to be downregulated in old petals, as would be predicted from the preliminary result of the SSH RT-PCR reaction (chapter 5). Despite the high level of replication of the spots, the tubulin gene did not give significant and reproducible results in the leaf microarray experiment (chapter 6), however, the preliminary results from the SSH RT-PCR show that the tubulin gene is more highly expressed in young leaves than in old (chapter 5). *LP H9* and *P1 F4* were both chosen as they were derived from the SSH libraries (chapter 5), from old wallflower leaves and old wallflower petals respectively, and so there is a representative wallflower clone derived from each organ being tested for its pattern of expression. Both have been sequenced (chapter 5) and show homology to a CCR4-associated protein and a peptide transporter, respectively. *LP H9*, as tubulin, gave a significant and reproducible results in the petal microarray experiment, but not the leaf (chapter 6), however, unlike the tubulin, it was upregulated in old petals. *P1 F4*, like *SAG12*, gave a significant and reproducible result in both the leaf and petal microarray experiments (chapter 6), and like *SAG12*, was upregulated in both tissues with increasing age. *LP H9* is involved with transcriptional control of genes in *Arabidopsis*, usually causing activation of genes (Denis and Malvar, 1990), and thus appears to be involved with the regulation of SAGs. Thus, establishing its expression pattern in wallflower petals will show whether it may also be involved with regulating gene expression in late development in these organs. It is likely that if the same

regulatory mechanisms are used, then the same processes may be at work in leaves and petals. Peptide transport is associated with protein hydrolysis and recycling in senescing leaves (Higgins and Payne, 1981), which is a defining characteristic of senescence. Thus, establishing the expression pattern of a peptide transporter gene (*P1 F4*) throughout petal development may indicate whether petals also transport peptides during late development, thus hydrolising recycling proteins, which would characterise senescence in petals.

As no genes are known to remain constantly expressed throughout development, *puv* primers, which amplify a 488 base pair region of the 18S rRNA, were tried as a normalisation control between samples of different developmental stages, as they should not vary in intensity with age.

7.2 Materials and Methods

RNA from each developmental stage of wallflower petals was quantified, and an equal amount of RNA from each stage used to make cDNA (described in sections 2.2.3 to 2.2.6, 2.2.9, 2.2.11 and 2.2.12).

Each of the PCR reactions described used 0.625 units of Taq polymerase (Qiagen Ltd., Crawley, UK), Qiagen buffer, 0.5µl of the appropriate cDNA, 1.5mM $MgCl_2$, 0.2mM dNTPs and 0.4mM each of the two primers under investigation (*SAG12 F* and *R*, and tubulin primers [*Pos 63* and *Neg 2*] see 5.2.5 for details; *LP H9 F*: 5'-GTTTGGACCGGGTTGCTC-3', *LP H9 R*: 5'-ACTCCGGCGTGTTTCACC-3', which amplify a 130bp fragment of the gene; *P1 F4 F*: 5'-AGAGCTTCGGAAGCGCTCTG-3', *P1 F4 R*: 5'-AGGTACCACTTTGCACATGC-3', which amplify a 219bp gene fragment). Normalisation controls were performed using the *puv* primers (*puv2*: 5'-TTCCATGCTAATGTATTCAGAG-3'; *puv4*: 5'-ATGGTGGTGACGGGTGAC-3'; Dempster *et al.*, 1999; which amplify a 488bp fragment of 18S rRNA). Reactions were cycled in a Perkin Elmer 2700 thermocycler for the appropriate number of cycles for that primer pair (established below), under the conditions 15 mins 95°C hotstart, {95°C 10 seconds, 65°C 30 seconds, 68°C 2.5 mines} for the *SAG12*, *LP H9* and *P1 F4* primers, and 15 mins 95°C hotstart, {95°C

10 seconds, 50°C 30 seconds, 68°C 2.5 minutes} for the tubulin and *puv* primers. Using the same number of cycles for each reaction done with a given primer pair is important for this method to be semi-quantitative (see section 7.2.1). The products were analysed on an ethidium bromide stained agarose gel (as described in section 2.2.10) and band intensities were quantified in arbitrary units using the Gene genius bioimaging system and GeneSnap software, both from SynGene (Synoptics Ltd., Cambridge, UK). The measurements are corrected for background using the 'auto background' function of the software, which accounts for and removes a proportion of the signal due to local background in the area immediately surrounding each band.

As a negative control, a PCR reaction containing a volume a water to replace the template cDNA was performed for each set of PCR reactions to ensure that there was no contamination in any of the PCR reagents. A negative control was also run for each individual PCR sample containing a volume of the corresponding control RT reaction which contained no RT enzyme (see section 2.2.12) as template, to ensure complete removal of genomic DNA during the DNAse treatment of the RNA prior to the RT reaction. This ensures that the band intensities measured are completely due to cDNA made from mRNA present in the original sample and not due to contaminating genomic DNA.

7.2.1 Cycle number optimisation

To establish the optimal cycle number, an equal amount of the cDNA from each developmental stage was mixed. Five 25µl PCR reactions were set up for each of the primer sets using 0.5µl of the mixed stage cDNA and one reaction was removed from the thermocycler at 18, 24, 30, 36 and 42 cycles for each primer set. The band intensities measured at each cycle number were plotted as a graph to establish the optimal cycle number for each reaction, and a cycle number from the middle of the exponential phase chosen for each primer pair as being optimal.

7.2.2 RT PCR

Once optimal cycle number was established for each pair of primers, the RT-PCR reactions were performed three times each for each pair of primers, using each petal developmental stage separately as a template.

7.3 Results

7.3.1 Cycle number optimisation

Band intensity was compared at each of the cycle numbers tested and a graph of intensity values plotted (figure 7.3.1.1). For each pair of primers, 32 cycles was found to be in the middle of the exponential phase of amplification, with the exception of the *puv* primers, where 27 cycles were optimal. However, using these optimised cycle numbers the expression of *SAG12* increased from very low expression to very high expression at an early stage of development (stage 1, see section 7.3.2) and the expression of *LP H9* showed little variation throughout development (see section 7.3.2). This may have been due to overcycling in both cases, and so no replicate reactions were run for these two primer pairs at 32 cycles, and the PCR reactions for both primer pairs were repeated using 27 cycles.

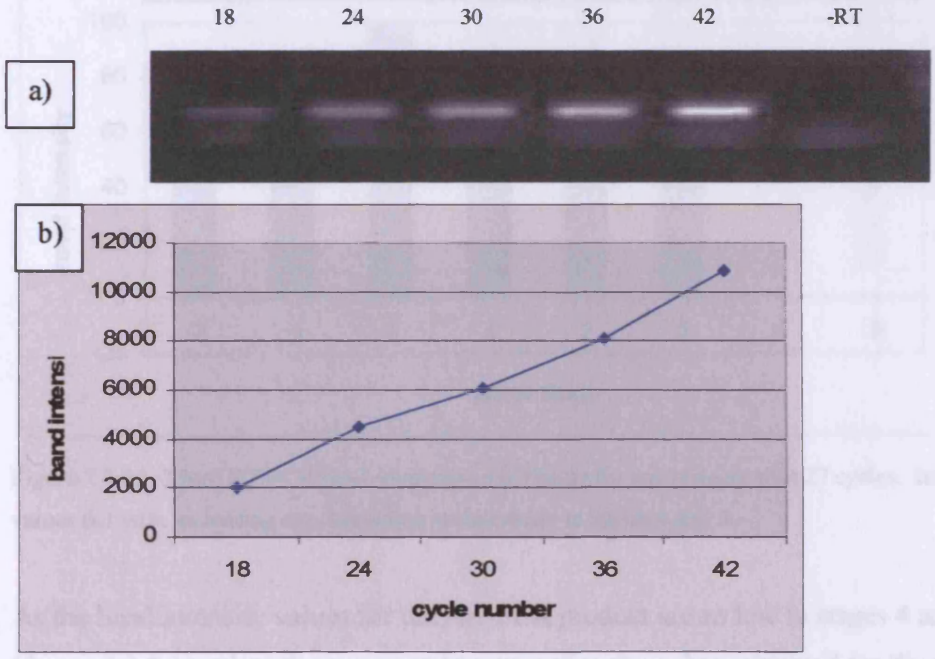


Figure 7.3.1.1 a) RT-PCR bands for *SAG12* reactions at 18, 24, 30, 36 and 42 cycles, along with the –RT control, which was made with –RT reactions for mixed stages and cycled for the maximum number of cycles (42). The bands become increasingly bright with an increased number of cycles. Nothing is amplified in the –RT control, even at this high number of cycles, indicating that there is no genomic DNA present in the cDNA. b) Graph of the intensity values for each band against the number of cycles. Each point on the graph is shown directly below its corresponding band in a).

7.3.2 RT PCR

Each primer pair was used under the optimal conditions established for their use as described in section 7.3.1, and the resulting band intensities calculated and plotted for each reaction. The intensity values returned from SynGene are arbitrary, and dependent on such factors as the exposure time and the aperture size for the gel image taken as well as DNA band intensity, and brightness and contrast can also be adjusted after the image has been taken, adding further variables. Thus, it is not possible to compare intensities between different images. To try and standardise the values for band intensity, all values in each developmental series for each primer pair were divided by the highest value in that series and multiplied by 100, giving the values for expression for each series as a percentage of the maximum expression within that series.

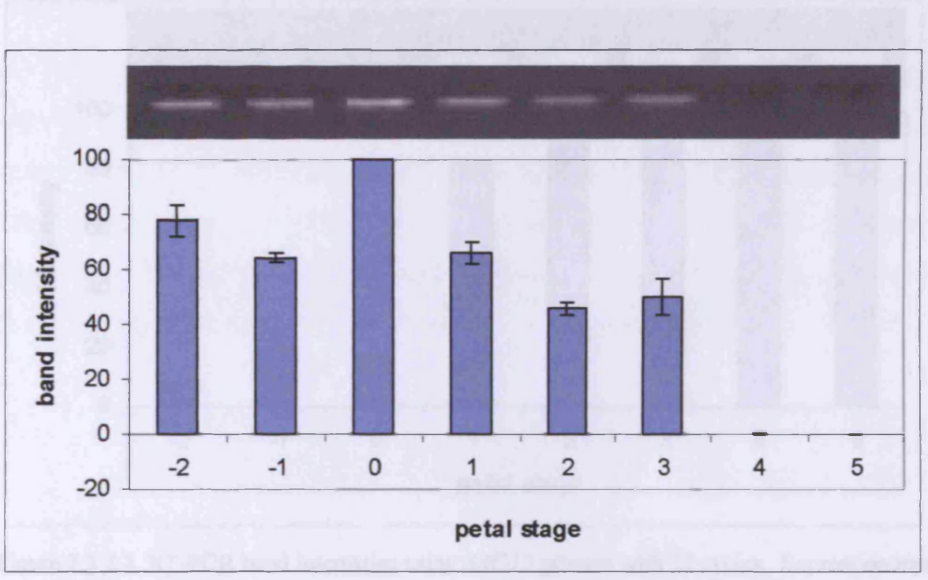


Figure 7.3.2.1 Mean RT-PCR band intensities ±SD using the *puv* primers with 27 cycles. Intensity values fall with increasing age, becoming undetectable in stages 4 and 5.

As the band intensity values for the *puv* PCR product are so low in stages 4 and 5 (figure 7.3.2.1), when these are used to normalise the values obtained for the other PCR products, the normalised expression profile of all of the genes is identical, being very low in stages -2 to 3 and artificially increased in the last two stages. Even values for tubulin, when normalised in this way, show very low expression in stages -2 to 3, then appear to be highly elevated in stages 4 and 5. Thus, the use of these results for the normalisation step was deemed inappropriate, and omitted. However, the

inclusion of *SAG12* in these experiments, for which expression data was already available from the northern analysis allowed a higher degree of confidence in the results from the other genes analysed.

SAG12 expression increased sharply at stage 1, when 32 cycles were used for the reaction, and remained high until the end of the developmental series (figure 7.3.2.2). Thus, 27 cycles (figure 7.3.2.3) was also tried for this primer pair, to see if fewer cycles gave further insight into the expression pattern of this gene. Reducing the number of cycles resulted in the sharp increase in expression occurring slightly later, at stage 2, expression continuing to rise into stage 3, then falling slowly through stages 4 and 5.

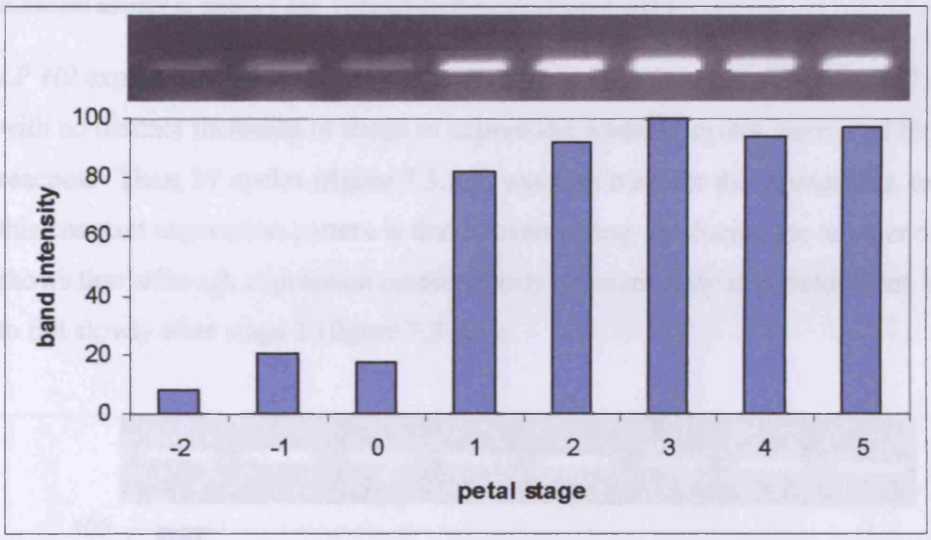


Figure 7.3.2.2 RT-PCR band intensities using *SAG12* primers with 32 cycles. Expression increases sharply at a relatively early stage (stage 1) and remains high.

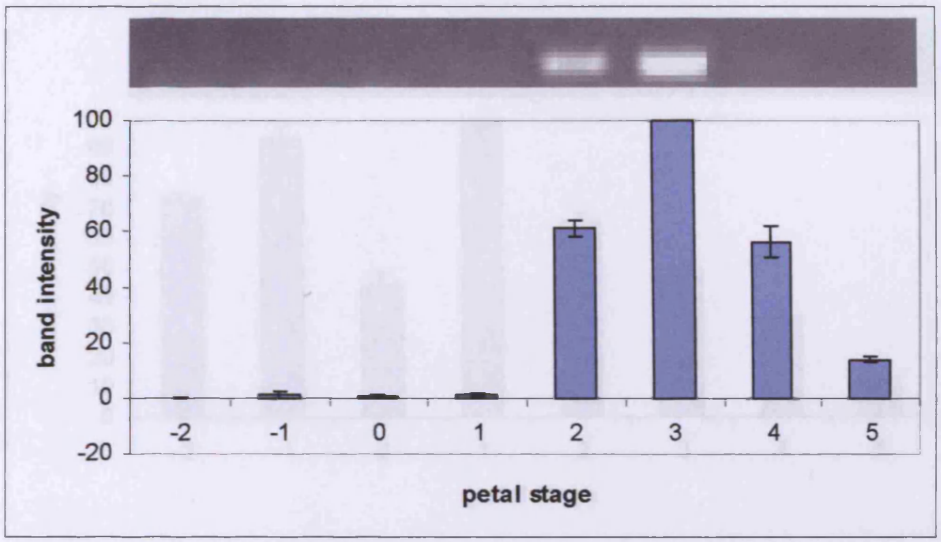


Figure 7.3.2.3 Mean RT-PCR band intensities ±SD using *SAG12* primers with 27 cycles. Expression increases sharply at stages 2 and 3, then falls through stages 4 and 5.

LP H9 expression remained fairly constant throughout development (figure 7.3.2.4), with no distinct increases or drops in expression when 32 cycles were used for the reaction. Thus, 27 cycles (figure 7.3.2.5) was also tried for this primer pair, to see if this constant expression pattern is due to overcycling. Reducing the number of cycles shows that although expression remains fairly constant early in development, it begins to fall slowly after stage 1 (figure 7.3.2.5).

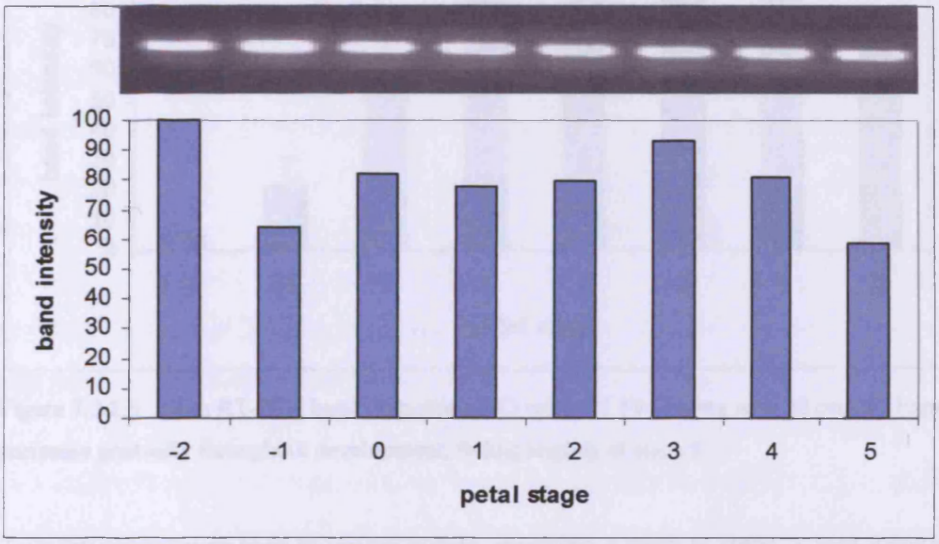


Figure 7.3.2.4 RT-PCR band intensities using *LP H9* primers with 32 cycles. Expression remains fairly constant throughout development

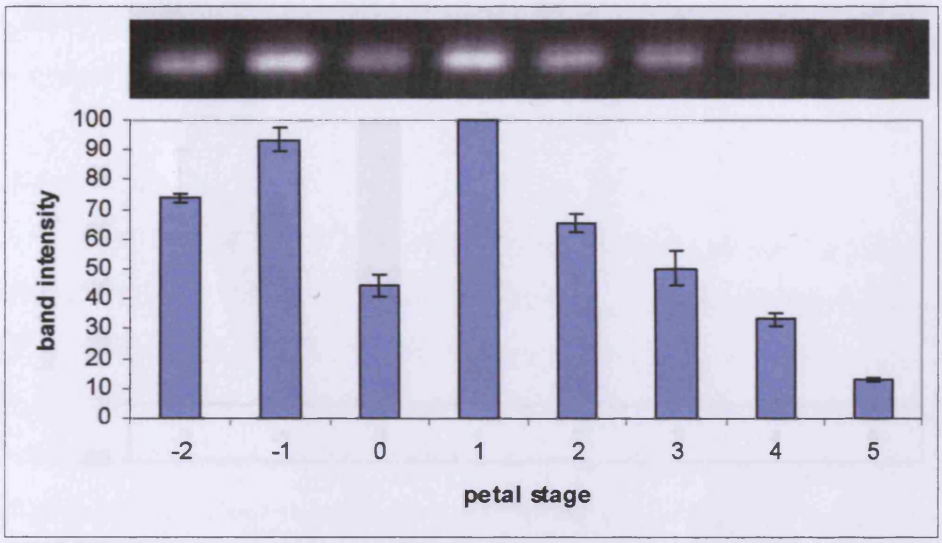


Figure 7.3.2.5 Mean RT-PCR band intensities ±SD using *LP H9* primers with 27 cycles. Expression remains fairly constant until stage 1, then decreases slowly from stage 2 to stage 5.

P1 F4 expression rose gradually from stage -2 until stage 4, then fell slightly at stage 5 (figure 7.3.2.6).

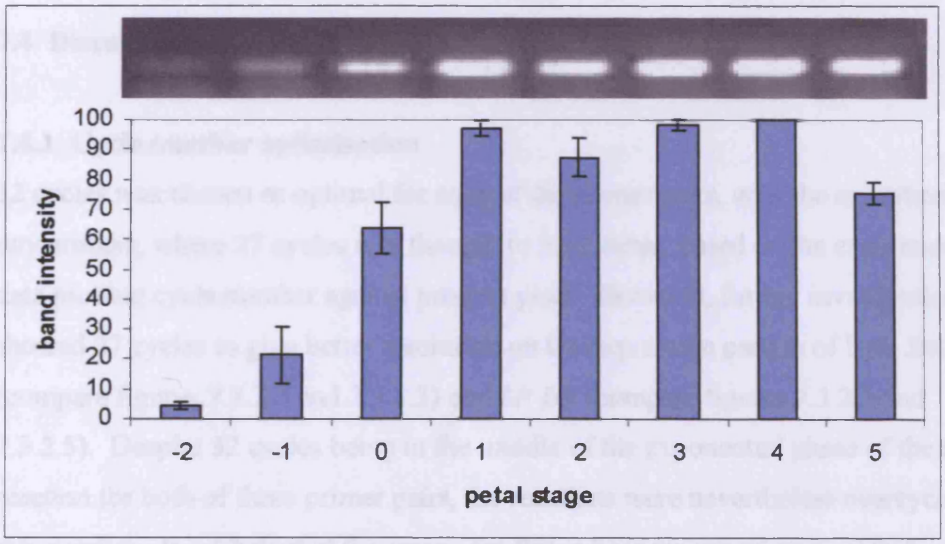


Figure 7.3.2.6 Mean RT-PCR band intensities ±SD using *P1 F4* primers with 32 cycles. Expression increases gradually throughout development, falling slightly at stage 5.

Tubulin expression rose in young petals, reaching a peak at stage 0, just prior to petal opening, then fell gradually throughout the rest of development (figure 7.3.2.7).

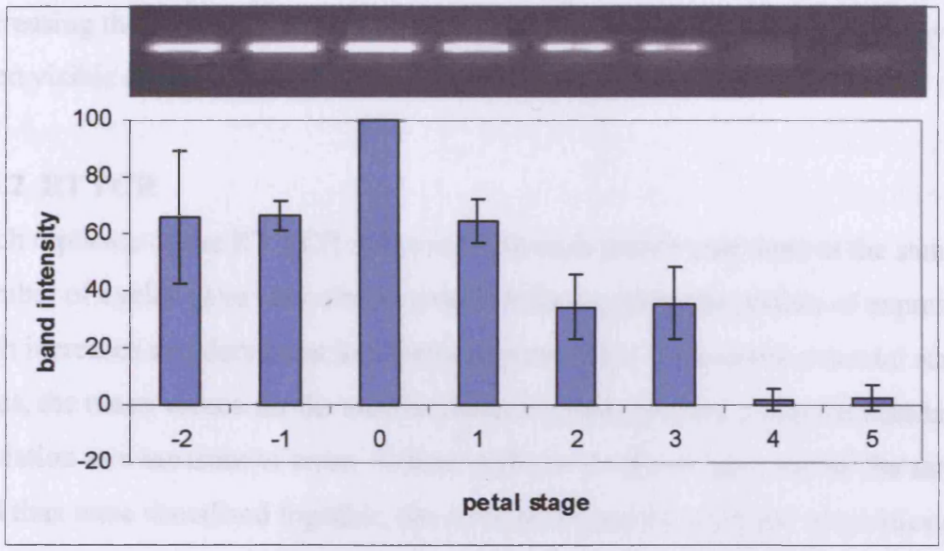


Figure 7.3.2.7 Mean RT-PCR band intensities ±SD using tubulin primers with 32 cycles. Expression increases in young petals, reaching a peak just before flower opening (stage 0), then decreases gradually as the petals get older.

The raw data for band intensities for each of the replicates of the test data are given in appendix 6.

7.4 Discussion

7.4.1 Cycle number optimisation

32 cycles was chosen as optimal for each of the primer pairs, with the exception of the *puv* primers, where 27 cycles was thought to be optimal, based on the experimental data plotting cycle number against product yield. However, further investigation showed 27 cycles to give better resolution on the expression pattern of both *SAG12* (compare figures 7.3.2.2 and 7.3.2.3) and *LP H9* (compare figures 7.3.2.4 and 7.3.2.5). Despite 32 cycles being in the middle of the exponential phase of the PCR reaction for both of these primer pairs, the reactions were nevertheless overcycled at this number. It is likely that the reason for this is because mixed stage cDNA was used for the optimisation, thus averaging the very low expression of these genes at certain developmental stage with the very high expression at other stages, and thus the reactions were overcycled in those developmental stages where expression was high. Similarly, it would appear likely that the reason for the unsuitability of the *puv* results for normalisation may be due to undercycling of the reaction. It is possible that by

increasing the cycle number for the *puv* reaction, that some PCR product may have been visible at stages 4 and 5.

7.4.2 RT PCR

Each replicate of the RT-PCR reactions with each primer pair done at the same number of cycles gave very similar results with regard to the pattern of expression, with increases and decreases in expression seen at the same developmental stage. Thus, the mean values for the standardised data were plotted, using the standard deviation as a measure of error. Where replicate reactions were run on the same gel, and thus were visualised together, the absolute values for the band intensities of the replicates were very close and showed very little variation, indicating that differences in band intensity values seen are likely to be a result of imaging differences, rather than differences in expression between replicate reactions. Should further RT-PCR experiments be run, the replicates for each reaction would be imaged together, eliminating this source of variability.

The band intensities for the *puv* primer products were found to be reasonably constant in early developmental stages, but began to fall later in development, and dropped sharply to an almost undetectable level in stages 4 and 5 (figure 7.3.2.1). As the band intensity values were so low in these stages, when used to normalise the values obtained for the other PCR reactions, the normalised expression profile of all of the genes was identical, being very low in stages -2 to 3 and artificially increased in the last two stages. From the gel images for these reactions (figures 7.3.2.2 to 7.3.2.7), it is evident that the normalised results are an artefact, and the expression patterns of the two genes with known expression profiles (tubulin and *SAG12*) clearly showed this to be the case. This primer pair under these PCR conditions is therefore unsuitable for the normalisation of these results, and this step was omitted. The most likely explanation for this artefact is that despite attempts at optimisation of the PCR reaction, the reaction was undercycled. Cycle number is difficult to estimate accurately, as proved by the fact that both the *SAG12* and *LP H9* cycle numbers needed adjusting.

SAG12 expression was shown to increase sharply at stage 2, continue to rise rapidly to stage 3, then decrease in expression in stages 4 and 5. This agrees with the results of

the microarray, which shows *SAG12* expression to increase in old petals relative to young petals (section 6.3.4) and correlates with the results of the northern analysis (section 5.3), where figure 5.3.3 b) also shows a sharp increase in *SAG12* expression at stage 2 of petal development. However, as shown by the northern blot in figure 5.3.3 b), *SAG12* expression is higher in stages 4 and 5 than it is in stages 2 and 3, which is an interesting difference to note. It is likely that the results of the northern analysis are the more reliable of the two techniques being compared, due to the problems encountered in these experiments in the normalisation of the PCR reactions. It is also possible that the products of the reverse transcription of degraded mRNAs are inhibiting the RT-PCR reactions for stages 4 and 5, resulting in an apparent decrease in expression in these two developmental stages. This is consistent with the very low band intensities obtained for the *puv* PCR product in these two developmental stages. Another possible explanation for this difference between the two techniques is that the stage 4 and 5 lanes of the northern were overloaded with RNA, due to the difficulties of estimating the concentration of degraded RNA. However, as published accounts of *SAG12* expression in leaves show that expression continues to rise from its initiation, throughout development and senescence (Lohman *et al.*, 1994), it would seem likely that the results of the northern blots in petals are more accurate, reflecting this pattern. It would be interesting here to compare the results of RT-PCR of *SAG12* in leaves with the results of the northern to see how those compare to the results obtained in petals. Thus, it is important to consider in the analysis or the other RT-PCR reactions that stages -2 to 3 gave the same results as northern analysis for the *SAG12* positive control, but that stages 4 and 5 appeared to be expressed at a lower level using RT-PCR than they did when analysed with northern blots. Increased expression of *SAG12* in late development is consistent with its role as a cysteine protease (Lohman *et al.*, 1994), involved with the breakdown of proteins, possibly for export of the resulting polypeptides from the petal, indicating senescence processes may be active in wallflower petals.

LP H9 expression remained constant until stage 1, and then gradually decreased from stage 2 to stage 5. However, if the expression in stages 4 and 5 were actually higher than seen by the result of the RT-PCR reactions, as was suggested may be the case by comparison of the *SAG12* RT-PCR results with the results of the northern analysis for *SAG12*, there would still be a drop in expression of this gene in stages 2 and 3, but

expression may increase again during very late development in stages 4 and 5. This is interesting, as the results of the microarray indicate that expression of this gene is increased in old petals, which would be consistent with the gene increasing in expression in stages 4 and 5. Even when comparing the average expressions as measured by RT-PCR for young petals (stages -2, -1 and 0) and old petals (stages 3, 4 and 5) (figure 7.3.2.5), expression is higher in the young petals than in is in old. The putative role of this gene in the regulation of transcription, and thus possibly in the regulation of SAG expression, would indicate that it may only increase slightly in expression, and this increase may be early in the senescence process. Figure 7.3.2.5 does appear to show a peak in expression at stage 1, however the difference between the expression level at stage 1 and some other developmental stage is small, and so it is difficult to tell using a semi-quantitative method whether or not this apparent peak is noteworthy. It would be interesting, therefore, to further study the expression of this gene in petals by northern analysis, to see whether the differences in expression shown in the microarray and RT-PCR experiments seen in the case of *SAG12* are also found with other genes.

P1 F4 shows a gradual increase in expression with increasing age throughout development, eventually dropping slightly in expression in stage 5. However, as this is a very small drop shown by a technique which is only semi-quantitative, it may not be noteworthy. Also, as the *SAG12* positive control RT-PCR showed a drop in expression in very late development, despite the results of the northern analysis showing this gene to continue increasing in expression until stage 5 (section 5.3), it is possible that the drop seen in the expression of *P1 F4* is also artefactual, and that expression of the gene continues increasing throughout development. The results of the RT-PCR for this gene agree with the results of the microarray (section 6.3.4), which show expression to increase in old petals compared to young petals. This is consistent with the role of this gene as a peptide transporter, indicating as peptides are broken down in late development by proteases (such as SAG12), the resulting polypeptides are transported, possibly for export from the organ and recycling to other parts of the plant.

Tubulin expression increases in petals in the closed bud, peaking just before flower opening (stage 0), then drops gradually as the petal continues to age. Even if, as

suggested by the comparison of the *SAG12* RT-PCR and northern results, the expression in stages 4 and 5 is artificially low, it is likely that this trend would still hold true. These results match the results obtained from the microarray experiment (section 6.3.4), which show tubulin expression to decrease in petals with increasing age and confirm the preliminary results seen by RT-PCR in chapter 5, where tubulin expression appeared to fall in old petals.

The problems of using a semi-quantitative method have been highlighted here by the comparison of the *SAG12* RT-PCR results with the results obtained from northern analysis of the *SAG12* clone and by the artificially low results obtained for the *puv* PCR product in very late development. However, at least for the earlier stages of development and for the tubulin expression, the RT-PCR results matched those from the microarray. Likewise, the RT-PCR results for clone *P1 F4* were consistent with those from the microarray. Thus, despite problems with the quality of the RT-PCR data for the later stages of development, the RT-PCR data for these clones is helpful in assessing the reliability of the array data, confirming that these clones which were used as positive controls for the array were showing the same overall pattern of expression. Results from the fourth clone, *LP H9*, however, were not as reassuring. Similar inconsistencies however have been reported in other microarray studies (e.g. Breeze *et al.*, 2004) and highlight the need for very careful evaluation of microarray data and RT-PCR data before firm conclusions are drawn.

8 Discussion and conclusions

8.1 Synthesis of results

In most cases, the results of the different experiments within this study have reinforced each other, thus pointing to the same conclusion. Nevertheless, results of certain experiments have contradicted each other, leading to some uncertainty. In this section, the results of the different experiments and the conclusions drawn from them are considered, where these conclusions support each other is discussed and possible reasons for contradictory evidence from different experiments are offered.

The results of the physiology work (chapter 3) show stage 2 of petal development and stage 4 of leaf development to be important stages, as it is at these times that chlorophyll levels (leaves only), fresh and dry weights, and protein contents of the organs all reached their peak. This has lead to these stages being defined as 'mature', and being considered comparable between the two organs. In agreement with these results, the *SAG12* northern blots from chapter 4 show a sharp increase in the expression of this gene in stage 2 of petals and stage 5 of leaves, with the RT-PCR results (27 cycles) of chapter 7 confirming this result in petals. Although there is no RT-PCR data for *SAG12* expression in wallflower leaves, the pattern of expression elucidated by northern analysis of wallflower leaves is similar to the pattern of *SAG12* expression in the leaves of other species, such as the closely related *Arabidopsis* (Lohman *et al.*, 1994). Thus, although this protease gene is present at low levels throughout petal development, it is activated in earnest in petals at the time when the protein content of the organ is maximal, then increases in expression as the protein levels fall with increasing age. Similarly, in daylily petals, protease activity increased on a per petal basis after flower opening, and continued to rise from this time throughout the remainder of development, with protein levels falling at the same time (Stephenson and Rubinstein, 1998). In *Alstroemeria* petals, protease activity measured by zymography increased just following the peak in petal protein content, with the increase in cysteine protease gene expression being simultaneous with the peak in petal protein content, when expressed on a per petal basis (Wagstaff *et al.*, 2002; 2001). In wallflower leaves, no *SAG12* expression was detected until stage 5, just after the peak in leaf protein content, and again, *SAG12* expression increases as

the protein levels drop in the senescing leaves. Similarly, in *Arabidopsis*, *SAG12* expression was first detected just following the peak in protein content and continued to increase with increasing age, as protein levels dropped further (Lohman *et al.*, 1994). Thus there is a correlation between the physiological parameter of protein content and the molecular level expression of a cysteine protease gene. Of course, this assumes that the protein is immediately active once the mRNA has been translated. Some data suggests that post-translational modification may be important during the HR (Xing *et al.*, 1997), and some cysteine proteases require cleavage to activate the proenzyme (Karrer *et al.*, 1993), although it is not known whether this type of regulation is important during senescence (Buchanan-Wollaston, 1997). However, the *Alstroemeria* data suggests that the cysteine protease genes at least are active very shortly after translation (Wagstaff *et al.*, 2002), with the daylily data supporting this, showing protein degradation to be concomitant with protease activity (Stephenson and Rubinstein, 1998). There is a slight difference in the relationship between protein levels and *SAG12* cysteine protease expression between petals and leaves, with a slight difference in timing noted between the protein peak and the onset of *SAG12* expression in leaves, whereas the events appear to be simultaneous in petals. A possible explanation for this is that petal development is likely to progress over a much shorter timescale that leaf development (Rubinstein, 2000), and thus, these processes are compressed in time in petals compared to leaves, so that processes that are separable in leaves appear inseparable in petals. As the definition of a stage is completely arbitrary, then a way to overcome this would be to sample the petal tissue at closer time intervals, which therefore may also reveal the subtlety in this tissue too. Furthermore, the results of the microarray (chapter 6) also show *SAG12* to be very highly upregulated in both old petals (stages 3, 4 and 5) and in old leaves (stages 5 and 6) compared to young tissue (petal stages -2,-1 and 0; leaf stage 3), with an average ratio of 386 in petals and 113 in leaves between the young and old tissues. The results for *SAG12* expression are very interesting, as this gene is highly senescence-specific in leaves (Weaver *et al.*, 1998), and is upregulated even more strongly in late petal development than it is during leaf senescence. The microarray also showed other proteolytic genes to be upregulated in both late petal and late leaf development, including other cysteine proteases and a variety of other types of proteases, indicating that there is a wide range of protease genes responsible for the drop in protein levels seen in the physiology results for both organs (chapter 3). This

contrasts with the results of Wagstaff *et al.* (2002), where zymography indicated that only cysteine proteases were active during late development in *Alstromeria* petals. However Stephenson and Rubeinstein (1998) found cysteine, serine and metalloproteinases to be active during the late development of daylily petals, and other gene expression studies have also shown a variety of proteases, including multiple cysteine proteases as well as serine and metalloproteinases, to be upregulated during late petal development (discussed in section 6.4.4). The results of the SSH work (chapter 5) show that a gene showing homology to a peptide transporter is present in a library of genes upregulated during late wallflower petal development, indicating that the breakdown products of the degraded protein may be exported from the petal for recycling at this time. The results of the microarray confirmed that this gene is upregulated in old petals and also in old leaves, and is again more highly upregulated during the late development of petals than leaves, with a mean ratio of 118 in petals and 9 in leaves. The RT-PCR results (chapter 7) for this gene further confirm it to be highly upregulated during late petal development, with expression increasing slowly up to stage 1, after which time expression remained constantly high. High upregulation of gene expression in old tissues compared to young could be on account of high expression in the old tissue or very low expression in young tissue. Whichever of these is the case, the fact that the gene is more highly expressed in old tissues than in young, when the overall levels of gene expression are falling indicates that the product of that gene is important for senescence, even if the absolute level of expression of the gene in old tissues is not very high.

Similarly, *RBCS* expression in leaves (shown by northern blotting in chapter 4) is at its strongest in stages 3 and 4, which are the stages where chlorophyll levels, fresh and dry weight, and protein content are at their highest (chapter 3). This stands to reason, as RBCS is a protein associated with photosynthesis, indicating that it, along with other proteins (including many photosynthetic proteins) and chlorophyll, is being degraded as photosynthesis ceases and senescence begins. This is also the case in *Arabidopsis* leaves (Stessman *et al.*, 2002). In the leaves of *Phaseolus vulgaris* *RBCS* expression and synthesis fall during senescence, as is the case for several other photosynthetic proteins (Crafts-Brandner *et al.*, 1996; Bate *et al.*, 1991), in parallel with a drop in total RNA and protein levels (Crafts-Brandner *et al.*, 1996), with similar results obtained in wheat leaves (Crafts-Brandner *et al.*, 1998). This also ties

in with the expression pattern of *SAG12* in leaves, which is only detectable in leaf stages 5 onwards. Thus, the drop in chlorophyll levels and *RBCS* expression from their maximum in stage 4, indicating the transdifferentiation of chloroplasts to gerontoplasts, combined with the expression of *SAG12* in stage 5 confirm the definition of this developmental stage as 'old' in leaves.

As well as a drop in protein levels, fresh and dry weight and leaf chlorophyll levels, a drop in the RNA content of both organs was noted during late development (chapter 3). In petals, RNA levels peaked at stage 0, then dropped very sharply by stage 1, remained fairly constant until stage 4, then fell again by stage 5. Leaf RNA levels rose between stages 1&2 and 3, remained constant until stage 5, then fell through stages 6 and 7. These results are similar to those obtained in other species (section 3.4.5). The leaf SSH library (chapter 5) contained a gene showing homology to a bifunctional nuclease, indicating that expression of this gene is upregulated in old leaf tissues compared to young leaves, as is the case in *Arabidopsis* (Perez-Amador *et al.*, 2000). The results of the microarray (chapter 6) confirmed that this gene is, indeed, upregulated in both old leaves and old petals, again to a much greater degree in petals (ratio = 29) compared to leaves (ratio = 7.6). The microarray also showed expression of an *Arabidopsis* RNAse gene to be upregulated with age in both wallflower petals (ratio = 7.5) and leaves (ratio = 3.8).

Petal dry weight falls to 67% of its maximum by stage 5 and leaf dry weight falls to 56% of its maximum by stage 7. In leaves, this may largely be due to remobilisation of nutrients, which may also be the case in petals; although metabolism provides an alternative explanation for both organs (see section 3.4.3 for discussion). As carbohydrates are an important constituent of plant organs, with levels of soluble sugars known drop due to their being exported during senescence in other species (Verlinden, 2003; Bieleski, 1995; Yamane *et al.*, 1993), it is reasonable to assume that some of the drop in dry weight seen in wallflowers may be due to a drop in the levels of carbohydrates. This corresponds to the increased expression of the *Arabidopsis*-derived β-glucosidase gene detected by the microarray in both old petals and old leaves of wallflower, hydrolysing carbohydrates to sugars, which may subsequently be exported.

8.2 Conclusions: Senescence or Ageing?

The work described in this thesis indicates that late development in petals shows similarities to events seen in senescing leaves in both physiology and gene expression. Late petal development in this and other species is an active process involving the induced expression of a large number of genes, many of which are also induced during leaf senescence (e.g. Breeze *et al.*, 2004; van Doorn *et al.*, 2003; Wagstaff *et al.*, 2002; Verlinden *et al.*, 2002; Channeliere *et al.*, 2002; Panavas *et al.*, 2000). This leads to the conclusion that late developmental processes in petals cannot be termed 'ageing', which was defined in section 1.1.2 as being a passive process. However, the possibility remains that if the active process in either petals or leaves were blocked by inhibitors of RNA or protein synthesis, passive ageing may occur as an underlying process. This may also be the case when active deterioration is prevented by the overproduction of cytokinins, as late development is only delayed, and deterioration of both petals and leaves is still eventually seen (Clark *et al.*, 2004; Chang *et al.*, 2003; Gan and Amasino, 1995). This would suggest that the PCD following on from leaf senescence may therefore be a pleitropic consequence of the nutrient salvage that occurs during senescence.

A reduction in levels of antioxidant enzymes, along with breakdown of the processes that protect against free-radical damage have been associated with ageing, and even suggested as a cause of ageing (e.g. Munne-Bosch and Alegre, 2002; Bieleski, 2002; Orendi *et al.*, 2001; Ye *et al.*, 2000*a*; Jimenez *et al.*, 1998). This is not the case in wallflower petals, with transcript levels of catalase and glutathione peroxidase increasing during late petal development 3.5-fold and 2-fold, respectively. There is also evidence to suggest that metallothioneins, with a member of this family, derived from *Arabidopsis*, shown to increase in expression 31.5-fold during late wallflower petal development, and which have been shown to be important in petals senescence of other species (Breeze *et al.*, 2004), may protect DNA from oxidative damage by free-radicals in mammalian systems (Chubatsu and Meneghini, 1993). GST gene expression levels also increase during late petal development between 5- and 20-fold, and this gene family also has a role to play in protecting the cell from oxidative damage (Cobbet and Goldsbrough, 2002; Dixon *et al.*, 2002; section 1.5.5.1). However, this increase seen in the levels of enzymes with antioxidant capability may

not be enough to contend with the increase in free-radicals, and it is likely that the expression of other antioxidant genes may decrease at this time. These may serve to accelerate the rate of late developmental processes in petals and leaves. Nevertheless, the fact that these genes are expressed in petals furthers the case that late developmental processes in wallflower petals are not passive, and therefore cannot be defined as 'ageing'.

Having concluded that wallflower petals do not age passively, and that the process is therefore active, is there any evidence for this process being senescence? Many of the genes known to be involved with the regulation of leaf senescence have been shown by the microarray experiment (chapter 6) to be upregulated during wallflower leaf senescence are similarly shown to be upregulated during late petal development in wallflowers. This indicates that petal late developmental processes are likely to be actively regulated and indicates similarities between the regulation of events in petals and in leaves. However, this alone does not indicate that petals senesce, as the same regulatory mechanisms are also shared by other forms of developmental and induced PCD (section 1.4). Equally, the physiological similarities seen between the two organs, and the large number of induced genes shared between the two late developmental processes do not actually prove shared senescence-processes to be the case. The physiological parameters measured are broad, and the similarities in, for example, protein levels, only shows that protein levels fall in old petals, as they do in old leaves, but not necessarily that they are being degraded or that the degradation is for the purpose of recycling. Even if degradation was occurring in both organs, the pathways of degradation may still differ, and there may also be selective protein degradation, such as through the ubiquitination of certain proteins (Courtney *et al.*, 1994), which may also differ between organs. Similarly, the loss of fresh and dry weight as the petal increases in age and wilts allows the possibility that dry weight is lost as macromolecules are broken down and exported and the loss of fresh weight could mean that these nutrients are exported in solution. However, the loss of dry weight may also be due to respiration and loss of fresh weight could be a result of transpiration. Some of the genes that are expressed at higher levels in both organs with increasing age do, however, indicate that there is active degradation of macromolecules during late petal development, as during leaf senescence (discussed fully in section 6.4.4), further indicating that senescence is a possibility, but again not

proving it to be the case. As is the case in leaf senescence, the upregulation of antioxidant genes and defence genes during late petal development shows the need to retain cellular viability at this time, further indicating that the process is actively genetically regulated.

Many genes upregulated during leaf senescence are also common to other late developmental processes culminating in PCD, but that are not involved with the recycling of nutrients. However, there are also genes that are expressed during leaf senescence that are not upregulated in other late developmental processes in leaves and *vice versa*. One gene that is exclusively expressed during developmental senescence in leaves is *SAG12* (Noh and Amasino, 1999*a*; Weaver *et al.*, 1998; Lohman *et al.*, 1994; Becker and Apel, 1993) and has been used as a molecular marker for senescence (Noh and Amasino, 1999*a*). This senescence-specific gene was also found to be upregulated during late petal development in wallflowers, putting forward a strong argument for senescence in wallflower petals. Due to the nature of the microarray study, no comment can be made about genes which were shown to be unique to either leaf senescence or to late petal development, as they may indeed be expressed exclusively in one of the two organs, or they may be expressed in both organs, but with poor reproducibility or statistical significance in one. In order to know whether such genes are exclusive to one organ or just poorly represented on the array on one of the organs, the results would have to be confirmed by another method, such as northern analysis or RT-PCR. There was also evidence for nutrient recycling during late development in wallflower petals, in the upregulation of the expression of genes involved with remobilisation - a peptide transporter and a lipid transfer protein. As nutrient recycling is the defining feature of senescence (section 1.1.1), this is a strong basis, particularly considering the supporting evidence, for the tentative conclusion that wallflower petals do, indeed, senesce. This conclusion remains tentative, however, as gene homology does not necessarily prove functional homology of the protein products.

With regard to the model of the petal as a modified senescing leaf, most physiological parameters peaked at stage 2 of petal development, which is almost as soon as the flower has opened, and fall from this time onwards. The only exception to this trend was the RNA content of the petals, which peaked just before flower opening and

declined at the time of opening, then remained low from that time onwards. Similarly, in leaves stage 4 sees the peak of these physiological parameters, which is also the time of full leaf expansion. Thus stage 2 of petal development and stage 4 of leaf development are both considered to be 'mature' stages, where the organ is fully performing its function and not showing any visible or physiological signs of deterioration. However, whereas *SAG12* could not be detected at all in northern blots of leaf RNA until stage 5, it is expressed in petals (albeit at very low levels) in all the developmental stages looked at, including stage -2 (chapter 4). Thus, late developmental processes begin very early in wallflower petals compared to in leaves, as was noted in *Alstroemeria* (Wagstaff *et al.*, 2003). However, although expressed at very low levels in young wallflower petals, *SAG12* expression increases dramatically in expression just after full petal expansion at stage 2 (shown by northern analysis, chapter 4 and RT-PCR, chapter 7). This is comparable to the results in leaves, both in terms of extent of expression, as expression from stage 5 onwards in leaf is very strong, as is the case in fully expanded petals; and also with regard to timing of expression, occurring shortly after full expansion in both leaves, and petals. There is also the consideration that, from the results of the microarray (chapter 6) 298 of the clones spotted on the microarray showed higher expression in old leaves and in old petals, whereas none showed higher expression in young petals and old leaves. Thus, it must be concluded that a young petal shows little similarity to a senescing leaf in terms of its physiology or gene expression, as far as the extent of the experiments carried out can determine.

8.3 Further work

Although much progress has been made in elucidating events during late development in wallflower petals with reference to leaf senescence, further work remains to be done to confirm and elaborate upon the results already obtained. In this section, some ideas for future experiments are proposed.

8.3.1 Short-term goals

It would be interesting to gain more data on each of the chapters presented in this thesis.

8.3.1.1 Physiology

For the physiology work, more parameters could be looked at, such as the activity of protease enzymes, to see whether an increase in protease activity can be seen at the time of increased transcription of protease enzymes. Zymography could further characterise protease activity and could be run with the use of specific inhibitors of certain classes of proteases to establish which classes are active during senescence. Zymography experiments in *Alstroemeria* showed that only cysteine proteases were active during late development of petals (Wagstaff *et al.*, 2002), whereas other classes of protease were also found to be upregulated at the transcriptional level in wallflowers (chapter 6). This approach could also indicate whether the activation of different protease enzymes is synchronous, or whether there may be a cascade, as is the case in animals. Comparison of timing of events shown by these zymography results with the molecular results already obtained could help elucidate whether these proteases are active as soon as they are translated, or whether post-transcriptional activation may be important.

Cell membrane integrity measurements were attempted for wallflowers by quantifying the electrolyte content of organs floating on distilled water (these data were incomplete, and therefore were not presented). Increased presence of electrolytes in the water indicates increased 'leakiness' of the cell membrane, and thus lipid degradation (van Doorn and Stead, 1994). Ion leakage has been noted in the petals of many species of flowers just prior to wilting, including daylilies, where it increases by a factor of four from fully mature open flowers until just before wilting (Bieleski and Reid, 1992) and *Iris*, where it also increases considerably during senescence (Celikel and van Doorn, 1995). It would therefore be useful to complete these studies in wallflower.

It would also be interesting to look at the chlorophyll content of the petals, to establish at what stage chlorophyll is broken down, and whether there is any still present in the mature petals, with its colour masked by that of the petal pigments. Further study of the pigments present in the petal would also be interesting, but is likely to bear little relevance to the comparison with leaf senescence as a recycling process, as there are no homologous events in wallflower leaves. However, an understanding of what pigments are present and whether or not they are broken down during senescence

would be interesting and may help explain the presence of certain gene transcripts in petals which may not be present in leaves.

Direct measurement of export of nutrients from petals during late development would be the best proof of senescence in these organs, as has been established in daylily (Bieleski, 1995). This would support the hypothesis that the peptide transporter and lipid transfer protein genes identified by the microarray experiment (chapter 6) are upregulated in old wallflower petals and leaves because they are involved with remobilisation of nutrients.

As RNA levels have already been measured, measurement of DNA levels would be interesting, as levels of both nucleic acids have been reported to decline with increasing age, with RNA levels appearing to decrease at a greater rate than DNA levels (Lesham *et al.*, 1986). DNA laddering could be looked for in both leaves and petals, which is seen during animal apoptosis (Bowen and Lockshin, 1981) and in some kinds of cell death in plants (Panavas *et al.*, 2000), either by looking for ladders on an ethidium bromide stained gel or by TUNEL assays (Orzaez and Granell, 1997).

8.3.1.2 Molecular work

To further the molecular work presented, sequencing of more of the SSH clones and searching for homologous genes from *Arabidopsis* or other species would be very informative. This approach would give a better idea of what processes are involved in late petal development, as more of the upregulated genes would have a putative identity, and some detail of their expression pattern would already be known from the microarray experiment. Studies in other plant species on a similar scale, using techniques including SSH and microarrays, in diverse species and systems have sequenced a large proportion of their clones (e.g. Andersson *et al.*, 2004; Breeze *et al.*, 2004; Gepstein *et al.*, 2003; Hu *et al.*, 2003; van Doorn *et al.*, 2003; van Zhong and Burns, 2003; Chen *et al.*, 2002*b*; Swidxinski *et al.*, 2002; Bohnert *et al.*, 2002; Hinderhofer and Zentgraf, 2001). The shared genes, upregulated in both old petals and leaves, would be interesting to sequence, as further showing regions of similarity between the two late developmental processes. Genes 'unique' to each of the two organs would also be interesting, as then further experiments could be carried out to ascertain whether they are, indeed, unique, or whether they only appeared to be, due

to poor reproducibility or statistical significance in one or the other of the organs. Sequencing more of the genes upregulated in old petals, but downregulated in old leaves would establish whether genes other than GSTs show this unusual pattern of expression, and may help shed light on why these genes appear to be differentially regulated in the two organs. Within each of these classes, the genes with the highest expression ratios between old and young tissues would be best sequenced first, as these have a greater certainty of involvement in senescence, as this species has not been studied previously. Sequencing would also give an indication of the degree of redundancy in the SSH library. Once sufficient sequence has been obtained, the genes could be clustered into functional groups, from which pathways involved in senescence signalling and execution could be derived.

Microarray experiments using probes prepared from each of the developmental stages compared against a reference stage would give more detailed information on gene expression patterns and the timepoints at which changes in expression occur, and it would then be possible to group genes with regard to their pattern of expression. Comparison of these detailed expression patterns between petals and leaves would be most informative. The expression patterns of more genes of particular interest could be confirmed either by northern blotting, by RT-PCR or by real-time RT-PCR. Microarray analysis does not show if gene expression is unique to one or the other of the organs under study, because the apparent absence of gene expression may be due to a genuine absence of expression, or it may be due to poor replication or a lack of statistical significance. Thus, the confirmation of the results by another method may identify genes that are unique to one process or the other. Use of a combination of methods to confirm the results obtained has been proven to be necessary. The work of Breeze *et al.* (2004) on *Alstroemeria* showed that different methods could give different results for expression patterns of the same gene, like the metallothioneins, and the confirmation of the wallflower microarray results obtained in chapter 6 by RT-PCR (chapter 7) in this study also showed differences in expression pattern dependent on the method used for the gene encoding the CCR4-associated protein.

8.3.1.3 The role of ethylene

The role of ethylene during late development in wallflowers could also be looked at, as this hormone is known to be a key regulator of late developmental events in the

petals and leaves of many species, including *Arabidopsis* (section 1.4.2.3.1). This could be done on the physiological level by treating wallflowers with ethylene and noting the effects compared to untreated control plants or by measuring ethylene evolution by wallflowers and relating the timing (if it occurs) with late developmental processes in the two organs. On a molecular level, the microarray used in chapter 6 could be probed with probes prepared from ethylene treated and untreated plants to establish differences in gene expression and how gene expression during ethylene-induced senescence compared with the patterns already observed during natural senescence. Similar work has already been done on ethylene-induced gene expression in *Arabidopsis* leaves(van Zhong and Burns, 2003), identifying many genes from diverse classes as being ethylene-regulated. Although these were not directly compared to natural senescence, many of the gene classes identified by this study as being ethylene-regulated have also been shown to be upregulated during senescence. This could help establish whether ethylene is involved in the regulation of wallflower late development and how it exerts its effect.

8.3.2 Long-term goals

8.3.2.1 Spatial studies

Microscopy studies at both the light microscopy and electron microscopy level could yield very interesting results on cellular and subcellular events during senescence and the connection of these events to the expression of particular senescence associated genes. The petals of a different species of wallflower (*Erysimum cherii*) and of *Arabidopsis thaliana* have both been subjected to ultrastructural study of the cells and plastids (Weston and Pyke, 1999; Pyke and Page, 1998), however, it would be interesting to make a direct comparison of petals and leaves. This approach could be taken further using *in situ* hybridisation to establish the cellular and subcellular locations of senescence associated genes of particular interest. This could show whether transcripts are targeted to certain regions of the organ or the cell and could show whether genes are expressed in different parts of each organ at different times, for example expressing at the edges of the organ earlier than near the vascular tissues. It could also indicate whether some genes involved with recycling are more highly expressed near the vascular tissue, as is the case in rice with glutamine synthetase, which may be involved with nitrogen remobilisation (Kamachi *et al.*, 1992*b*).

8.3.2.2 Functional studies

All of the molecular work done so far has looked at increased transcription of genes and has identified the genes with increased expression in senescing wallflower petals and leaves by sequence homology. However, sequence homology does not guarantee functional equivalence. Thus, it would be interesting to look at some of the genes of interest either in knockout mutant plants or in transgenic plants where that gene is being overexpressed or silenced. In order to do this, the wallflower-derived gene of interest could be looked at in mutant *Arabidopsis* plants or *Arabidopsis* plants could be transformed to overexpress the gene or to express it in the antisense orientation so that the gene is silenced. Similar transgenic studies have shown that genes which are upregulated during senescence are functionally involved in the process (Roitsch *et al.*, 2003; He and Gan, 2002; Xu *et al.*, 2002; Miller *et al.*, 2000; Aida *et al.*, 1998; Fan *et al.*, 1997; Michael *et al.*, 1993). Ideally, however, over- or under-expression of the wallflower gene would be looked at in a wallflower background. In order to do this, a transformation system would have to be pioneered in wallflowers. This may be difficult to achieve in practice, as the closely related *Brassica* species have proved recalcitrant to transform.

8.3.2.3 Proteomics and post-translational control

Another interesting direction for future work would be to take a proteomic approach to look at changes in protein expression throughout development. Combined with the genomic approach already taken, this would give a lot of information on translational and post-translational regulation of senescence and how changes in gene expression equate to changes in the protein populations present in senescing organs. Post-translational regulation of glutamine sythetase in senescing leaves has been shown (Finnemann and Schjoerring, 2000), some cysteine proteases require activation by cleavage of the proenzyme (Karrer *et al.*, 1993), and ubiquitination in known to be involved in late petal development (Courtney *et al.*, 1994), but it is presently unknown whether early transcription of genes into inactive proteins which are later post-translationally activated is an important mechanism of regulation during senescence. This combined genomic-proteomic approach would help to answer that question.

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Appendix 1

A1 Optimisation of RNA extraction method

A1.1 Introduction

Isolation of intact RNA is a basic requirement for many molecular studies, especially those involving gene expression. However, extracting high-quality RNA from plant material can be difficult due to certain properties of cellular structure and metabolic activity in plants. Potential obstacles include:

- The presence of a durable cell wall.
- The relatively high abundance of polysaccharides, which can have chemical properties similar to nucleic acids, and so may copurify with the RNA. Polysaccharide contamination can also hinder resuspension of precipitated RNA, interfere with absorbance based quantification and may inhibit enzymatic manipulations, poly(A)+ RNA selection and/or electrophoretic migration (Gao *et al.*, 2001; Wilkins and Smart, 1996; Schultz *et al.*, 1994; Bahloul and Burkard, 1993; Graham, 1993).
- The accumulation of phenolics, terpenes, tannins and other secondary metabolites, some of which can bind nucleic acid when the tissues are lysed (Gao *et al.*, 2001; Dong and Dunstan, 1996; Wang and Vodkin, 1994; Schultz *et al.*, 1994; Katterman and Shattuck, 1983), changing the chemical properties that form the basis of the isolation procedure. Such compounds can also copurify with the RNA, causing possible complications with quantification and further manipulation of the RNA (Gao *et al.*, 2001; Lal *et al.*, 2001; Wilkins and Smart, 1996; Schultz *et al.*, 1994; Graham, 1993).

The extent to which such factors interfere with RNA extraction can vary significantly between plant species, tissue types, environmental conditions and developmental stages. To overcome these difficulties, all of the researchers quoted above, and many others in addition, have developed new methods or have modified existing methods to extract RNA of a suitably high quality for work on the species, tissue type, environmental condition or developmental stage of interest. The choice of method

will also depend on the purpose for which the RNA will be used, accounting for the amount and purity of the RNA required for that specific use.

Freezing the tissue in liquid nitrogen and grinding it to a fine powder with a mortar and pestle is an effective way to disrupt the cell wall. However, the tissue must be kept frozen until the addition of an RNAse-denaturing buffer, so that ribonucleases remain inactive. The properties of certain buffers as inhibitors of RNAses are important, as endogenous endonucleases are ubiquitous to all developmental stages, and although they are normally compartmentalised, the homogenisation of the tissue allows their release. However, this is a particularly important factor when working on senescing tissue, as activities of nucleases increase markedly during late development (e.g. Gepstein *et al.*, 2003; Blank and McKeon, 1991; 1989; Lesham *et al.*, 1986; Panavas and Rubinstein, 1998; Panavas *et al.*, 1999). As these nucleases are active, the RNA has already started to be degraded, and thus, the RNA extracted from late developmental stages is likely to be of poorer quality than that extracted from younger tissue. Therefore, particular care needs to be taken to ensure that this problem is not exacerbated during the extraction procedure.

Carbohydrates and secondary metabolites can be difficult to separate from RNA. Polysaccharides can be removed by such methods as column chromatography. However, there are many types of secondary plant metabolites, and the different types may interfere with RNA extraction in different ways. The most obvious secondary metabolite produced by wallflowers are the pigments in the fully developed petals, and glucosinolates are often found in species within the family Brassicaceae.

A1.1.1 Hot borate RNA extraction

Hunter and Reid (2001) found that a modification of the hot borate RNA isolation method of Wan and Wilkins (1994) suitable for RNA extraction from petal tissue in order to study changes in gene expression during flower senescence in daffodils (*Narcissus pseudonarcissus* L. 'Dutch Master') and Wagstaff *et al.* (2002) had success using the same method in the study of senescence of *Alstroemeria* petals. Thus, wallflower leaf and petal RNA was extracted by this method, along with *Alstroemeria* leaf and petal RNA as a positive control.

A1.1.2 Tri reagent RNA extraction

Wallflower leaf and petal RNA were extracted by this method, as well as *Arabidopsis* leaf RNA as a positive control, as this method has previously worked for *Arabidopsis*. The basis on which this method works is described in section 2.2.3.

A1.1.2.1 Tri reagent RNA optimisation for northern blotting

As the original Tri reagent method did not produce sufficient RNA of suitable quality for northern blotting, modifications were made to the original protocol, including increasing the length of incubation steps, further phenol chloroform extractions and longer centrifugations.

A1.1.2.2 Tri reagent RNA optimisation for microarray probes

Furthermore, the phenol: chloroform purification was not suitable for extracting RNA for making microarray probes, as any residual organic compounds in the RNA would adversely affect the reaction. Thus, Qiagen (Qiagen Ltd., Crawley, UK) RNeasy columns were tried for purifying the Tri reagent-extracted RNA. These use minispin columns to selectively bind the RNA to a silica-gel-based membrane in the presence of a high salt buffer and ethanol, contaminants are removed by washing and with on-column DNase digestion, before the RNA is eluted.

A1.1.2.3 Tri reagent RNA optimisation for increased speed

As the column purification had proved successful, and the Tri reagent extraction procedure was so time-consuming, the complete Qiagen Plant RNeasy extraction kit (Qiagen Ltd., Crawley, UK) was tried.

A1.2 Materials and methods

Many RNA extraction methods were tried, and then modified to try to improve yield and quality of the RNA. All solutions and chemicals were obtained from Sigma, unless otherwise stated and all of the equipment was sterilised and treated with RNAse ZAP prior to use. All centrifugation steps were carried out in a DuPont instruments Sorvall RC-5B refrigerated superspeed centrifuge with an SS-34 rotor or a Beckman Coulter Allegra 2IR microcentrifuge with the stated rotor.

A1.2.1 Hot borate RNA extraction

5 ml of hot borate buffer (see list of buffers, section 2.1) per sample was heated to 80°C, 1g of plant material was ground to a fine powder in liquid nitrogen using a sterile pestle and mortar, and the powdered plant material was added to the hot buffer and vortexed for 30 seconds. 37.5 µl of proteinase K (20mg/ml) was added to each sample and incubated at 42°C for 90 mins with shaking. 400µl of 2M potassium chloride was added to each sample, incubated on ice for 30 mins with shaking, and centrifuged at 11 K rpm for 30 mins. The resulting pellet was resuspended in 800µl of sdw.

To clean the RNA, an equal volume of chloroform was added to each sample, vortexed for 30 seconds, and microcentrifuged at 4°C and 13 K rpm for 5 mins using the F2402H rotor. The top, aqueous layer was retained. 80µl of 3M sodium acetate (pH 8.0) and 800µl of isopropanol were added to each of the samples and microcentrifuged at 4°C and 13 K rpm for 30 mins using the F2402H rotor. The resulting pellet was washed with 200µl of 80% ethanol and resuspended in 50µl of sdw.

A1.2.2 Tri reagent RNA extraction

Initially, the manufacturers' protocol was followed. Following disruption of the tissue in liquid nitrogen, ten volumes of Tri reagent was added to each unit mass of tissue and the samples homogenised using an IKA labortechnik T25 basic polytron motorised homogeniser. The samples were incubated at room temperature for 5 mins to permit complete dissociation of nucleoprotein complexes, centrifuged at 11K rpm for 5 mins at 4°C to remove cellular debris, and 0.2 volumes of chloroform was added to the supernatant for each volume of Tri reagent and vortexed to mix. The mixture was again incubated at room temperature for 5 mins and centrifuged at 11 K rpm for 15 mins at 4°C, retaining the colourless upper aqueous phase. The RNA was precipitated solution by the addition of 0.5 volumes of isopropanol per volume of Tri reagent initially used, incubation at room temperature for 5 mins and centrifuged at 11K rpm for 8 mins at 4°C. The pellet was ethanol washed (as described in section 2.2.6) and dissolved in 150µl of sdw.

A1.2.2.1 Tri reagent RNA extraction optimisation for northern blotting

Modifications of the original protocol were tried in order to improve yield and remove contaminants:

- Increasing the length of the incubation steps was found to increase yield.
- Replacing the ethanol wash with phenol: chloroform purification was found to give cleaner RNA.
- Repeating the phenol: chloroform purification until no interface formed between the aqueous and organic layers was found to be necessary for complete removal of contaminants. This routinely took 10-15 repeats.
- To try to obtain cleaner RNA, the step with the addition of chloroform to separate the RNA, DNA and protein phases was repeated and the second chloroform layer also back extracted. However, no difference in purity or yield was observed following this modification.
- It was found that longer centrifugation steps were necessary to precipitate the RNA from the solutions used, thus increasing yield. This may be as the volumes used for the wallflower extraction were scaled-up from those quoted in the manufacturers' original protocol.
- Back extraction of the organic and interface layers with sdw at the stage when RNA, DNA and protein are separated by the addition of chloroform, and of the phenol: chloroform layer and chloroform layer during the phenol: chloroform purification step also increased yield.

The optimised final protocol is described in sections 2.2.3 to 2.2.6.

A1.2.2.2 Tri reagent RNA extraction optimisation for microarray probes

Qiagen (Qiagen Ltd., Crawley, UK) RNeasy columns were used for purifying the Tri reagent-extracted RNA, according to the manufacturers' protocol. The RNA was bound to a silica-gel-based membrane in the presence of a high salt buffer and ethanol, contaminants are removed by washing and with on-column DNase digestion, and the RNA is eluted in 30µl of water.

A1.2.2.3 Tri reagent RNA optimisation for increased speed

The complete Qiagen Plant RNeasy extraction kit (Qiagen Ltd., Crawley, UK) was tried, firstly in accordance with the manufacturers' instructions, and then some

modifications were made to improve the performance of the kit. After grinding the plant tissue in liquid nitrogen, a highly denaturing lysis buffer is added (buffer RLT), and the samples centrifuged through a QIAshredder homogeniser (supplied) to remove insoluble material and reduce the viscosity of the lysate. The resulting cleared lysate is then cleaned on the RNeasy columns.

The QIAshredder column was found to clog up easily with cell debris, consequently not clearing the lysate efficiently. Hence, the extraction worked better if the debris were pelleted by a short microcentrifugation step and not added to the column. Reducing the amount of starting material used (from the maximum recommended 100mg to 30-50mg) also improved yield for the same reason.

A1.3 Results and discussion

A1.3.1 Hot borate RNA extraction

Although RNA was successfully extracted using this method, it was of poor quality from both leaves and petals (see figure A1.3.1.1) and was not good enough to use for northern blotting. The leaf RNA was of better quality than the petal RNA for both the *Alstroemeria* and wallflower.

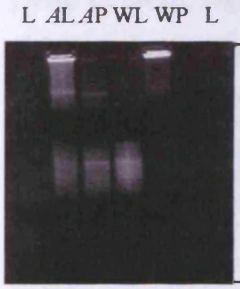

Figure A1.3.1.1 Example of the RNA extracted by the hot borate method from *Alstroemeria* leaf (*A*L) and petal (*A*P) and wallflower leaf (WL) and petal (WP). The RNA is smeared and the two rRNA bands cannot be distinguished, indicating that the RNA is of poor quality. The wells appear bright, probably due to contaminants being present in the RNA.

A1.3.2 Tri reagent RNA extraction

Good quality RNA was obtained by this method; however yield was poor and the RNA still appeared to contain contaminants, as a fluorescent substance remained in the wells of the gel and did not run with the RNA. Thus, protocol needed modification to improve yield and RNA purity.

A1.3.2.1 Tri reagent RNA extraction optimisation for northern blotting

After modification, this method consistently gave very good quality RNA (figure A1.3.2.1.1) from both leaves and petals. This was used for northern blotting. However, following all the modifications, this turned out to be a very long and time-consuming procedure.

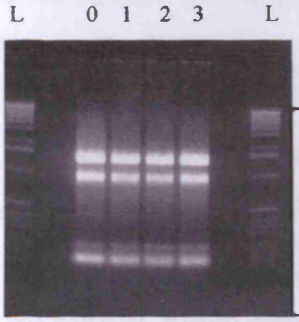

Figure A1.3.2.1.1 Example of wallflower petal RNA from different developmental stages extracted by the Tri reagent method. The RNA is of very good quality with no degradation of the rRNA bands. The small bands at the bottom of the lanes represent tRNA, and are sharp bands, with the absence of large, indistinct bands again showing a lack of degradation.

A1.3.2.2 Tri reagent RNA extraction optimisation for microarray probes

This turned out to be a quick and efficient way of purifying the RNA, and this RNA was used for microarray probe making.

A1.3.2.3 Tri reagent RNA extraction optimisation for increased speed

This method worked well for leaf and young petals (stages -2, -1 and 0), giving good yields of clean, high quality RNA. However, this method proved unsuitable for the older petal stages, possibly due to the petal pigments reacting with the extraction buffer. Also, as the kit is only available as a 'mini kit' and not in any larger sizes, despite returning good yields, the absolute amount of RNA extracted was nevertheless small as only a small amount of starting material (30-50mg) could be used. Consequently, this method was considered unsuitable for extraction of RNA from wallflowers.

Thus, despite the fact that methods successfully used for RNA extraction from petals and leaves in other species being partially effective in the case of wallflowers (Wagstaff *et al.*, 2002; Hunter and Reid, 2001; Chomczynski and Sacchi, 1987; Wan and Wilkins, 1994), new adaptations were needed to successfully extract high-quality RNA from wallflower organs, and slightly different methods were required depending on the downstream use of the RNA.

A1.4 Preparation of consumable plasticware for RNA extraction

A1.4.1 Introduction

As a large number of Eppendorf tubes were used during the procedure, a test was devised to see whether it is necessary to treat them all with RNAseZAP, or whether certified RNAse-free tubes could be used untreated (as autoclaving does not denature RNAses effectively).

A1.4.2 Materials and methods

6 certified RNAse- free tubes were treated in different ways:

1. No RNAse, rinsed with 500µl of Sigma RNAseZAP, rinsed twice with sdw.
2. No RNAse, rinsed with 500µl of Ambion RNAseZAP, rinsed twice with sdw.
3. Untreated
4. Rinsed with 2µl RNAse in 500µl of sdw, rinsed with 500µl of Sigma RNAseZAP, rinsed twice with sdw.
5. Rinsed with 2µl RNAse in 500µl of sdw, rinsed with 500µl of Ambion RNAseZAP, rinsed twice with sdw.
6. Rinsed with 2µl RNAse in 500µl of sdw, rinsed twice with sdw.

1µl of mixed age petal RNA was put into each tube with 9µl of RNA loading dye and run on an agarose gel (as described in section 2.2.10) to test the integrity of the RNA.

A1.4.3 Results and discussion

The experiment to test whether or not the certified RNAse-free tubes could be used without treating them with RNAseZAP gave a positive result (see figure A1.4.3.1). The RNA from the tubes that were not treated with RNAse was of similar quality, regardless of whether or not they were rinsed with RNAseZAP and regardless of which brand of RNAseZAP was used. The RNA from the tubes treated with RNAse and RNAseZAP (regardless of brand used) was badly degraded and the RNA from the tube treated with RNAse and rinsed only with water was completely degraded. This shows that the RNAseZAP does prevent degradation of RNA by RNAse, but does not completely inhibit it. However, a lot of RNAse was used in this experiment, so it is likely that RNAseZAP would help in eliminating small amounts of contamination. On the basis of these results, it was decided to use the certified RNAse-free tubes

without rinsing in RNAseZAP – saving a day in preparation work for each set of four RNA extractions. RNAseZAP was still used for treating surfaces and equipment.

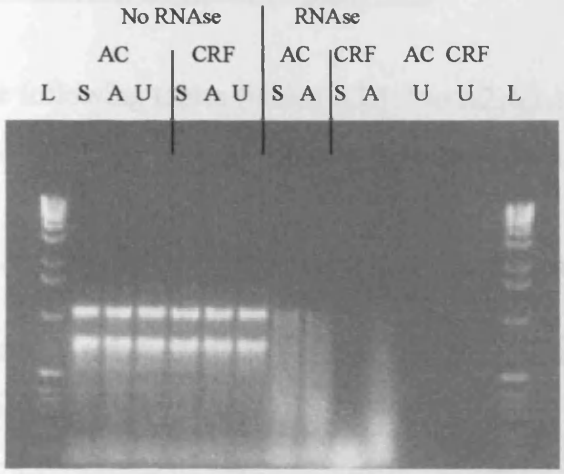

Figure A1.4.3.1 RNA quality from differentially treated autoclaved (AC) and certified RNAse free (CRF) Eppendorf tubes. Tubes were treated with sigma (S) or ambion (A) RNAseZAP, or remained untreated (U). Good quality RNA was obtained from all the tubes that were not treated with RNAse, indicating that there is no need to treat the tubes with RNAseZAP. Following treatment with RNAse, both with and without treatment with RNAseZAP, the RNA is degraded, although less so after RNAseZAP treatment.

Appendix 2

A2 Raw data for physiology work

The following tables (tables A2.1.1 to A2.4.2.1) summarise the raw data obtained for each of the physiological parameters measured.

A2.1 Raw data for chlorophyll measurements

Stage	lf weight (µg)	n.o. of leaves	lf area (mm2)	Au 645	Au 652	Au 663	Total Ch (µg/ml)	Ch A (µg/ml)	Ch B (µg/ml)
replicate 1									
1	35	2.7	113	0.090	0.120	0.210	3.336	2.424	1.074
2	58	2.7	235	0.190	0.280	0.500	7.784	5.837	2.001
3	53	1.3	177	0.150	0.220	0.380	6.116	4.421	1.649
4	56	1.3	194	0.230	0.320	0.550	8.896	6.364	2.682
5	54	1.3	272	0.220	0.310	0.520	8.618	6.010	2.594
6	53	1.3	276	0.160	0.220	0.360	6.116	4.140	1.972
7	32	2.0	263	0.060	0.080	0.110	2.224	1.235	0.857
replicate 2									
1	12	2.7	67	0.06	0.07	0.11	1.946	1.235	0.857
2	19	2.7	80	0.08	0.1	0.18	2.780	2.070	0.986
3	48	1.3	190	0.18	0.25	0.45	6.950	5.229	2.007
4	51	1.3	178	0.2	0.28	0.48	7.784	5.556	2.324
5	41	1.3	168	0.13	0.18	0.3	5.004	3.459	1.567
6	17	1.3	95	0.04	0.04	0.06	1.112	0.654	0.634
7	33	2.0	136	0.03	0.04	0.05	1.112	0.554	0.452
Averages									
1&2	31	2.7	123.75	0.105	0.1425	0.25	3.9615	2.8915	1.2295
3	50.5	1.3	183.5	0.165	0.235	0.415	6.533	4.825	1.828
4	53.5	1.3	186	0.215	0.3	0.515	8.34	5.96	2.503
5	47.5	1.3	220	0.175	0.245	0.41	6.811	4.7345	2.0805
6	35	1.3	185.5	0.1	0.13	0.21	3.614	2.397	1.303
7	32.5	2.0	199.5	0.045	0.06	0.08	1.668	0.8945	0.6545

Table A2.1.1 Raw data for chlorophyll measurements. The number of leaves used for making the extract and their weight and surface area are shown, along with the absorbencies measured at 645, 652 and 633 nm. These data were used to calculate the amount of chlorophyll (total chlorophyll, chlorophyll a and chlorophyll b) present in the extract using the equations of Bruinsma (1963; see section 3.2.2).

Stage	TCh ext (µg)	ChA ext (µg)	ChB ext (µg)	TCh (µg/lf)	ChA (µg/lf)	ChB (µg/lf)	ChA+ChB (µg/lf)	TCh-(ChA+B)
replicate 1								
1	33.360	24.240	10.740	12.356	8.978	3.978	12.956	-0.600
2	77.840	58.370	20.010	28.830	21.619	7.411	29.030	-0.200
3	61.160	44.210	16.490	47.046	34.008	12.685	46.692	0.354
4	88.960	63.640	26.820	67.621	48.374	20.387	68.761	-1.140
5	86.180	60.100	25.940	64.711	45.128	19.478	64.605	0.105
6	61.160	41.400	19.720	47.046	31.846	15.169	47.015	0.031
7	22.240	12.350	8.570	10.949	6.080	4.219	10.299	0.650
replicate 2								
1	19.460	12.350	8.570	7.207	4.574	3.174	7.748	-0.541
2	27.800	20.700	9.860	10.296	7.667	3.652	11.319	-1.022
3	69.500	52.290	20.070	53.462	40.223	15.438	55.662	-2.200
4	77.840	55.560	23.240	59.168	42.233	17.665	59.898	-0.730
5	50.040	34.590	15.670	37.574	25.973	11.766	37.739	-0.165
6	11.120	6.540	6.340	8.554	5.031	4.877	9.908	-1.354
7	11.120	5.540	4.520	5.474	2.727	2.225	4.953	0.522
Averages								
1&2	39.615	28.915	12.295	14.672	10.709	4.554	15.263	-0.591
3	65.330	48.250	18.280	50.254	37.115	14.062	51.177	-0.923
4	83.400	59.600	25.030	64.154	45.846	19.254	65.100	-0.946
5	68.110	47.345	20.805	52.392	36.419	16.004	52.423	-0.031
6	36.140	23.970	13.030	27.800	18.438	10.023	28.462	-0.662
7	16.680	8.945	6.545	8.340	4.473	3.273	7.745	0.595

Table A2.1.2 Derived data for chlorophyll measurements. The amount of chlorophyll (total chlorophyll, chlorophyll a and chlorophyll b) in each extract (10ml) was calculated, and used to calculate the amount of chlorophyll (total chlorophyll, chlorophyll a and chlorophyll b) in µg per leaf. The amount of chlorophyll a and chlorophyll b summed should be the same as the amount of total chlorophyll. This was checked and the difference between the two values was used as a measure of error.

A2.2 Raw data for fresh and dry weight measurements

A2.2.1 Petal fresh and dry weights

Stage	N.o. pls	foil weight (g)	FW inc.foil (g)	DW inc.foil (g)	Total FW (g)	Total DW (g)	FW/petal (g)	DW/petal (g)	FW/petal (mg)	DW/petal (µg)
replicate 1										
-2	58	0.4280	0.5040	0.4440	0.0760	0.0160	0.0013	0.0003	1.3	276
-1	58	0.4700	0.5770	0.4940	0.1070	0.0240	0.0018	0.0004	1.8	414
0	70	0.4570	0.6320	0.5120	0.1750	0.0550	0.0025	0.0008	2.5	786
1	95	0.4750	1.0060	0.5660	0.5310	0.0910	0.0056	0.0010	5.6	958
2	68	0.4790	0.8720	0.5450	0.3930	0.0660	0.0058	0.0010	5.8	971
3	66	0.4690	0.8060	0.5290	0.3370	0.0600	0.0051	0.0009	5.1	909
4	74	0.4570	0.7150	0.5100	0.2580	0.0530	0.0035	0.0007	3.5	716
5	97	0.4320	0.6680	0.4940	0.2360	0.0620	0.0024	0.0006	2.4	639
replicate 2										
-2	42	0.1690	0.2478	0.1847	0.0788	0.0157	0.0019	0.0004	1.9	374
-1	52	0.1690	0.2816	0.1955	0.1126	0.0265	0.0022	0.0005	2.2	510
0	54	0.1790	0.3699	0.2210	0.1909	0.0420	0.0035	0.0008	3.5	778
1	84	0.2230	0.6124	0.3023	0.3894	0.0793	0.0046	0.0009	4.6	944
2	68	0.1630	0.5380	0.2527	0.3750	0.0897	0.0055	0.0013	5.5	1319
3	59	0.2410	0.5310	0.3013	0.2900	0.0603	0.0049	0.0010	4.9	1022
4	51	0.3280	0.5425	0.3700	0.2145	0.0420	0.0042	0.0008	4.2	824
5	67	0.2050	0.3605	0.2558	0.1555	0.0508	0.0023	0.0008	2.3	758
replicate 3										
-2	20	0.2876	0.3125	0.2937	0.0249	0.0061	0.0012	0.0003	1.2	305
-1	20	0.3553	0.3869	0.3645	0.0316	0.0092	0.0016	0.0005	1.6	460
0	20	0.4822	0.5268	0.4970	0.0446	0.0148	0.0022	0.0007	2.2	740
1	20	0.4676	0.5671	0.4829	0.0995	0.0153	0.0050	0.0008	5.0	765
2	20	0.4589	0.5690	0.4784	0.1101	0.0195	0.0055	0.0010	5.5	975
3	20	0.4149	0.5035	0.4310	0.0886	0.0161	0.0044	0.0008	4.4	805
4	20	0.3192	0.3845	0.3379	0.0653	0.0187	0.0033	0.0009	3.3	935
5	20	0.4303	0.4732	0.4460	0.0429	0.0157	0.0021	0.0008	2.1	785

Table A2.2.1.1 Raw data for petal fresh and dry weight measurements. The petals were weighed in foil, so the weight of the foil on its own was measured, along with the weight of the foil containing fresh petals and the foil containing dried petals. The number of petals weighed is also given. The fresh and dry weights of the petals were calculated by subtracting the weight of the foil from the weight of the foil-wrapped fresh and dry petals. These values were then divided by the total number of petals to give the mean weight per petal.

	Average			Standard deviation		
Stage	FW/petal (mg)	DW/petal (µg)	DW/petal (mg)	FW/petal (mg)	DW/petal (µg)	DW/petal (mg)
-2	1.5	318	0.318	0.3	50	0.050
-1	1.9	461	0.461	0.3	48	0.048
0	2.8	768	0.768	0.7	24	0.024
1	5.1	889	0.889	0.5	108	0.108
2	5.6	1088	1.088	0.2	200	0.200
3	4.8	912	0.912	0.3	109	0.109
4	3.7	825	0.825	0.5	109	0.109
5	2.3	727	0.727	0.1	78	0.078

Table A2.2.1.2 Derived data for petal fresh and dry weights. The mean fresh and dry weights per petal were calculated from the three replicates, and the standard deviation used to measure error.

A2.2.2 Leaf fresh and dry weights

Stage	N.o. leaves	foil weight (g)	FW inc.foil (g)	DW inc.foil (g)	Total FW (g)	Total DW (g)	FW/leaf (g)	DW/leaf (g)	FW/leaf (mg)	DW/leaf (mg)
replicate 1										
1&2	20	0.6735	0.9442	0.7332	0.2707	0.0597	0.01354	0.00299	13.54	2.99
3	20	0.6367	1.3473	0.7871	0.7106	0.1504	0.03553	0.00752	35.53	7.52
4	20	0.6489	1.4393	0.8092	0.7904	0.1603	0.03952	0.00802	39.52	8.02
5	20	0.5721	1.3117	0.7219	0.7396	0.1498	0.03698	0.00749	36.98	7.49
6	20	0.6115	1.2413	0.7414	0.6298	0.1299	0.03149	0.00650	31.49	6.50
7	20	0.5234	0.9232	0.6237	0.3998	0.1003	0.01999	0.00502	19.99	5.02
replicate 2										
1&2	20	0.9832	1.2329	1.0430	0.2497	0.0598	0.01249	0.00299	12.49	2.99
3	20	0.6870	1.5266	0.8572	0.8396	0.1702	0.04198	0.00851	41.98	8.51
4	20	0.6893	1.6891	0.8689	0.9998	0.1796	0.04999	0.00898	49.99	8.98
5	20	0.6917	1.4721	0.8423	0.7804	0.1506	0.03902	0.00753	39.02	7.53
6	20	0.7318	1.2822	0.8716	0.5504	0.1398	0.02752	0.00699	27.52	6.99
7	20	0.6136	1.0033	0.7229	0.3897	0.1093	0.01949	0.00547	19.49	5.47
replicate 3										
1&2	20	0.4234	0.5630	0.4731	0.1396	0.0497	0.00698	0.00249	6.98	2.49
3	20	0.3973	1.0871	0.5775	0.6898	0.1802	0.03449	0.00901	34.49	9.01
4	20	0.4195	1.0693	0.5996	0.6498	0.1801	0.03249	0.00901	32.49	9.01
5	20	0.4118	1.0318	0.5713	0.6200	0.1595	0.03100	0.00798	31.00	7.98
6	20	0.4121	0.7617	0.5124	0.3496	0.1003	0.01748	0.00502	17.48	5.02
7	20	0.4129	0.5834	0.4933	0.1705	0.0804	0.00853	0.00402	8.53	4.02

Table A2.2.2.1 Raw data for leaf fresh and dry weight measurements. The leaves were weighed in foil, so the weight of the foil on its own was measured, along with the weight of the foil containing fresh leaves and the foil containing dried leaves. The number of leaves weighed is also given. The fresh and dry weights of the leaves were calculated by subtracting the weight of the foil from the weight of the foil-wrapped fresh and dry leaves. These values were then divided by the total number of leaves to give the mean weight per leaf.

	Average		Standard deviation	
Stage	**FW/leaf (mg)**	**DW/leaf (mg)**	**FW/leaf (mg)**	**DW/leaf (mg)**
1&2	11.00	2.82	2.87	0.24
3	37.33	8.35	3.31	0.62
4	40.67	8.67	7.19	0.46
5	35.67	7.67	3.40	0.22
6	25.50	6.17	5.90	0.84
7	16.00	4.83	5.29	0.60

Table A2.2.2.2 Derived data for leaf fresh and dry weights. The mean fresh and dry weights per leaf were calculated from the three replicates, and the standard deviation used to measure error.

A2.3 Raw data for protein content

A2.3.1 Petal protein content

A2.3.1.1 Standards

Standards mg ml-1	0.1	0.2	0.4	0.6	0.7	1	1.2	2	2.5
Standards ug/ well	1	2	4	6	7	10	12	20	25
std1	0.342	0.445	0.673	0.786	0.833	1.022	1.245	1.656	1.889
std2	0.308	0.419	0.586	0.728	0.711	0.936	1.146	1.523	1.773
std3	0.315	0.418	0.566	0.707	0.750	0.969	1.158	1.562	1.886
Std Mean	0.322	0.427	0.608	0.740	0.765	0.976	1.183	1.580	1.849
Std SD	0.018	0.015	0.057	0.041	0.062	0.043	0.054	0.068	0.066

Table A2.3.1.1.1 Absorbance values for protein standard solutions for the first replicate petal plate. The concentrations of the protein standard solutions are given, along with the equivalent amount of protein present in the well containing that standard solution. The absorbance was measured in triplicate for each standard, and the values obtained for each replicate are given, along with the average of the three replicates and the standard deviation.

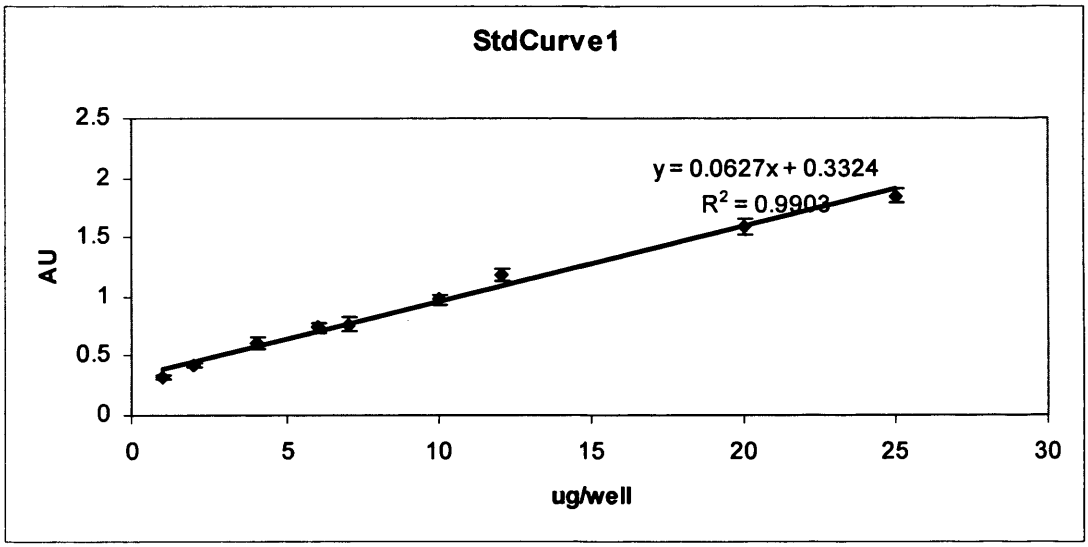

Figure A2.3.1.1.1 Standard curve produced from the data in table A2.3.1.1.1. The average absorbance values were plotted against the protein content of the well, with the error bars representing the standard deviation. A trendline was plotted through the points, and the equation of the trendline is given along with its R^2 value. This standard curve was used to calculate the protein content of the petal samples of the first replicate plate.

Standards mg ml-1	2.5	2	1.2	1	0.7	0.6	0.4	0.2	0.1
Standards ug/ well	25	20	12	10	7	6	4	2	1
std1	1.938	1.634	1.044	0.871	0.790	0.647	0.510	0.365	0.274
std2	1.974	1.443	1.052	0.860	0.668	0.627	0.493	0.335	0.261
std3	1.999	1.474	0.977	0.841	0.670	0.650	0.478	0.337	0.264
Std Mean	1.970	1.517	1.024	0.857	0.709	0.641	0.494	0.346	0.266
Std SD	0.031	0.103	0.041	0.015	0.070	0.013	0.016	0.017	0.007

Table A2.3.1.1.2 Absorbance values for protein standard solutions for the second, third and fourth replicate petal plates. The concentration of the protein standard solutions are given, along with the equivalent amount of protein present in the well containing that standard solution. The absorbance was measured in triplicate for each standard, and the values obtained for each replicate are given, along with the average of the three replicates and the standard deviation.

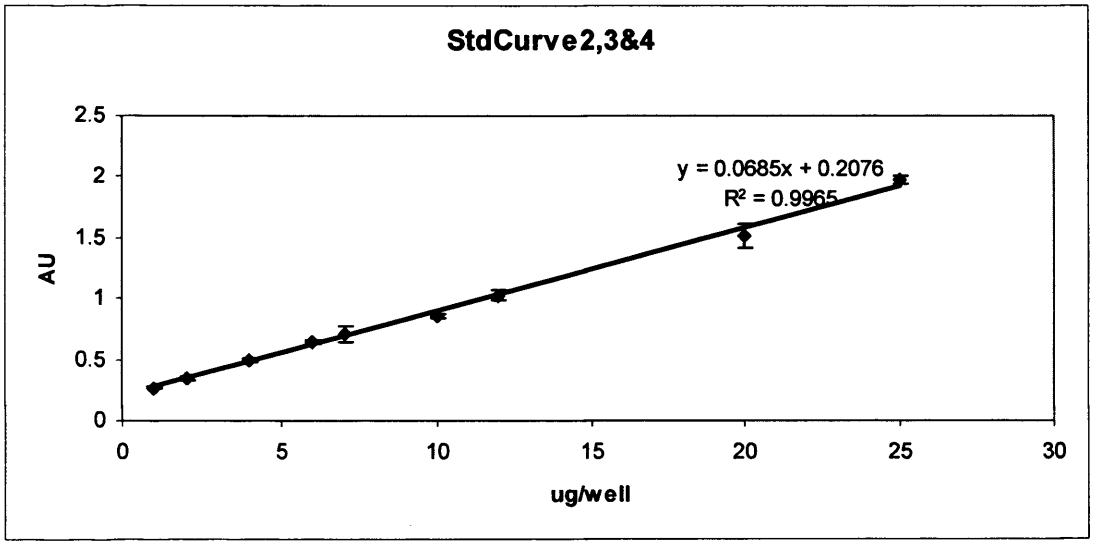

Figure A2.3.1.1.2 Standard curve produced from the data in table A2.3.1.1.2. The average absorbance values were plotted against the protein content of the well, with the error bars representing the standard deviation. A trendline was plotted through the points, and the equation of the trendline is given along with its R^2 value. This standard curve was used to calculate the protein content of the petal samples of the second, third and fourth replicate plates.

Standards mg ml-1	0.05	0.1	0.2	0.4	0.6	0.8	1	1.2	2	2.5
Standards ug/ well	0.5	1	2	4	6	8	10	12	20	25
std1	0.139	0.164	0.219	0.314	0.421	0.504	0.613	0.823	0.927	1.219
std2	0.138	0.164	0.213	0.329	0.448	0.623	0.725	0.898	0.958	1.298
std3	0.143	0.164	0.207	0.432	0.511	0.571	0.540	0.826	1.101	1.399
Std Mean	0.140	0.164	0.213	0.358	0.460	0.566	0.626	0.849	0.995	1.305
Std SD	0.003	0.000	0.006	0.064	0.046	0.060	0.093	0.042	0.093	0.090

Table A2.3.1.1.3 Absorbance values for protein standard solutions for the fifth replicate petal plate. The concentration of the protein standard solutions are given, along with the equivalent amount of protein present in the well containing that standard solution. The absorbance was measured in triplicate for each standard, and the values obtained for each replicate are given, along with the average of the three replicates and the standard deviation.

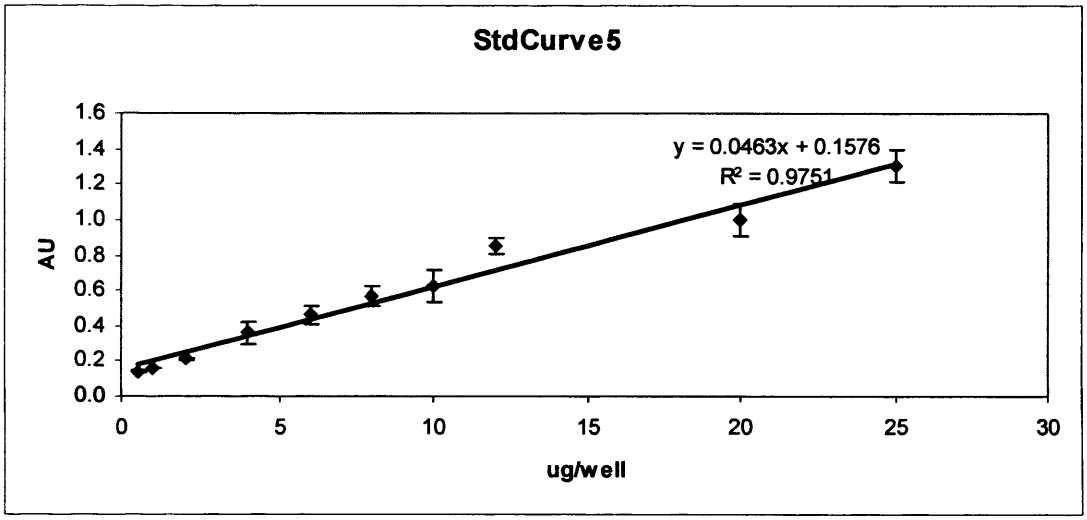

Figure A2.3.1.1.3 Standard curve produced from the data in table A2.3.1.1.3. The average absorbance values were plotted against the protein content of the well, with the error bars representing the standard deviation. A trendline was plotted through the points, and the equation of the trendline is given along with its R^2 value. This standard curve was used to calculate the protein content of the petal samples of the fifth replicate plate.

A2.3.1.2 Samples

stage	-2	-1	0	1	2	3	4	5
plate 1								
AU 1	0.584	0.693	0.771	0.947	2.098	1.794	1.442	0.783
AU2	0.553	0.635	0.800	1.026	2.100	1.711	1.063	0.732
AU3	0.647	0.681	1.161	1.298	2.001	1.543	1.596	1.032
sampleprot/well1	4.064	5.785	7.016	9.794	27.960	23.162	17.606	7.205
sampleprot/well2	3.575	4.869	7.473	11.040	27.991	21.852	11.624	6.400
sampleprot/well3	5.059	5.595	13.171	15.333	26.429	19.200	20.037	11.135
petalprot1	121.929	173.539	210.471	293.805	838.791	694.850	528.182	216.153
petalprot2	107.251	146.077	224.203	331.211	839.738	655.551	348.730	192.005
petalprot3	151.759	167.857	395.132	460.000	792.863	576.005	601.100	334.052
plate 2								
AU 1	0.697	0.356	0.482	0.489	0.686	0.595	0.554	0.441
AU 2	0.379	0.319	0.407	0.429	0.637	0.560	0.571	0.428
AU 3	0.416	0.335	0.502	0.452	0.624	0.552	0.560	0.468
sampleprot/well1	7.156	2.194	4.027	4.129	6.996	5.672	5.075	3.431
sampleprot/well2	2.528	1.655	2.936	3.256	6.283	5.163	5.323	3.241
sampleprot/well3	3.067	1.888	4.318	3.591	6.094	5.046	5.163	3.824
petalprot1	214.693	65.809	120.822	123.878	209.890	170.159	152.258	102.921
petalprot2	75.851	49.654	88.076	97.681	188.496	154.877	159.680	97.245
petalprot3	92.005	56.640	129.554	107.723	182.820	151.384	154.877	114.709
plate 3								
AU 1	0.407	0.400	0.597	0.722	0.970	0.827	0.669	0.500
AU 2	0.394	0.341	0.507	0.569	0.768	0.660	0.621	0.464
AU 3	0.329	0.332	0.455	0.514	0.736	0.654	0.569	0.468
sampleprot/well1	2.936	2.834	5.701	7.520	11.130	9.048	6.749	4.289
sampleprot/well2	2.747	1.975	4.391	5.294	8.190	6.618	6.050	3.765
sampleprot/well3	1.801	1.844	3.634	4.493	7.724	6.531	5.294	3.824
petalprot1	88.076	85.020	171.032	225.608	333.887	271.452	202.468	128.681
petalprot2	82.400	59.260	131.737	158.807	245.692	198.538	181.511	112.963
petalprot3	54.020	55.330	109.033	134.793	231.721	195.919	158.807	114.709
plate 4								
AU 1	0.324	0.319	0.417	0.460	0.694	0.590	0.542	0.478
AU 2	0.338	0.315	0.449	0.499	0.681	0.601	0.598	0.455
AU 3	0.359	0.327	0.452	0.524	0.734	0.648	0.589	0.493
sampleprot/well1	1.728	1.655	3.081	3.707	7.113	5.599	4.901	3.969
sampleprot/well2	1.932	1.597	3.547	4.275	6.924	5.759	5.716	3.634
sampleprot/well3	2.237	1.772	3.591	4.639	7.695	6.443	5.585	4.187
petalprot1	51.837	49.654	92.442	111.216	213.383	167.976	147.018	119.075
petalprot2	57.950	47.908	106.414	128.244	207.707	172.778	171.468	109.033
petalprot3	67.119	53.147	107.723	139.159	230.847	193.299	167.539	125.624
plate 5								
AU 1	0.392	0.860	0.753	0.816	1.024	0.889	0.878	0.649
AU 2	0.383	0.719	0.853	1.063	1.042	1.019	0.783	0.705
AU 3	0.390	0.740	0.886	1.102	1.061	0.890	0.804	0.696
sampleprot/well1	5.065	15.166	12.869	14.219	18.709	15.798	15.552	10.607
sampleprot/well2	4.871	12.136	15.012	19.545	19.096	18.606	13.517	11.819
sampleprot/well3	5.014	12.585	15.742	20.406	19.509	15.814	13.966	11.634
petalprot1	151.964	454.991	386.074	426.582	561.261	473.930	466.565	318.208
petalprot2	146.121	364.082	450.370	586.349	572.894	558.184	405.522	354.574
petalprot3	150.426	377.536	472.255	612.181	585.272	474.408	418.976	349.013

Table A2.3.1.2.1 (previous page) The absorbencies of the three replicates of each petal sample (AU1-3) are given for each of the five replicate plates. Using the standard curves (figures A2.3.1.1.1 to A2.3.1.1.3), the amount of protein in the wells equating to their absorbencies were calculated (sampleprot/well1-3). This was used to calculate the amount of protein in each petal by multiplying the protein content of each well by the amount of extract made and dividing this by the number of petals used to make the extract.

Stage	-2	-1	0	1	2	3	4	5
Mean protein/petal (ug)	107.560	147.100	213.022	262.483	415.684	340.621	284.313	185.931
SD	47.255	138.500	139.907	180.014	255.194	204.944	159.907	101.047
SE	12.629	37.016	37.392	48.111	68.204	54.774	42.737	27.006

Table A2.3.1.2.2 The mean protein content per petal was calculated from the 15 replicate measurements (3 replicates on 5 replicate plates). The standard deviation and standard error of the mean were calculated, and the latter used as a measure of error.

A2.3.2 Leaf protein content

A2.3.2.1 Standards

Standards mg ml-1	0.065	0.13	0.3	0.6	0.8	1	1.2	2	2.5
Standards ug/ well	0.65	1.3	3	6	8	10	12	20	25
std1	0.242	0.294	0.476	0.710	0.746	0.937	0.996	1.485	1.465
std2	0.255	0.305	0.440	0.715	0.727	0.926	0.993	1.452	1.518
std3	0.250	0.301	0.440	0.703	0.751	0.941	1.008	1.468	1.524
Std Mean	0.249	0.300	0.452	0.709	0.741	0.935	0.999	1.468	1.502
Std SD	0.007	0.006	0.021	0.006	0.013	0.008	0.008	0.017	0.032

Table A2.3.2.2.1 Absorbance values for protein standard solutions for the first replicate leaf plate. The concentration of the protein standard solutions are given, along with the equivalent amount of protein present in the well containing that standard solution. The absorbance was measured in triplicate for each standard, and the values obtained for each replicate are given, along with the average of the three replicates and the standard deviation.

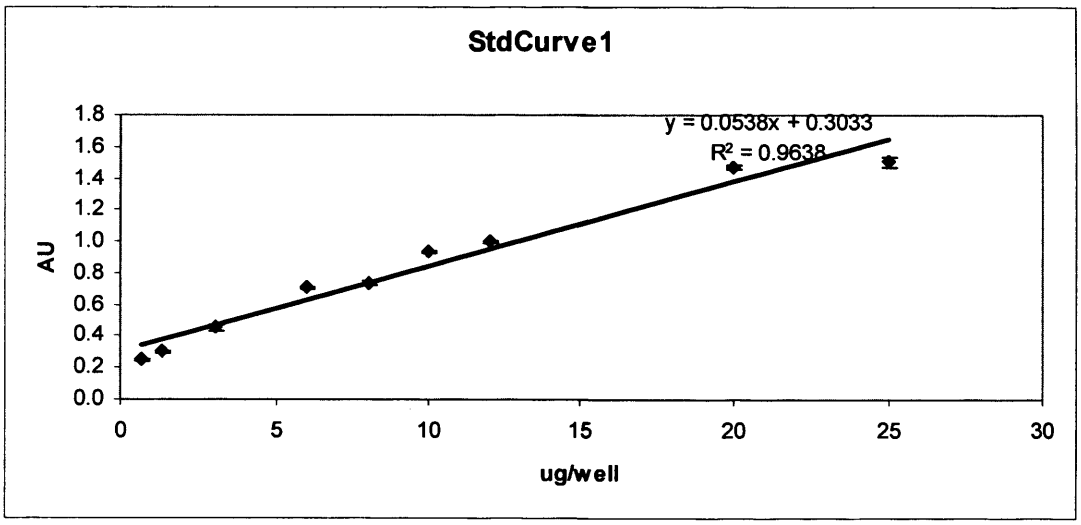

Figure A2.3.1.2.1 Standard curve produced from the data in table A2.3.1.2.1. The average absorbance values were plotted against the protein content of the well, with the error bars representing the standard deviation. A trendline was plotted through the points, and the equation of the trendline is given along with its R^2 value. This standard curve was used to calculate the protein content of the leaf samples of the first replicate plate.

Standards mg ml-1	0.065	0.13	0.3	0.6	0.8	1	1.2	2	2.5
Standards ug/ well	0.65	1.3	3	6	8	10	12	20	25
std1	0.272	0.348	0.455	0.681	0.752	0.974	1.088	1.600	1.603
std2	0.369	0.411	0.477	0.803	0.903	1.098	1.159	1.644	1.619
std3	0.402	0.471	0.609	0.868	0.906	1.022	1.171	1.644	1.594
Std Mean	0.348	0.410	0.514	0.784	0.854	1.031	1.139	1.629	1.605
Std SD	0.068	0.062	0.083	0.095	0.088	0.063	0.045	0.025	0.013

Table A2.3.2.2.2 Absorbance values for protein standard solutions for the second replicate leaf plate. The concentration of the protein standard solutions are given, along with the equivalent amount of protein present in the well containing that standard solution. The absorbance was measured in triplicate for each standard, and the values obtained for each replicate are given, along with the average of the three replicates and the standard deviation.

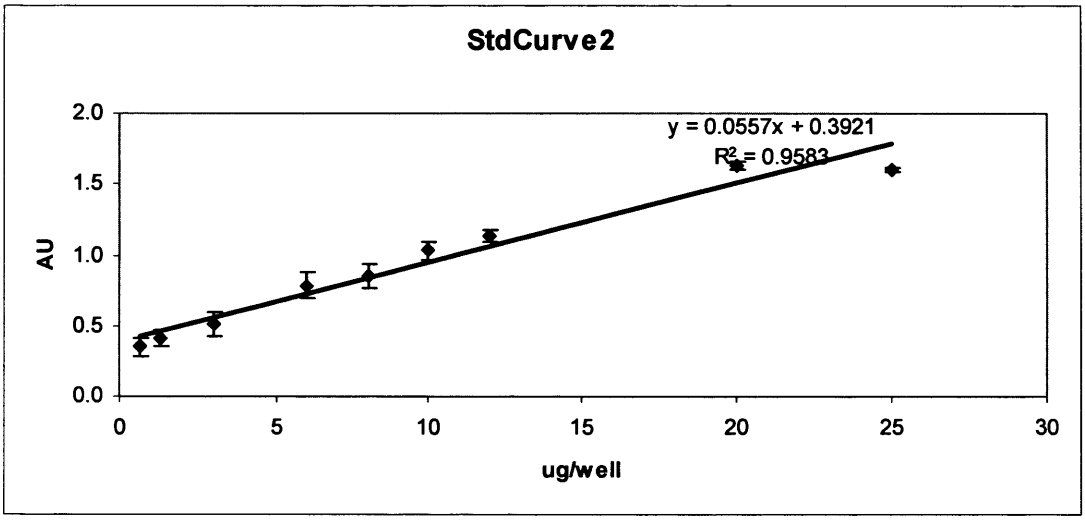

Figure A2.3.1.2.2 Standard curve produced from the data in table A2.3.1.2.2. The average absorbance values were plotted against the protein content of the well, with the error bars representing the standard deviation. A trendline was plotted through the points, and the equation of the trendline is given along with its R^2 value. This standard curve was used to calculate the protein content of the leaf samples of the first second plate.

Standards mg ml-1	0.065	0.13	0.3	0.6	0.8	1	1.2	2	2.5
Standards ug/ well	0.65	1.3	3	6	8	10	12	20	25
std1	0.229	0.397	0.570	0.862	0.839	1.070	1.146	1.581	1.558
std2	0.351	0.439	0.602	0.849	0.888	1.064	1.156	1.610	1.573
std3	0.398	0.471	0.636	0.880	0.895	1.070	1.171	1.606	1.666
Std Mean	0.326	0.436	0.603	0.864	0.874	1.068	1.158	1.599	1.599
Std SD	0.087	0.037	0.033	0.016	0.031	0.003	0.013	0.016	0.059

Table A2.3.2.2.3 Absorbance values for protein standard solutions for the third replicate leaf plate. The concentration of the protein standard solutions are given, along with the equivalent amount of protein present in the well containing that standard solution. The absorbance was measured in triplicate for each standard, and the values obtained for each replicate are given, along with the average of the three replicates and the standard deviation.

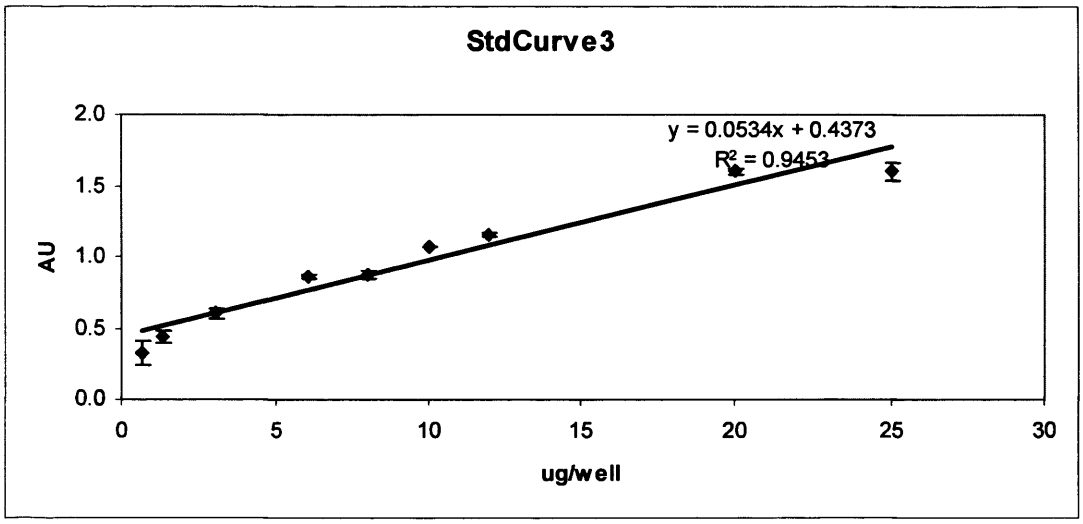

Figure A2.3.1.2.3 Standard curve produced from the data in table A2.3.1.2.3. The average absorbance values were plotted against the protein content of the well, with the error bars representing the standard deviation. A trendline was plotted through the points, and the equation of the trendline is given along with its R^2 value. This standard curve was used to calculate the protein content of the leaf samples of the third replicate plate.

Standards mg ml-1	0.065	0.13	0.3	0.6	0.8	1	1.2	2	2.5
Standards ug/ well	0.65	1.3	3	6	8	10	12	20	25
std1	0.352	0.478	0.630	0.917	0.967	1.179	1.243	1.735	1.683
std2	0.424	0.502	0.666	0.945	0.976	1.175	1.245	1.738	1.805
std3	0.443	0.505	0.673	0.936	0.993	1.217	1.271	1.730	1.784
Std Mean	0.406	0.495	0.656	0.933	0.979	1.190	1.253	1.734	1.757
Std SD	0.048	0.015	0.023	0.014	0.013	0.023	0.016	0.004	0.065

Table A2.3.2.2.4 Absorbance values for protein standard solutions for the fourth and fifth replicate leaf plates. The concentration of the protein standard solutions are given, along with the equivalent amount of protein present in the well containing that standard solution. The absorbance was measured in triplicate for each standard, and the values obtained for each replicate are given, along with the average of the three replicates and the standard deviation.

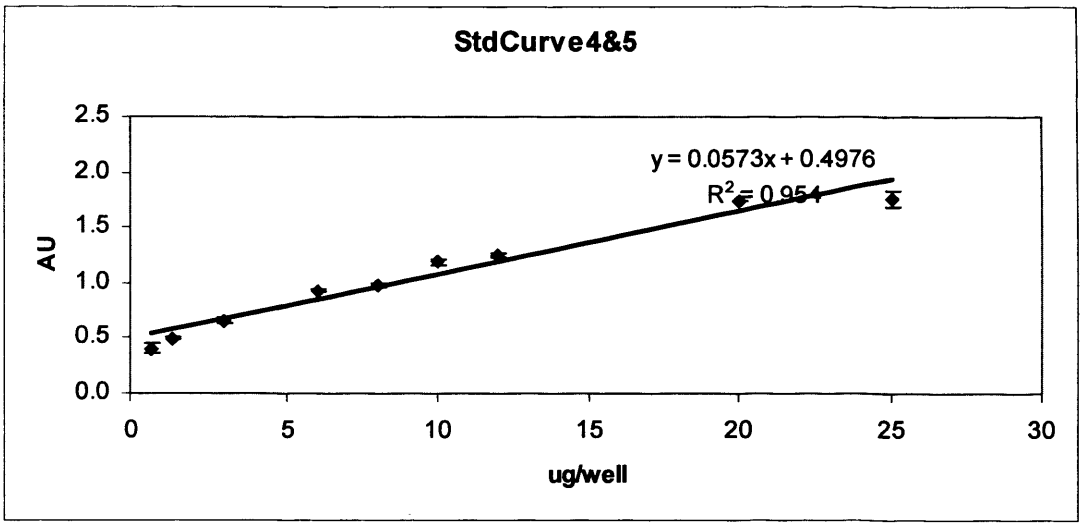

Figure A2.3.1.2.4 Standard curve produced from the data in table A2.3.1.2.4. The average absorbance values were plotted against the protein content of the well, with the error bars representing the standard deviation. A trendline was plotted through the points, and the equation of the trendline is given along with its R^2 value. This standard curve was used to calculate the protein content of the leaf samples of the fourth and fifth replicate plates.

A2.3.2.2 Samples

stage	1+2	3	4	5	6	7
plate 1						
AU 1	0.787	1.483	1.900	1.552	0.664	0.385
AU 2	0.982	1.605	1.750	1.632	0.626	0.354
AU 3	0.653	1.724	1.758	1.555	0.622	0.500
sampleprot/well1	8.982	21.923	29.672	23.202	6.707	1.517
sampleprot/well2	12.608	24.198	26.900	24.695	5.996	0.948
sampleprot/well3	6.494	26.402	27.042	23.273	5.925	3.650
leafprot1	1347.345	3288.375	4450.860	3480.345	1006.065	227.520
leafprot2	1891.260	3629.655	4034.925	3704.310	899.415	142.200
leafprot3	974.070	3960.270	4056.255	3491.010	888.750	547.470
plate 2						
AU 1	1.161	1.924	1.935	1.812	1.030	0.794
AU 2	1.209	1.921	1.954	2.044	1.172	0.569
AU 3	1.022	2.003	1.868	2.108	1.295	0.652
sampleprot/well1	13.798	27.507	27.709	25.491	11.446	7.213
sampleprot/well2	14.672	27.440	28.045	29.658	14.000	3.181
sampleprot/well3	11.312	28.918	26.499	30.800	16.218	4.659
leafprot1	2069.760	4126.080	4156.320	3823.680	1716.960	1081.920
leafprot2	2200.800	4116.000	4206.720	4448.640	2100.000	477.120
leafprot3	1696.800	4337.760	3974.880	4620.000	2432.640	698.880
plate 3						
AU 1	1.209	2.082	2.142	1.802	1.200	1.152
AU 2	1.217	2.115	2.386	1.870	1.182	1.143
AU 3	1.253	2.076	2.344	1.981	1.226	0.696
sampleprot/well1	14.442	30.801	31.918	25.553	14.275	13.381
sampleprot/well2	14.610	31.416	36.496	26.837	13.940	13.214
sampleprot/well3	15.280	30.690	35.715	28.903	14.777	4.839
leafprot1	2166.333	4620.208	4787.708	3832.958	2141.208	2007.208
leafprot2	2191.458	4712.333	5474.458	4025.583	2090.958	1982.083
leafprot3	2291.958	4603.458	5357.208	4335.458	2216.583	725.833
plate 4						
AU 1	1.287	2.193	2.223	1.653	1.435	1.117
AU 2	1.535	2.190	2.235	1.744	1.511	1.123
AU 3	1.396	2.166	2.442	1.681	1.432	0.841
sampleprot/well1	13.772	29.589	30.118	20.173	16.364	10.809
sampleprot/well2	18.109	29.536	30.329	21.760	17.686	10.915
sampleprot/well3	15.676	29.113	33.927	20.649	16.311	5.995
leafprot1	2065.745	4438.310	4517.660	3025.880	2454.560	1621.385
leafprot2	2716.415	4430.375	4549.400	3263.930	2652.935	1637.255
leafprot3	2351.405	4366.895	5088.980	3097.295	2446.625	899.300
plate 5						
AU 1	1.484	2.278	2.293	1.805	1.538	1.008
AU 2	1.523	2.335	2.369	1.851	1.596	1.035
AU 3	1.147	1.893	2.111	1.650	1.384	0.529
sampleprot/well1	17.210	31.070	31.334	22.818	18.162	8.905
sampleprot/well2	17.898	32.075	32.657	23.611	19.167	9.381
sampleprot/well3	11.338	24.352	28.160	20.120	15.464	0.547
leafprot1	2581.520	4660.490	4700.165	3422.630	2724.350	1335.725
leafprot2	2684.675	4811.255	4898.540	3541.655	2875.115	1407.140
leafprot3	1700.735	3652.745	4224.065	3017.945	2319.665	81.995

Table A2.3.2.2.1 (previous page) The absorbencies of the three replicates of each leaf sample (AU1-3) are given for each of the five replicate plates. Using the standard curves (figures A2.3.1.2.1 to A2.3.1.2.4), the amount of protein in the wells equating to their absorbencies were calculated (sampleprot/well1-3). This was used to calculate the amount of protein in each leaf by multiplying the protein content of each well by the amount of extract made and dividing this by the number of leaves used to make the extract.

Stage	1+2	3	4	5	6	7
Mean protein/leaf (ug)	2062.019	4250.281	4565.210	3675.421	2064.389	991.536
SD	482.321	449.772	479.848	507.626	651.888	648.335
SE	128.906	120.207	128.245	135.669	174.224	173.275

Table A2.3.2.2.2 The mean protein content per leaf was calculated from the 15 replicate measurements (3 replicates on 5 replicate plates). The standard deviation and standard error of the mean were calculated, and the latter used as a measure of error.

A2.4 Raw data for RNA content

A2.4.1 Petal RNA

age	n.o. petals	weight (g)	total ul RNA	conc RNA (ug/ul)	total RNA (ug)	ugRNA/pl	ugRNA/gFW
-2	193	0.22	30	1.5	45	0.233	204.545
-1	187	0.27	30	3.5	105	0.563	388.889
0	153	0.31	30	3.8	114	0.744	367.742
1	146	0.77	50	0.7	35	0.240	45.455
2	161	0.82	50	1.2	60	0.373	73.171
3	259	1.03	50	1.7	85	0.329	82.524
4	142	0.41	30	1.6	48	0.338	117.073
5	500	0.9	50	1	50	0.100	55.556

Table A2.4.1.1 Raw data for petal RNA content. The number of petals used for the each RNA extraction and their weights are given. The volume in which the extracted RNA was resuspended is given along with the concentration of the RNA. These to values were multiplied to give the total amount of RNA extracted. This value was divided by the number of petals used for the extraction to calculate the amount of RNA per petal, and divided by the fresh weight of the petals used for the extraction to calculate the amount of RNA present per g fresh weight of petals.

A2.4.2 Leaf RNA

age	n.o. leaves	weight (g)	total ul RNA	conc RNA (ug/ul)	total RNA (ug)	ugRNA/lf	ugRNA/gFW
1&2	40	0.759	50	1.55	77.5	1.94	93.78
3	21	1	50	2.6	130	6.19	130.00
4	20	1	50	2.4	120	6.00	120.00
5	21	1	50	2.4	120	5.71	120.00
6	21	1	50	1.4	70	3.33	70.00
7	36	0.9	100	0.4	40	1.11	44.44

Table A2.4.2.1 Raw data for leaf RNA content. The number of leaves used for the each RNA extraction and their weights are given. The volume in which the extracted RNA was resuspended is given along with the concentration of the RNA. These two values were multiplied to give the total amount of RNA extracted. This value was divided by the number of leaves used for the extraction to calculate the amount of RNA per leaf, and divided by the fresh weight of the leaves used for the extraction to calculate the amount of RNA present per g fresh weight of leaves.

Appendix 3

A3 *Arabidopsis* senescence associated genes printed on the microarray

Clone	PROTEIN ENCODED	*Arabidopsis* gene
138	Alpha tubulin	At4g14950
139	Actin	At1g49240
ACC2	ACC synthase	At4g26200
ACO2	ACC oxidase	At1g62380
At103-A	Glutathione S transferase	At2g29450
AtWRKY15	WRKY factor	At2g23320
AtWRKY17	WRKY factor	At2g24570
AtWRKY4	WRKY factor	At1g13960
AtWRKY53	WRKY factor	At4g23810
AtWRKY6	WRKY factor	At1g62300
B113	RBCS	At1g67090
B72	Cytochrome P450	At2g45570
Bax 1	Cell death factor	At5g47120
CAB	CHLOROPHYLL A/B	At2g34420
CAT1	catalase	At1g20630
CAT2	catalase	At4g35090
CAT3	catalase	At1g20620
CCH	Copper homeostasis factor	At3g56240
CST	Citrate synthase	At2g44350
Cyp71B3	cytochrome P450	At3g26220
Cyp71B4	cytochrome P450	At3g26280
Cyp83A1	cytochrome P450	At4g13770
Cyp91A2	cytochrome P450	At4g37430
DHOR	Dihydro-orotate reductase	At3g17810
EAT1	ACC oxidase	At1g05010
ERD 16	Ubiquitin extension CEP52	At3g52590
Erd1	Clp protease	At5g51070
ERD6	Sugar transporter ATZF1	At1g08930
GST1	GST Atpm24.1	At4g02520
HIN1	Harpin induced	At2g35980
Lox1	lipoxygenase	At1g17420
Lox2	lipoxygenase	At1g55020
Lox3	lipoxygenase	At3g45140
LSC141	Xylosidase?	At5g49360
LSC213	POP dikinase	At4g15530/At4g15520
LSC215	xyloglucan fucosyl trans ?	At2g15390
LSC222	chitinase	At2g43570
LSC223	unknown	At2g38640
LSC226	cytochrome P450	At4g31500
LSC30	ferritin	At5g01600
LSC301	12-OPDR	At1g76680
LSC320	unknown	At1g27330
LSC322	Aquaporin MIPC	At2g39010
LSC323	Isopropylmalate dehase	At5g03290
LSC326	Gamma VPE	At4g32940
LSC327	unknown	At1g27020
LSC331	flavonol glucosyl transfera	At4g01070
LSC332	Unknown	At2g17710
LSC334	NADPH quin oxidoreduct	At4g21580
LSC336	unknown	BAC F4N2

LSC337	Histone H1-3	At2g18050
LSC418	Cu amine oxidase	At4g12280
LSC460	GS	At5g37600
LSC54	metallothionein	At1g07600
LSC550	cytochrome P450	At1g64950
LSC639	ABC transporter	At4g04770
LSC648	hydrolase/lipase	At5g13800
LSC7	cysteine protease	At5g60360
LSC760	aspartic protease	At1g11910
LSC790	cysteine protease	At4g39090
LSC803	glutathione peroxidase	At4g11600
LSC812	Carnitine racemase	At1g65520
LSC813	unknown	At3g17800
LSC813A	Acid phosphatase	At3g17790
LSC814	DNA J homolog	At5g06910
LSC815	SRP?	At4g30600
LSC88	Wali 7 like/stress response	At5g19140
LSC94	PR1a	At2g14610
nClpP3	Cys protease	At1g66670
nClpP5	Cys protease	At1g49970
PDF1..2	defensin	At5g44420
PED1	Thiolase	At2g33150
PLC1	Phospholipase C	At4g38530
PLD (1)	Phospholipase D	At3g15730
PLD (3)	Phospholipase D	At4g35790
PR21	Zeaxanthin epoxidase	At5g67030
RD21a	Cys protease	At1g47128
RNS1	RNAse	At2g02990
RNS2	RNAse	At2g39780
SAG 15	ERD 1	At5g51070
Sag 18	Unknown	At1g71190
SAG 21	LEA pro	At4g02380
SAG 27	Nitrilase 2	At3g44300
SAG12	cysteine protease	At5g45890
SAG13	ADH	At2g29350
SAG14	Blue Cu binding	At5g20230
Sen 4	xyloglucan transferase	At4g30270
Sen5	unknown	At3g15450
SRG3	b- glucosidase	At3g02040
STP4	sugar transporter	At3g19930
UBIQ	Polyubiquitin UBQ4	At5g03240

Table A3.1 Identities of the *Arabidopsis* clones known to be upregulated during leaf senescence that were printed onto the microarray (chapter 5), along with details of the protein encoded by the gene and the unique identifying *Arabidopsis* gene number.

Appendix 4

A4 Optimisation of the microarray experiment

As a microarray experiment contains several elements (described in chapter 6), any or all of the elements of the array experiment can be sub-optimal, resulting in unusable data. As this proved to be the case in the initial attempt, a series of experiments was embarked upon to establish where the problem lay.

A4.1 Experiment 1

A4.1.1 Materials and methods

The first array experiment used a probe made from young and old total RNA (defined in section 6.2.2) with the cDNA post-labelling method using CyDyes, as described in section 6.2.2.2. The RNA used was made as described in section 2.2.3 and purified as described in section 2.2.8, and it is important to note that the messageAmp aRNA amplification kit (section 6.2.2.1) was not used. Once made, the quality of the probe was checked by running 0.5µl of each labelled cDNA on an agarose gel which was set on a microscope slide (as described in section 2.2.10, with the exception that ethidium bromide was not used, as the CyDye used to label the cDNA is fluorescent, and thus ethidium bromide was not needed to visualise the DNA). The gel was scanned using an Affymetrix 428 array scanner with the supplied software (Affymetrix, Santa Clara, CA, USA) at 532nm (Cy3) and 633nm (Cy5). This probe was hybridised to one of the microarray slides (prepared as described in section 6.2.1) as described in section 6.2.3 and scanned, as described in section 6.2.4.

A4.1.2 Results and discussion

From the gel run of the probe, the probe appeared to be of high quality. Both channels of the probe labelled well, and high incorporation of the CyDyes into the cDNA could be seen as a smear of variably sized cDNAs on the gel, with a low amount of unincorporated dye seen as a smaller smear. However, when the slide was scanned, no spots were visible at all, including the autofluorescent marker spots. This strongly suggested that there were no spots present on the slide for the probe to

hybridise to, indicating that there may have been a problem with the printing or the fixing of the slides.

In order to resolve this problem, details of the printing and fixing procedure were checked with the molecular biology support unit, and the remaining slides from the same print run were stained with sybr green, as described in section 6.2.1, to check for the presence of spots on the remaining slides. Spots were visible, indicating that the remaining slides were useable.

A4.2 Experiment 2

A4.2.1 Materials and methods

For the second array experiment, a probe was made from young and old total RNA (defined in section 6.2.2) using the cDNA direct-labelling method using CyDyes, as this protocol is quicker than that for the post-labelling method, and cheaper, as a kit was not required. The petal RNA used was prepared as described in section 2.2.3, purified as described in section 2.2.8, and mixed as defined in section 6.2.2 as old and young. 20µg of 'old' or 'young' petal total RNA (10µl) was mixed with 1µl of anchored oligo dT in an amber microcentrifuge tube on ice and incubated at 70°C for 5 mins. The reaction mixture was allowed to cool to room temperature and incubated for a further 10 mins to allow annealing of the primers to the template RNA. Once annealed, the reaction mixture was returned to the ice and 4µl of 1st strand RT buffer (Invitrogen, Groningen, the Netherlands; supplied with RT enzyme), 2µl of 0.1M DTT, 1µl of dUTP nucleotide mix and 1µl of dUTP CyDye labelled nucleotide was added to it and mixed. This reaction was incubated at 42°C for 2 mins before adding 2µl of superscript II reverse transcriptase enzyme (Invitrogen, Groningen, the Netherlands) and incubating at 42°C for a further 50 mins then at 70°C for 15 mins to inactivate the enzyme. The mRNA template was degraded by the addition of 2µl of 2.5M sodium hydroxide with incubation at 37°C for 15 mins. This was neutralised by the addition of 10µl of 2M HEPES free acid. The probe was cleaned using the QIAquick PCR purification kit (Qiagen Ltd, Crawley, UK), as described in section 2.2.14. The probe was checked on a gel, as described in section A4.1.1 and hybridised to one of the microarray slides (prepared as described in section 6.2.1) as described in section 6.2.3 and scanned, as described in section 6.2.4.

A4.2.2 Results and discussion

From a comparison of the gels run of the probes made in section A4.1.1 and A4.2.1, this probe did not appear as good as the probe made in section A4.1.1, but was still acceptable for use. This was probably due to the different labelling methods used, as post-labelling is generally considered to be a better method than direct labelling (see section 6.1.1.2 for a discussion). Also, a kit was used in section A4.1.1, which came with an optimised protocol, rather than using different reagents from different manufacturers which had not been optimised for use in this way, as was done here. Despite this probe not appearing to be as good as the previous probe, the hybridisation did work. However, the hybridisation was poor, and the intensity of the spots was therefore weak and no usable data was obtained. This may have been due to the dye bias encountered when using the direct labelling method (see section 6.1.1.2), and so the experiment was repeated having swapped the dyes used to label the young and the old RNA. This did not improve the results. It can therefore be concluded that either the spots on the slide have printed poorly, that the probe used is not of high enough quality, or that both the slide and the probe are sub-optimal. Thus, the quality of the slide and of the probe need to be assessed independently.

A4.3 Experiment 3

A4.3.1 Materials and methods

In order to test whether the slides were acceptable, a slide was sent to collaborators working at HRI Wellesbourne, where they had a similar microarray system working well on *Arabidopsis*. They used *Arabidopsis* RNA using the same methods as described in section 6.2.2 to prepare the probe, and used this probe to hybridise to the wallflower microarray slide (which also contains some spots derived from *Arabidopsis* genes) using the same method as described in section 6.2.3 and scanned the slide, as described in section 6.2.4.

A4.3.2 Results and discussion

The *Arabidopsis* probe hybridised successfully to the wallflower array, giving usable, but still weak, signals from most of the spots. Thus, it can be concluded that, although the slides are acceptable, in order for them to give usable results, the probe used must be of very high quality.

A4.4 Experiment 4

A4.4.1 Materials and methods

Thus, for the next step in the optimisation process, fresh RNA was extracted and used immediately to minimise problems with RNA quality due to storage for excessive periods of time. The protocol described in section 6.2.2 was followed to make the probe, using the messageAmp aRNA kit to improve the efficiency of the probe labelling reaction as there was more aRNA (equivalent to mRNA) present in the reaction than in the total RNA previously used, and using the post-labelling kit to improve the quality of the final probe compared to the direct-labelled probe made without a kit.

A4.4.2 Results and discussion

This method worked well, and was used to generate the results presented in chapter 6.

Appendix 5

A5 Ratios and t-test P-vales obtained for each clone on the microarray

The following table lists each of the clones spotted on the microarray, along with the value for ratio of old/young, the t-test P-value, and the mean raw values for old and young for the 12 replicate spots for both the petal and the leaf experiments. Where there are no values given, that clone was removed from the analysis following Imagene analysis for being empty (sections 6.2.4.1 and 6.3.3.1). All numbers are given to 4 decimal places.

Systematic	Petal ratio	petal t-test P-value	mean old petal	mean young petal	Leaf ratio	leaf t-test P-value	mean old leaf	mean young leaf
138	0.2873	0.6125	324.8333	1015.7500	0.8688	0.6482	579.9166	661.0000
139	0.4197	0.6841	645.9583	1205.0000	0.8142	0.4825	743.3751	799.4584
ACC2					0.0100	no reps.	-60.0000	200.5000
ACO2	3.0318	0.0000	12445.3750	4195.3750	1.1248	0.5513	7914.2090	6530.9590
APHPC1	17.2821	0.0076	379.3333	19.4583	3.1764	0.0414	223.7222	77.7222
APHPH9	3.0415	0.0001	4056.0420	1322.8334	1.0796	0.6896	4901.9165	4476.1665
APHPH9h	4.2626	0.0000	4514.7085	1053.8713	0.8709	0.2151	5610.7744	6299.4100
APHPH9n	3.8654	0.0000	9042.7380	2351.2004	0.3388	0.3314	9686.7680	10737.0170
APL1B4	7.9790	0.0039	455.6667	53.2083	4.6664	0.0091	263.4167	50.1042
APL1B5	29.0486	0.0090	492.9375	15.8750	7.6234	0.0057	523.6250	81.0000
APL1F6	2.0004	0.4394	271.0417	148.4167	0.8419	0.6040	315.5833	379.8750
APL1H3	4.0394	0.0000	1135.2917	266.2500	1.3144	0.1497	947.9166	696.0416
APP1D11	5.7979	0.0000	1434.4584	270.5000	2.2799	0.0102	513.2083	231.5625
APP1F10	5.6331	0.0003	4340.5830	749.7083	0.3411	0.0164	2836.9580	7117.8330
APP1F4	67.7231	0.0000	1115.7500	14.5000	9.3505	0.0007	560.3125	49.3958
APP1F4h	141.4174	0.0004	8531.6310	69.5198	8.6033	0.0000	1822.6069	211.7805
APP1F4n	144.6308	0.0001	9948.3980	77.6641	9.6387	0.0075	2097.0728	218.8229
APP1F8	19.5872	0.0000	2484.0835	126.5417	0.3648	0.0205	2022.7500	5629.2915
APP2B1	12.2710	0.0000	614.8333	48.5417	13.3044	0.0000	525.3333	37.7500
APP4D6	4.3792	0.5304	178.7500	50.5833	1.5661	0.1693	547.3959	358.0625
APP5B10	10.4446	0.0028	439.0417	40.2500	2.4196	0.0317	270.0625	104.8333
APP6C7	2.0936	0.7406	214.1250	81.6250	1.2616	0.6099	138.2083	104.9583
At103-A	0.3732	0.3981	2533.8333	6776.6250	0.8799	0.5961	4146.4585	4583.1250
AtWRKY15	1.0192	0.9708	1042.4584	1015.6667	1.0906	0.7170	1002.2084	943.5417
AtWRKY17	1.7058	0.6101	385.0833	208.7083	2.2842	0.1349	477.2500	263.7917
AtWRKY4	2.1798	0.0041	5729.9165	2635.4583	2.0229	0.0002	4595.9585	2169.5208
AtWRKY53	1.5526	0.4979	639.0834	401.9167	0.7727	0.3127	1368.4584	1772.1250
AtWRKY6	8.7602	0.0738	507.1667	54.3333				
B								
B113	0.5563	0.8675	148.8125	233.6042	0.2802	0.0194	101.0938	304.9479
B72	3.6334	0.0001	3556.2708	941.7499	1.6282	0.0327	3002.7085	1878.7917
Bax1	1.7730	0.1810	2035.5834	1003.0833	0.8098	0.4078	2249.2083	2611.2917
CAB	0.5204	0.6897	489.0208	969.7291	0.3731	0.0636	625.9167	1752.4583
CAT1	3.5689	0.0000	5106.5000	1431.0000	1.1288	0.5289	3082.1665	2706.7500
CAT2	1.3834	0.8319	456.1250	213.9583	0.7853	0.6583	803.1041	738.1458
CAT3	1.2373	0.5478	7982.4580	6405.1250	0.8362	0.4384	6942.9165	7767.9165
CCH	4.0291	0.0000	14937.8750	3693.9167	1.5134	0.0204	11651.3750	7739.4590
CST	1.6617	0.2492	1097.9583	632.8333	0.8491	0.6564	616.8750	543.9792
Cyp71B3								
Cyp71B4	7.5106	0.0520	299.9583	43.6667	1.9057	0.1307	217.9583	117.4167
Cyp83A1	4.4702	0.1495	298.7083	90.1667	2.2338	0.0423	304.0000	138.0000
Cyp91A2					3.7483	0.1613	197.0000	50.8333
DHOR	1.6758	0.2020	1323.9583	748.6250	1.1397	0.6424	603.1458	512.0208
EAT1	5.8336	0.0046	3174.1250	563.0834	1.7971	0.1908	2680.4165	1395.0208
Erd1	7.5026	0.1522	490.9583	52.4167	2.5963	0.1488	247.4167	73.2917
ERD16	1.1174	0.9673	662.0000	528.0000	0.6167	0.4858	385.5833	595.7916
ERD6	3.3152	0.2833	236.1875	71.2500	1.0626	0.8918	187.6875	153.9375
GST1	9.4091	0.0002	4341.5415	470.7917	0.2842	0.0104	3644.2502	11885.7920
HIN1	5.7201	0.1884	334.1250	55.7083	5.1748	0.0001	541.6666	98.3333
lambda					10.2000	no reps.	102.0000	10.0000

Lox1	2.6440	0.1541	347.7292	148.0208	1.3120	0.3337	575.5000	391.6250
Lox2	1.3059	0.8013	452.9167	334.4167	0.4712	0.1908	396.2917	436.2083
Lox3	0.2241	0.7443	69.5625	395.8125	0.8358	0.5804	484.2500	608.3333
LSC141	17.0924	0.0000	6239.7920	385.1250	2.8515	0.0001	1632.7916	588.7500
LSC213	4.8795	0.0078	828.2500	181.2083	1.3964	0.2407	506.2917	369.4167
LSC215	1.5461	0.1835	4240.6875	2741.9375	1.0406	0.8689	3145.5000	2996.7083
LSC222	2.3294	0.0055	1966.4167	748.0000	1.0615	0.7857	2011.2500	1824.0834
LSC223	4.8038	0.0000	3089.2085	685.2500	1.2961	0.1709	2923.7500	2172.1667
LSC226	3.4269	0.0111	701.8334	231.4792	0.6059	0.1100	2054.9167	3298.8750
LSC30	1.2002	0.6920	2022.9583	1743.9167	3.6868	0.0003	4606.2295	1327.3750
LSC301	2.1243	0.0080	3552.0833	1768.1250	1.4864	0.0215	4142.9165	2790.3750
LSC320	0.7682	0.6515	4823.7080	6173.6250	0.5712	0.0808	3976.9585	6038.5830
LSC322	0.2596	0.8030	187.7500	576.4167	0.2886	0.1074	72.9167	292.4583
LSC323	1.4857	0.2243	4378.6250	2974.0415	1.0822	0.6837	4138.3750	3723.4167
LSC326					9.2353	no reps.	157.0000	17.0000
LSC327	3.7363	0.0452	560.7083	210.2917	0.8416	0.5587	787.3749	930.9583
LSC331	13.4463	0.0000	2272.9583	179.5000	1.3578	0.3324	2073.7915	1576.2500
LSC332	6.9256	0.0019	463.0000	69.5833	1.4508	0.3488	223.5417	161.4167
LSC334	1.9919	0.0607	1314.2916	683.7084	1.1611	0.4973	979.8124	844.8334
LSC336	3.9546	0.0370	464.0417	135.5208	0.7007	0.5899	157.3125	240.0625
LSC337	20.2440	0.0010	5373.9585	316.9583	5.6873	0.0002	5039.1250	834.7708
LSC418	2.3664	0.5598	163.4375	78.7083	2.2644	0.5070	131.5000	59.0000
LSC460	2.6581	0.0003	11309.2920	4241.4165	1.2971	0.1359	7673.6250	5868.1670
LSC54	31.5423	0.0002	365.0625	11.2917	1.7135	0.1260	309.0000	190.7083
LSC550	5.2662	0.2216	584.6041	110.6042	3.2195	0.0381	541.9375	231.0000
LSC639	1.8046	0.1544	1053.8750	529.1250	1.8144	0.0510	761.1250	443.5417
LSC648	5.2353	0.0000	3569.6667	712.0833	2.6030	0.0068	3056.4165	1199.5834
LSC7	6.1992	0.0038	854.8542	169.2292	0.9144	0.7146	1137.8959	1254.3333
LSC760	3.9404	0.0118	2532.9167	775.8541	1.0208	0.9370	2101.3335	2019.4584
LSC790	2.9720	0.0000	8108.8750	2944.0835	1.4830	0.0410	7146.1665	4860.9795
LSC803	1.7942	0.0297	12404.7090	6926.2085	0.9108	0.6627	10545.2920	11392.1250
LSC812	2.4910	0.0020	2785.5417	1124.6250	0.8894	0.6786	2027.2083	1915.9375
LSC813	1.4719	0.2379	3775.0835	2557.1250	0.9825	0.9324	4448.4580	4567.9580
LSC813A	2.2609	0.5440	158.3750	76.2083	1.5855	0.4918	127.4375	85.2500
LSC814	1.3426	0.5296	863.2084	632.5417	1.3473	0.3332	1053.6250	832.5416
LSC815	2.9194	0.0093	1929.0833	706.1250	0.8815	0.5805	1977.2917	2216.1665
LSC88								
LSC94	4.2051	0.0001	881.5208	224.3958	0.5099	0.0491	4058.8750	7828.8750
M								
N								
nClpP3	2.1324	0.3250	612.1042	323.1042	0.9919	0.9816	1224.3750	1051.4584
nClpP5	1.5200	no reps.	2927.5000	1926.0000	0.1797	0.3069	43.2500	243.2500
NO DNA								
PDF1..2	2.9419	0.7432	184.0000	56.0000	1.4781	0.5707	276.4375	183.5625
PED1	3.2607	0.2188	1403.1875	443.6041	1.7005	0.2867	1480.3750	794.3125
Plate1 A1								
Plate1 A10	1.4272	0.9127	135.7500	95.2500	1.1871	0.7911	185.7500	141.4167
Plate1 A11	3.6420	no reps.	442.5000	121.5000	5.0825	0.0313	179.7222	48.3333
Plate1 A12	3.3721	0.4524	336.2500	86.3750	1.8332	0.4442	120.2500	64.5833
Plate1 A13								
Plate1 A14	278.4540	0.0014	16914.9160	96.0417	138.4276	0.0000	12938.5000	95.5417
Plate1 A15	22.4005	0.0002	10822.1670	462.8750	4.6532	0.0016	1698.6250	388.4167
Plate1 A16	41.8794	0.0000	1781.4166	43.4167	5.9256	0.0282	661.8125	98.6875
Plate1 A17	0.9197	0.8955	846.0001	843.8750	1.0168	0.9614	858.5417	914.1250
Plate1 A18	1.3114	0.4376	4386.4585	3378.5835	2.5642	0.0000	8875.2920	3543.7920
Plate1 A19	34.6162	0.0001	2944.9165	89.2917	4.3971	0.0049	3748.1250	832.8334
Plate1 A2	12.2257	0.1690	260.7500	22.5000	0.3810	0.1075	262.5000	419.2917
Plate1 A20	3.2682	0.2097	254.0417	72.1458	1.6024	0.1391	437.8542	288.2083
Plate1 A21	1.1549	no reps.	82.0000	71.0000	2.1339	0.3188	120.5556	51.2222
Plate1 A22	30.9000	no reps.	309.0000	10.0000				
Plate1 A23	24.0435	no reps.	553.0000	23.0000				
Plate1 A24	1.2276	0.8779	288.2708	283.9375	1.3042	0.6205	488.7708	370.3958
Plate1 A3	1.0096	0.9966	190.8542	192.0625	1.2177	0.7215	322.3889	249.3889

Plate1 A4								
Plate1 A5								
Plate1 A6	1.7850	0.1065	2953.6250	1738.9375	1.2023	0.6150	1569.0416	1693.5000
Plate1 A7	22.3130	0.0000	557.8333	29.0000	23.9136	0.0002	3515.8890	155.2222
Plate1 A8					1.6166	0.5743	98.0000	59.4167
Plate1 A9								
Plate1 B1	10.8461	0.0067	3965.6250	466.1667	1.6576	0.0110	2812.9790	1639.7084
Plate1 B10								
Plate1 B11								
Plate1 B12								
Plate1 B2					4.4164	0.2889	83.2500	21.0000
Plate1 B3	0.7704	0.8355	216.3333	289.3750	1.8328	0.1865	438.9444	241.0000
Plate1 B4	6.1313	0.0000	12424.8330	2016.8750	1.6441	0.0072	10141.9160	6000.3750
Plate1 B5	6.1190	no reps.	257.0000	42.0000				
Plate1 B6	2.5653	0.8206	115.8750	47.8750	3.2538	0.0006	663.1111	209.0556
Plate1 B7	1.7222	0.1158	958.1666	562.3750	1.6346	0.0156	1958.2500	1221.1250
Plate1 B8	373.1050	0.0000	6909.9165	23.9583	363.8443	0.0000	6218.3330	17.8333
Plate1 B9	2.8876	no reps.	244.0000	84.5000	1.3765	0.7057	92.6667	67.3333
Plate1 C1								
Plate1 C10	4.3571	no reps.	305.0000	70.0000	1.6875	0.5962	114.0833	74.9167
Plate1 C11	2.8740	no reps.	182.5000	63.5000	0.8536	0.8511	120.0000	113.8889
Plate1 C12	12.0648	0.0081	678.5417	93.7083	23.5400	0.0000	3462.1250	166.5000
Plate1 C13	1.8176	0.6068	871.9167	525.4167	1.4850	0.2039	619.1459	469.9375
Plate1 C14					6.1287	0.0047	173.3333	24.5833
Plate1 C15	4.0941	no reps.	174.0000	42.5000	8.0455	no reps.	177.0000	22.0000
Plate1 C16	1.1279	0.8362	688.8334	669.3750	0.8394	0.5387	674.3333	796.0833
Plate1 C17	1.1544	0.8075	1857.7917	1413.2500	1.7750	0.0274	2335.7083	1280.2917
Plate1 C18	1.1875	0.6822	3967.2500	3353.7917	2.4044	0.0001	5317.0000	2286.0835
Plate1 C19	4.0681	0.0000	8887.5830	2287.8335	1.6817	0.0088	7121.8960	4585.7085
Plate1 C2								
Plate1 C20	1.6661	0.4319	431.7500	270.3333	0.6918	0.7061	485.5000	479.8750
Plate1 C21	1.7072	0.7939	357.8125	197.1250	1.1626	0.8801	514.0000	648.3125
Plate1 C22	1.3796	0.6159	508.9167	364.8333	0.8273	0.6182	761.7500	847.0209
Plate1 C23	0.8955	0.9414	232.0208	297.9792	0.7217	0.4125	785.2083	1197.3541
Plate1 C24	0.5488	0.6714	347.7083	674.5833	1.1439	0.7883	585.4166	587.8750
Plate1 C3	1.9328	0.3814	329.6042	184.1250	1.2961	0.4810	397.0833	313.0000
Plate1 C4	20.3991	0.0244	295.8333	14.5000	5.1048	no reps.	158.2500	31.0000
Plate1 C5								
Plate1 C6	11.0144	0.3328	253.0000	23.6667	1.0046	0.9951	219.3333	158.0000
Plate1 C7					3.8971	0.1378	164.6667	43.1667
Plate1 C8	55.2088	0.0001	1364.0417	27.3333	53.3716	0.0014	1124.1667	26.1111
Plate1 C9								
Plate1 D1	30.6870	0.0000	1282.6666	47.8333	19.5405	0.0000	720.8749	31.6667
Plate1 D10	278.0650	0.0000	3483.2083	12.5833	197.1771	0.0000	2788.9792	14.9792
Plate1 D11								
Plate1 D12								
Plate1 D2					30.4000	no reps.	304.0000	10.0000
Plate1 D3					37.2500	no reps.	372.5000	10.0000
Plate1 D4	1.5926	0.8813	239.3333	134.3333	1.2815	0.7766	166.0833	152.2500
Plate1 D5	2.2669	0.7856	116.3333	52.0000	1.7894	0.4804	124.2500	75.4167
Plate1 D6	1.6292	0.2949	644.2083	428.3750	1.5497	0.0206	1475.9583	960.7916
Plate1 D7	5.4417	0.2338	181.6250	63.7708	2.5550	0.2314	104.8333	41.7500
Plate1 D8	454.3864	0.0000	14784.2080	33.6250	269.2525	0.0000	10156.6250	39.9167
Plate1 D9								
Plate1 E1								
Plate1 E10	15.9292	0.0001	981.9166	78.9792	7.1983	0.0018	302.2500	47.8125
Plate1 E11	24.2892	0.0091	463.8125	12.4167	10.3649	0.0004	207.8333	17.8333
Plate1 E12	2.5211	0.6172	147.2778	104.8889	3.5852	0.0083	213.1667	61.5000
Plate1 E13	44.6371	0.0001	954.9792	24.3750	2.3898	0.2495	508.3125	137.5833
Plate1 E14	19.3596	0.0391	786.0416	49.2500	8.9552	0.0102	1069.3959	145.3333
Plate1 E15	4.3291	0.0318	460.6458	142.7083	6.0824	0.0005	773.6667	128.6250
Plate1 E16	2.7364	0.2776	306.0208	106.7917	2.8653	0.0210	643.3333	210.8750
Plate1 E17	1.0684	0.9176	1410.5416	1307.4792	1.2274	0.2707	2093.3335	1763.4167

Plate1 E18	0.8205	0.7449	1427.2500	1745.8959	1.0208	0.9196	2276.6458	2220.7292
Plate1 E19	1.3492	0.4290	2513.2500	1865.5000	1.4587	0.0267	4164.2915	2868.6250
Plate1 E2	34.4500	no reps.	344.5000	10.0000				
Plate1 E20	2.6975	0.0415	558.7500	218.3333	1.6920	0.1471	958.2708	601.5833
Plate1 E21	1.0442	no reps.	118.0000	113.0000	0.4999	0.3462	94.1875	200.9792
Plate1 E22	0.8836	0.8037	5652.6665	6421.6665	0.9292	0.7483	8045.2710	8541.9790
Plate1 E23	6.6094	no reps.	211.5000	32.0000				
Plate1 E24	11.3611	no reps.	818.0000	72.0000	12.2625	0.0084	375.7500	24.5000
Plate1 E3	86.4423	0.0007	4355.5000	64.6250	2.4823	0.0012	3056.2500	1218.7500
Plate1 E4	3.5152	no reps.	174.0000	49.5000	3.9700	0.0200	173.7083	39.0000
Plate1 E5	1.7801	no reps.	502.0000	282.0000	8.0400	no reps.	201.0000	25.0000
Plate1 E6	3.1086	0.2825	237.2708	80.5625	0.7712	0.6710	177.2083	244.5417
Plate1 E7					4.8215	0.2215	174.2500	34.0000
Plate1 E8	4.8289	0.0030	604.5833	157.4583	2.8431	0.0510	647.7083	266.7083
Plate1 E9					3.6098	no reps.	148.0000	41.0000
Plate1 F1	83.6581	0.0017	6936.0830	95.9167	23.0873	0.0000	4558.5000	219.6667
Plate1 F10					2.0294	no reps.	69.0000	34.0000
Plate1 F11					2.3806	0.4876	73.2500	31.6250
Plate1 F12								
Plate1 F2					9.1642	no reps.	307.0000	33.5000
Plate1 F3	2.5860	0.5519	219.1945	74.7222	2.2667	0.3401	134.0833	55.8333
Plate1 F4	17.7000	no reps.	177.0000	10.0000	4.0312	0.0980	163.7500	40.2500
Plate1 F5								
Plate1 F6								
Plate1 F7	4.4069	0.0013	759.3750	245.8750	1.8640	0.0540	1462.6667	917.1945
Plate1 F8	12.2380	0.2293	282.7500	20.6667	3.4594	0.0361	281.8333	105.5208
Plate1 F9	2.3643	0.5720	147.4792	60.3958	1.5064	0.2644	238.7708	155.5417
Plate1 G1								
Plate1 G10	1.1115	0.8833	589.2084	534.4584	1.9525	0.0379	733.2084	417.1042
Plate1 G11	3.5508	0.7346	161.2500	45.2500	1.2311	0.8106	3009.6665	1141.6666
Plate1 G12	5.8854	0.2979	268.4375	60.3750	2.9208	0.0204	533.6250	189.6667
Plate1 G13	106.3486	0.0011	18413.9180	231.6250	2.7153	0.0204	9631.6875	3514.9792
Plate1 G14	1.9926	0.5776	206.2917	97.9167	6.2908	0.0207	924.5208	195.0417
Plate1 G15	2.2198	0.0619	632.0417	282.0000	2.5293	0.0111	674.1250	278.1667
Plate1 G16	10.5707	0.0140	309.9167	29.5000	26.6380	0.0000	1897.2084	76.3750
Plate1 G17	4.6501	0.0724	348.0208	72.5625	21.0913	0.0000	2558.8750	155.0417
Plate1 G18	4.8524	0.0000	5265.3545	1069.0416	2.4833	0.0007	2884.7083	1201.0209
Plate1 G19	2.7039	0.0008	5417.0830	2031.2084	1.3474	0.2245	4541.2500	3369.1250
Plate1 G2	3.6742	0.7751	115.0000	52.5000	9.1162	0.0413	158.3333	17.9167
Plate1 G20	0.6518	0.5711	3349.8333	5072.8745	0.9793	0.9233	2764.3333	2986.8125
Plate1 G21	0.6509	0.6510	551.5208	840.5625	1.3625	0.3181	586.9583	451.2084
Plate1 G22	0.9059	0.8833	1315.0000	1575.4584	0.5948	0.1433	800.5208	1300.7500
Plate1 G23					1.4087	0.6285	1080.3750	559.2500
Plate1 G24	177.3136	0.0006	24232.1680	191.2500	85.8697	0.0001	22880.8340	302.7500
Plate1 G3	1.0639	0.9546	336.7917	323.1458	0.5131	0.2078	257.5208	461.3333
Plate1 G4	7.9979	0.3775	254.0000	42.6667	6.3011	0.0004	368.1042	85.0833
Plate1 G5	2.9959	0.5756	243.1250	69.6875	13.0594	0.0129	797.3125	74.1875
Plate1 G6								
Plate1 G7	3.2597	no reps.	295.0000	90.5000	3.0745	0.1442	196.8333	53.8333
Plate1 G8	2.2886	0.2050	364.1250	161.7083	2.7928	0.0226	320.7778	148.2778
Plate1 G9	24.3512	0.0091	383.7917	13.2917	18.8200	0.0001	306.4583	12.0833
Plate1 H1	2.9843	0.5331	222.8125	66.8750	9.2372	0.0094	324.3125	34.3750
Plate1 H10	379.5480	0.0002	9932.9160	29.5833	165.9320	0.0000	9454.6045	62.1875
Plate1 H11	4.4577	0.4925	148.1458	34.3125	9.1363	0.0013	370.5556	42.9444
Plate1 H12	433.5104	0.0005	11401.1460	38.7500	229.2481	0.0001	6894.1670	35.0000
Plate1 H2								
Plate1 H3					2.0045	no reps.	441.0000	220.0000
Plate1 H4	1.6033	0.8887	137.5000	85.5000	0.3672	0.4794	117.0000	325.5000
Plate1 H5	0.9163	0.8580	1947.0416	2024.3334	0.7939	0.4093	1407.5834	1698.7083
Plate1 H6	1.0347	0.9561	651.0834	618.7500	1.1598	0.5541	757.6250	644.9584
Plate1 H7	0.8259	0.7980	444.5000	548.7916	0.9625	0.8865	652.2917	660.5000
Plate1 H8	7.1618	0.0088	497.2500	73.5208	1.0112	0.9886	78.8750	91.5000
Plate1 H9	4.1591	0.4966	248.9444	45.8333	2.6687	0.0288	250.2083	96.0625

Plate1 I1								
Plate1 I10	10.1250	no reps.	162.0000	16.0000	18.6000	no reps.	186.0000	10.0000
Plate1 I11	15.6729	0.1040	460.7500	32.0000	2.5875	0.2864	102.6667	38.8333
Plate1 I12	3.7948	no reps.	508.5000	134.0000	1.4840	0.5665	121.0833	80.5000
Plate1 I13	3.3925	0.6512	253.3750	68.2500	2.6125	0.1958	189.0000	75.3333
Plate1 I14	8.3971	0.2812	214.6667	23.3333	5.1491	0.0019	209.7083	39.4792
Plate1 I15	1.5875	0.4347	483.6250	302.7083	1.1162	0.6704	680.6041	585.5000
Plate1 I16	232.8976	0.0009	6565.4795	29.5833	59.8189	0.0009	2198.6667	57.5417
Plate1 I17	5.9089	0.0129	449.4583	90.3333	22.2263	0.0001	4079.2083	189.5833
Plate1 I18	0.9451	0.9027	5748.0420	6114.4165	0.9833	0.9372	10701.2910	10483.0000
Plate1 I19	1.8688	0.1983	852.5834	449.3542	1.5321	0.1247	1128.2500	742.8333
Plate1 I2	1.2795	no reps.	325.0000	254.0000	6.2000	no reps.	62.0000	10.0000
Plate1 I20	69.9653	0.0014	16683.5210	271.9167	2.3194	0.0212	11777.3760	5205.3960
Plate1 I21	3.0670	0.1031	384.7500	156.1667	1.7174	0.0679	582.8750	338.1667
Plate1 I22	1.3654	0.5011	1216.7083	862.7084	1.0199	0.9288	1171.7709	1153.9375
Plate1 I23								
Plate1 I24	2.2398	0.8879	89.5000	40.5000	16.2420	0.0001	1107.6875	104.5625
Plate1 I3	4.3235	0.5979	144.8750	32.7500	15.1657	0.0004	480.6250	35.7500
Plate1 I4	4.2236	0.7759	243.0000	105.7500	1.6617	0.5012	280.3333	271.0000
Plate1 I5					16.4000	no reps.	246.0000	15.0000
Plate1 I6	15.5672	0.0072	431.8542	26.3750	3.4557	0.1761	405.4167	254.0833
Plate1 I7								
Plate1 I8	2.7396	0.5079	235.0833	127.7500	1.9220	0.4506	194.9583	156.0625
Plate1 I9	0.4700	0.7445	131.2708	342.5208	0.4145	0.1078	168.6458	405.1667
Plate1 J1	148.5332	0.0009	4807.7085	42.9167	50.4449	0.0006	2904.1667	63.1458
Plate1 J10								
Plate1 J11	36.5128	0.0002	4012.4585	134.6250	37.6495	0.0018	2693.2917	112.8750
Plate1 J12								
Plate1 J2	96.1950	0.0000	1688.6667	10.2500	29.9934	0.0017	489.0833	13.1667
Plate1 J3	3.1083	0.4570	395.5625	97.2917	3.9444	0.1340	374.8333	66.4167
Plate1 J4	19.6799	0.0408	213.3889	10.5833	17.1977	0.0001	702.4375	26.4583
Plate1 J5								
Plate1 J6	0.9930	0.9910	533.1875	546.8750	0.8260	0.6052	301.9583	403.8542
Plate1 J7	3.7032	0.0236	543.2084	146.7708	3.0221	0.0004	768.8125	268.0833
Plate1 J8	5.1497	0.2271	207.0208	44.7917	2.7090	0.0653	231.1875	87.4583
Plate1 J9	214.7614	0.0016	8116.3335	47.6250	55.5129	0.0000	8988.0420	178.7083
Plate1 K1								
Plate1 K10	1.6684	0.8870	165.7500	88.2500	1.4991	0.4447	242.8125	189.2500
Plate1 K11	27.5000	no reps.	275.0000	10.0000	3.5793	0.1375	224.0000	69.5625
Plate1 K12	9.2227	0.0221	535.1250	69.0000	7.4189	0.0000	976.9375	144.0000
Plate1 K13	23.9701	0.1658	274.5000	10.0000	1.9901	0.4091	168.6667	85.2500
Plate1 K14	1.8021	0.0702	3871.5420	2147.6875	1.6269	0.0592	3040.0415	1694.9166
Plate1 K15	17.6151	0.0000	7055.7915	445.0000	12.1512	0.0044	5206.3330	477.7500
Plate1 K16	361.9293	0.0009	11322.5210	43.0417	76.1025	0.0000	6316.1250	86.7917
Plate1 K17	1.1778	0.7872	883.6250	822.0000	0.8575	0.6036	1686.6251	1673.2709
Plate1 K18	3.8708	0.0000	4548.4170	1219.0417	2.2551	0.0002	3575.5415	1615.1667
Plate1 K19	11.5819	0.0054	8402.1250	881.8333	5.7504	0.0000	5810.7080	1054.7500
Plate1 K2	3.2249	0.7671	157.6875	44.0000	12.9678	0.0183	327.0417	38.9167
Plate1 K20	1.3844	0.6355	512.7083	395.1458	0.7828	0.4588	676.4375	782.4584
Plate1 K21	0.4085	0.8017	81.2500	206.5000	0.7873	0.6655	115.1667	166.8958
Plate1 K22	5.5768	0.0000	6899.9170	1269.4166	0.8742	0.5692	6228.5420	6791.1045
Plate1 K23					0.3941	no reps.	240.0000	609.0000
Plate1 K24	19.0437	0.1654	443.7500	21.6250	26.8543	0.0000	409.0208	14.9583
Plate1 K3	16.6809	0.0165	2073.7083	179.2083	6.9027	0.0060	1509.8541	264.3750
Plate1 K4	1.3323	0.9496	97.3750	111.7500	0.9067	0.9070	65.1250	101.9375
Plate1 K5	8.5536	0.3384	223.6667	26.6667	5.1585	0.0932	318.8750	57.6250
Plate1 K6	11.9272	0.1921	230.6875	17.0000	5.3258	no reps.	237.0000	44.5000
Plate1 K7	1.5985	0.8121	327.0000	234.0000	0.8482	0.8409	254.0000	258.8333
Plate1 K8	1.7776	0.9046	158.7500	105.6875	3.4182	0.0874	154.5000	62.1667
Plate1 K9	11.8000	no reps.	236.0000	20.0000	21.5000	no reps.	215.0000	10.0000
Plate1 L1	3.5170	0.7422	263.0000	62.5000	9.4333	0.0141	239.9583	24.0833
Plate1 L10	8.4683	0.0002	1025.1250	141.6042	1.4619	0.3141	778.1666	569.0417
Plate1 L11								

Plate1 L12	9.2804	0.0172	354.0417	46.9375	8.9416	0.0003	420.4583	58.6667
Plate1 L2					6.3206	0.3126	162.2500	20.0000
Plate1 L3					3.4634	no reps.	366.2500	105.7500
Plate1 L4	1.6825	no reps.	106.0000	63.0000	7.7326	no reps.	332.5000	43.0000
Plate1 L5	1.7252	0.3011	685.6875	459.7917	0.6226	0.2234	346.8125	528.2500
Plate1 L6	152.4739	0.0048	7435.7085	99.4583	43.0615	0.0006	8318.0420	229.4167
Plate1 L7	184.2691	0.0000	6243.3540	36.6458	141.4938	0.0031	4453.6250	26.6042
Plate1 L8	4.6461	0.0000	2299.5835	502.0000	6.0936	0.0046	2231.0415	433.2083
Plate1 L9	0.6471	0.9259	280.0625	333.9375	2.4096	0.0060	414.2083	181.1250
Plate1 M1								
Plate1 M10	1.5110	0.8618	238.5000	224.7500	1.1227	0.7303	332.5000	303.6389
Plate1 M11	6.0280	no reps.	753.5000	125.0000	0.8910	0.8817	103.4167	118.8333
Plate1 M12	12.6865	0.0123	425.7778	44.0556	7.5748	0.0013	1887.0000	284.3542
Plate1 M13	0.1148	0.9700	38.7500	255.5000	0.8715	0.9001	60.7500	43.5000
Plate1 M14	2.3367	0.0471	1547.2500	850.1666	1.5969	0.0408	1258.7500	834.0834
Plate1 M15	3.0194	0.3100	302.1458	74.3333	1.8253	0.0807	284.7083	162.5833
Plate1 M16	4.1935	0.2624	488.7292	73.9167	2.8963	0.1438	158.5417	79.2292
Plate1 M17	39.9634	0.0031	7922.8745	219.9167	3.9251	0.0006	4754.4585	1213.0834
Plate1 M18	0.9671	0.9408	6748.2085	7091.4170	0.9855	0.9436	11350.4160	11034.5420
Plate1 M19	1.2434	0.6723	1624.5417	1217.0000	1.7013	0.0564	1630.7916	1003.2500
Plate1 M2	0.9457	no reps.	391.5000	414.0000				
Plate1 M20	0.6734	0.6724	2244.6665	3696.7500	0.7857	0.3791	2707.7500	3306.2915
Plate1 M21	3.3176	0.8922	183.2500	51.8750	4.2096	0.0213	228.2708	53.8542
Plate1 M22	1.8436	0.3329	1540.2083	883.2084	1.3098	0.2854	975.4583	734.2916
Plate1 M23	1.4060	0.5433	706.9583	489.1875	1.0745	0.9047	512.9584	447.4375
Plate1 M24	0.9757	0.9576	2952.5417	3061.6248	0.8309	0.4753	4318.8335	4880.4170
Plate1 M3	1.1698	0.9680	174.0000	119.1250	1.4431	0.6930	221.5625	112.8750
Plate1 M4	1.1948	no reps.	92.0000	77.0000				
Plate1 M5								
Plate1 M6	82.7457	0.0003	4906.3960	78.1667	4.2541	0.1963	2779.4585	322.4583
Plate1 M7	14.3117	0.1868	203.5000	15.2500	13.3244	0.0045	194.9722	12.1389
Plate1 M8	3.1247	0.3560	301.6875	111.5208	5.2470	0.0097	297.7500	78.2500
Plate1 M9	1.7806	0.8326	140.8333	69.9583	1.6362	0.2493	195.4583	111.3750
Plate1 N1	6.7636	0.3155	295.6250	39.2500	7.4966	0.0007	183.4167	27.6458
Plate1 N10	4.0634	0.0247	2325.8958	734.7083	1.2978	0.3419	2158.5210	1610.4375
Plate1 N11					3.9000	no reps.	78.0000	20.0000
Plate1 N12					10.7500	no reps.	172.0000	16.0000
Plate1 N2	0.9715	0.9624	858.7499	901.5417	1.2879	0.4594	1310.3542	848.3750
Plate1 N3	1.0796	0.9807	470.7292	416.3125	1.0588	0.9039	584.3125	479.4167
Plate1 N4	0.7713	0.9414	666.3750	611.5000	2.0450	0.6794	814.0000	759.7500
Plate1 N5					6.0357	no reps.	169.0000	28.0000
Plate1 N6					6.2488	0.0147	567.1667	57.1667
Plate1 N7	1.1004	0.8241	4659.4165	4465.8750	0.8652	0.5734	4739.8960	5363.5835
Plate1 N8	8.1929	0.5858	184.2500	22.5000	4.3928	0.0649	247.2500	65.3750
Plate1 N9	204.9492	0.0055	8109.5000	85.5417	81.9184	0.0000	6629.1250	94.4583
Plate1 O1								
Plate1 O10	3.7391	0.7597	143.7500	38.0000	7.7320	0.0219	389.0000	38.2500
Plate1 O11					2.0440	no reps.	836.0000	409.0000
Plate1 O12	1.9085	0.7990	154.3750	171.4167	4.6219	0.1234	847.4167	181.5000
Plate1 O13	0.6358	0.7138	462.8750	903.3750	0.5537	0.0971	702.2500	1145.2709
Plate1 O14	2.0562	0.1967	554.7292	268.3958	5.2951	0.0000	1655.8334	313.7083
Plate1 O15	1.6431	0.2980	840.7916	516.6250	1.3232	0.3394	546.5000	447.6250
Plate1 O16	383.6104	0.0018	11410.7090	48.0833	68.3171	0.0001	9280.8760	156.2083
Plate1 O17	3.6511	0.0082	2752.0000	641.0417	2.7205	0.0001	3156.3335	1166.6667
Plate1 O18								
Plate1 O19	1.3035	0.6045	898.0000	771.2917	0.9108	0.7046	1150.1250	1140.3334
Plate1 O2								
Plate1 O20	1.2879	0.5961	1833.3750	1425.5208	1.1917	0.4926	1447.4166	1195.2917
Plate1 O21	38.1506	0.0842	388.1250	10.0000	7.3387	0.0613	173.3125	14.8750
Plate1 O22	3.3411	0.0317	461.5417	153.0000	1.5560	0.0646	646.6667	421.0833
Plate1 O23	1.3638	0.4887	2877.7708	2102.9583	2.7875	0.0020	2595.4167	987.8334
Plate1 O24					1.4874	no reps.	177.0000	119.0000
Plate1 O3	17.4112	0.4009	178.0000	10.0000	2.4529	0.4745	191.0000	126.7500

Plate1 O4	11.8500	no reps.	118.5000	10.0000	7.6272	0.1088	184.3889	31.7778
Plate1 O5	1.0187	0.9779	1720.8334	1640.9792	0.8829	0.6584	1944.7084	2043.2085
Plate1 O6								
Plate1 O7	0.0100	no reps.	-1.5000	219.0000	15.1000	no reps.	151.0000	10.0000
Plate1 O8	5.0500	no reps.	202.0000	40.0000	6.8125	no reps.	218.0000	32.0000
Plate1 O9								
Plate1 P1	49.8473	0.0000	810.8750	16.7917	1.3182	0.3840	504.1250	349.0000
Plate1 P10								
Plate1 P11								
Plate1 P12								
Plate1 P2					9.7160	0.1337	297.5000	21.7500
Plate1 P3								
Plate1 P4	16.8058	0.0274	340.6875	24.3125	2.8063	0.4662	83.2500	29.7500
Plate1 P5	85.2559	0.0000	6252.8960	83.0417	44.3800	0.0000	5648.0625	141.4792
Plate1 P6	1.1586	0.7746	1970.7917	1584.3333	0.9097	0.8162	1850.4166	1746.1250
Plate1 P7	8.8850	0.0898	384.1250	39.6250	17.8045	0.0133	1325.1875	74.4375
Plate1 P8					4.5667	no reps.	205.5000	45.0000
Plate1 P9	1.1020	0.9636	353.0000	323.3333	1.9418	0.1825	419.2083	255.2083
Plate2 A1	0.1454	no reps.	69.0000	474.5000				
Plate2 A10	10.4194	no reps.	323.0000	31.0000				
Plate2 A11								
Plate2 A12								
Plate2 A2	36.6736	0.0025	799.9166	28.1250	2.5328	0.0602	381.6458	185.3958
Plate2 A3	87.7830	0.0000	5093.3335	60.1875	3.3202	0.0191	3703.7498	1138.6666
Plate2 A4	0.0100	no reps.	-29.5000	299.5000				
Plate2 A5	17.6365	0.4314	204.0000	11.0000				
Plate2 A6					0.0864	0.3427	102.7500	784.5000
Plate2 A7								
Plate2 A8								
Plate2 A9								
Plate2 B1	117.5758	0.0025	4819.8750	53.3750	5.6878	0.0128	2378.9165	462.4375
Plate2 B10	0.9299	0.9553	301.3333	297.2500	1.1558	0.7726	487.3958	370.2708
Plate2 B11	22.5827	0.0000	618.7917	47.3333	2.7967	0.0073	1351.3541	590.7917
Plate2 B12	2.5025	0.2176	298.9167	140.3542	6.8558	0.0000	1649.8334	255.1667
Plate2 B2	25.4126	0.0103	312.9167	11.3750	10.7966	0.0055	150.7917	12.4167
Plate2 B3	8.1006	0.0291	424.2500	54.3333	4.6883	0.1539	151.6111	34.8889
Plate2 B4	11.9330	0.0049	836.4584	51.0417	2.2128	0.0545	212.4583	96.2500
Plate2 B5	5.8276	0.2198	642.5834	77.9167	0.7577	0.6105	667.4584	682.6250
Plate2 B6	1.0476	0.9222	6740.0835	6396.7080	0.8078	0.3790	5194.4795	6109.7500
Plate2 B7	1.5749	0.2633	1489.5416	990.0001	1.2410	0.2767	1775.2500	1440.8750
Plate2 B8	1.5206	0.4471	1041.3541	834.7083	1.3239	0.1204	2281.0417	1755.2500
Plate2 B9	0.4882	0.4454	1406.1666	2878.5000	0.9920	0.9689	1656.7084	1664.5417
Plate2 C1	42.7489	0.0032	8618.2080	304.7917	6.8128	0.0000	2370.3750	377.2084
Plate2 C10	103.1593	0.0001	4306.4170	52.6667	2.2588	0.0132	2608.7915	1208.6666
Plate2 C11	2.5140	0.8162	193.5000	59.5000	3.5406	0.0151	448.4792	103.9583
Plate2 C12								
Plate2 C2	15.7780	0.0480	236.1667	15.5000	16.7500	no reps.	167.5000	10.0000
Plate2 C3	88.1038	0.0000	2605.8750	32.7917	4.1041	0.0009	1340.9375	294.3333
Plate2 C4	49.4894	0.0105	1601.2500	46.9583	1.8048	0.3862	1197.0833	633.3333
Plate2 C5	287.9785	0.0003	15352.0410	60.5000	63.0696	0.0006	7623.0420	140.9167
Plate2 C6	188.3001	0.0012	21660.8950	154.0625	73.1017	0.0002	20423.1050	335.5833
Plate2 C7	3.3142	0.0225	528.2708	183.6875	0.5120	0.0610	1117.5000	2179.8748
Plate2 C8	2.1722	0.8933	207.2500	81.7500	4.0693	0.1110	207.8750	103.8750
Plate2 C9	2.2645	0.2138	852.1250	558.5000	2.1598	0.2670	652.1875	465.6042
Plate2 D1								
Plate2 D10	2.7077	0.4972	333.5000	136.2500	5.4803	0.0231	479.4375	62.1250
Plate2 D11	4.3707	0.3210	351.6667	82.8333	0.4788	0.1532	218.2708	408.5000
Plate2 D12	17.1257	0.0004	3288.9585	248.5833	4.0456	0.0055	2537.4165	679.3750
Plate2 D2	3.4331	0.3695	179.6667	55.0000	5.5484	0.0035	374.1042	61.3542
Plate2 D3	3.4475	0.3355	291.8333	94.8333	0.2746	0.3076	59.6667	289.4167
Plate2 D4	9.8938	0.0001	4682.8750	442.6250	4.1194	0.0000	1243.8750	298.8333
Plate2 D5	4.3574	0.0007	755.5417	186.4583	2.5550	0.0110	599.8334	261.8333
Plate2 D6	3.3642	0.0027	1437.9584	421.0417	2.5207	0.0000	1878.2083	752.1250

Plate2 D7	2.1465	0.1117	915.5000	448.0833	1.5326	0.0276	1082.1667	710.3750
Plate2 D8	4.1452	0.0004	788.8333	190.1667	13.7014	0.0004	3937.4583	300.2083
Plate2 D9	1.2804	0.7451	496.2917	388.2292	2.0069	0.0689	566.7292	300.0000
Plate2 E1	0.4800	no reps.	397.0000	827.0000				
Plate2 E10	33.3846	no reps.	434.0000	13.0000				
Plate2 E11								
Plate2 E12								
Plate2 E2	0.1499	no reps.	41.0000	273.5000				
Plate2 E3								
Plate2 E4	3.4464	no reps.	289.5000	84.0000				
Plate2 E5	6.1869	no reps.	331.0000	53.5000				
Plate2 E6					0.0100	no reps.	1.5000	1005.0000
Plate2 E7								
Plate2 E8								
Plate2 E9	10.4490	no reps.	256.0000	24.5000				
Plate2 F10	1.6386	0.8849	169.5625	173.3750	1.3335	0.5985	124.5625	81.4583
Plate2 F11	4.1219	0.1009	287.2917	81.6667	1.7299	0.1217	389.4583	205.4167
Plate2 F12	8.4879	0.0000	3083.2500	392.1667	15.3332	0.0001	11841.8340	820.1250
Plate2 F2	14.1841	0.4667	146.1250	10.0000	4.4686	0.2184	145.8333	32.5000
Plate2 F3	13.0187	0.0732	515.6250	33.3750	5.4440	0.0469	129.0000	24.0000
Plate2 F4	64.5730	0.0002	1185.9584	20.3333	2.9723	0.1333	700.7084	235.8333
Plate2 F5	5.1375	0.0014	1613.6667	338.8542	1.6047	0.5381	1154.9167	999.5417
Plate2 F6	1.0151	0.9916	404.7917	350.2917	5.8802	0.0005	1235.4792	301.9792
Plate2 F7	1.8665	0.3575	576.7291	304.5833	1.0297	0.9203	474.1667	463.8750
Plate2 F8	1.9152	0.8638	291.2083	151.9583	6.1860	0.0074	1454.4583	258.5833
Plate2 F9	1.1652	0.7831	988.6666	955.1250	1.9015	0.0696	1353.2500	788.3333
Plate2 G1	18.2445	0.0000	545.3125	39.2917	0.2095	0.0072	307.7500	1624.0417
Plate2 G10	15.2529	0.0000	2713.6250	194.2917	0.3310	0.0143	2020.3334	6062.2920
Plate2 G12	42.1790	0.0000	561.3333	12.1250	2.2323	0.0309	433.8125	203.3750
Plate2 G2	6.9592	0.5048	210.5000	35.0000	0.3013	0.2033	84.9167	373.6667
Plate2 G3	51.9080	0.0002	18459.9160	408.6667	1.0465	0.8181	19473.4570	18449.4160
Plate2 G4	41.5431	0.0009	1607.1666	50.2917	0.8258	0.5402	926.9375	1014.5208
Plate2 G5	499.0744	0.0009	15165.2090	40.8333	81.7675	0.0000	9721.8955	148.8333
Plate2 G6	16.2914	0.0004	4762.7500	295.2500	0.2848	0.0142	3367.0000	10445.7705
Plate2 G7	12.8314	0.0000	2072.5417	176.2917	1.8487	0.0025	4283.2080	2463.7502
Plate2 G8	13.5603	0.0003	7357.2500	568.1667	2.2833	0.0001	10795.4790	5037.6040
Plate2 G9	49.0130	0.0000	3313.3333	74.6250	8.6853	0.0105	1206.6459	89.6250
Plate2 H1	7.5149	0.0033	776.9374	79.9792	3.6264	0.0156	917.6458	187.8542
Plate2 H10	1.8107	0.6875	186.8333	116.4792	3.9724	0.0023	361.1875	104.3333
Plate2 H11	0.3125	0.9190	136.2500	345.5000	0.5073	0.5246	143.8750	210.1250
Plate2 H12	2.7393	0.0444	638.0833	273.6250	0.6515	0.1573	1039.1667	1537.6250
Plate2 H2	4.0393	0.1250	572.7291	159.8750	2.2135	0.0819	487.4167	214.8542
Plate2 H3	63.9995	0.0000	2530.0835	39.7083	3.9334	0.0145	451.2500	120.7500
Plate2 H4	19.5409	0.0000	4179.6670	205.4583	1.9677	0.0059	806.7917	434.1667
Plate2 H5	6.7325	0.0000	4858.6040	694.0833	3.7344	0.0000	3256.8335	872.7917
Plate2 H6	2.1930	0.0616	735.7084	353.9167	1.1169	0.6310	717.1666	631.7500
Plate2 H7	1.2512	0.6897	739.9584	661.5833	0.7710	0.4303	705.6250	908.3333
Plate2 H8	1.2635	0.8492	350.5417	246.5000	2.0092	0.0341	769.6250	337.8333
Plate2 H9	1.2100	0.6717	1367.6250	1192.5000	1.6184	0.3462	1491.2917	1076.0833
Plate2 I1	4.9646	0.3100	236.5417	45.5000	5.9911	0.0002	413.0625	62.5000
Plate2 I10	10.8568	0.0000	1341.5834	110.7917	0.2997	0.0126	1183.2916	3956.6252
Plate2 I11	11.8642	0.0000	1346.5000	139.5000	2.8680	0.0012	806.9584	281.0417
Plate2 I12								
Plate2 I2	1.8617	0.0901	1373.7083	682.0416	1.8268	0.3274	611.2083	476.1667
Plate2 I3	84.2897	0.0001	6022.8340	73.4167	1.1712	0.3969	6306.2915	5480.6665
Plate2 I4	0.7724	0.8736	564.4792	736.5417	0.5017	0.4629	309.5208	597.7292
Plate2 I5	71.7164	0.0030	1275.4792	24.4167	0.7884	0.4811	958.1666	1262.3333
Plate2 I6	8.7630	0.2634	307.0000	32.6250	0.9496	0.9443	152.3750	255.0000
Plate2 I7					7.4875	0.1755	131.7500	19.0000
Plate2 I8	17.8533	0.0000	2983.9375	186.5417	0.2914	0.0109	2160.8335	7336.7920
Plate2 I9	13.8580	0.0006	2796.9375	238.8333	0.4533	0.0348	2820.1665	6179.4585
Plate2 J1	31.3784	0.0000	6233.4580	204.8750	2.6931	0.0000	3701.7498	1340.5833
Plate2 J10					6.1000	no reps.	61.0000	10.0000

Plate2 J11	1.6860	0.7250	152.4444	94.1667	1.9866	0.1554	191.1667	75.3125
Plate2 J12	15.3983	0.2309	241.7500	17.2500	13.1259	0.0000	452.1111	35.5000
Plate2 J2	1.0697	0.9275	519.0000	601.7083	1.6426	0.1957	503.4167	337.5417
Plate2 J3	8.9697	0.0077	1570.1250	250.1667	3.1343	0.0620	306.8958	112.4792
Plate2 J4	2.4628	0.2331	371.0000	150.0833	2.9893	0.0063	310.2083	106.2500
Plate2 J5	58.1556	0.0000	4596.3125	85.8958	4.2260	0.0341	657.8750	133.5625
Plate2 J6	6.0396	0.0000	4361.2915	739.0416	2.4404	0.0000	3512.0833	1399.8958
Plate2 J7	0.8816	0.8172	2867.6667	3265.0417	0.9805	0.9242	3842.7083	3863.6250
Plate2 J8	3.6897	0.2700	320.4583	112.3958	2.2881	0.0847	502.2500	253.2500
Plate2 J9	2.2266	0.0767	1115.5000	522.4375	4.9259	0.0000	2474.0000	500.4167
Plate2 K1	2.9769	0.1915	250.1667	113.2917	2.2178	0.1479	136.1250	96.0000
Plate2 K11	16.5726	0.0003	3253.6040	234.6250	0.5547	0.3861	2447.2917	5449.4585
Plate2 K12					18.1500	no reps.	181.5000	10.0000
Plate2 K2	6.5335	0.2423	241.0833	29.3333	11.6785	0.0004	655.1250	37.7083
Plate2 K3	26.5011	0.0010	3085.1250	143.2083	5.2626	0.0000	5720.1250	1024.1459
Plate2 K5	20.9592	0.0100	301.5625	13.3333	8.3343	0.0073	211.7500	19.8750
Plate2 K6	26.5702	0.0000	11889.5000	492.2917	3.5856	0.0145	5784.6245	1480.5834
Plate2 K7	82.5977	0.0000	2628.4583	36.4583	5.3773	0.0000	1349.3750	258.4167
Plate2 K9	42.5294	0.0000	807.1667	22.5417	2.5328	0.2985	124.0833	68.5833
Plate2 L1	52.3493	0.0001	1870.2500	34.9583	2.8163	0.0062	807.2708	258.6667
Plate2 L10	24.0985	0.0126	3057.8333	164.8333	3.0710	0.0134	2346.5210	887.0000
Plate2 L11	1.8713	0.9463	120.0000	112.2500	0.7946	no reps.	44.5000	56.0000
Plate2 L12	9.5452	0.0011	448.3542	78.4583	6.7851	0.0000	1082.6666	161.8750
Plate2 L2	3.4394	0.6303	183.1458	47.5625	1.0880	0.9116	104.0000	102.3750
Plate2 L3					0.1253	no reps.	22.5000	179.5000
Plate2 L4	13.6264	0.0003	5758.2500	422.9167	6.3831	0.0005	2648.2500	440.3333
Plate2 L5	8.3757	0.0000	6759.7085	819.5417	2.4100	0.0035	4143.5000	1662.4167
Plate2 L6	7.3593	0.0000	4552.7500	605.8750	2.9643	0.0000	3320.5000	1103.4167
Plate2 L7	2.5033	0.1838	447.6042	183.2708	2.2605	0.2430	643.0000	387.3333
Plate2 L8	1.7287	0.5370	417.1250	234.3750	1.8925	0.0168	530.6666	274.3333
Plate2 L9	2.9906	0.3800	208.5833	110.8542	0.5702	0.2507	137.1458	226.9167
Plate2 M1	33.1315	0.0012	719.5000	26.1667	6.8495	0.0456	122.9375	19.5000
Plate2 M10	11.3823	0.0000	2739.0625	302.1458	0.2932	0.0112	1931.3750	6281.5420
Plate2 M11	40.1000	no reps.	401.0000	10.0000				
Plate2 M12								
Plate2 M2	202.5680	0.0000	14917.2295	90.5625	89.0988	0.0004	6678.3750	113.5833
Plate2 M3	14.0300	0.0005	534.4166	35.2500	1.3044	0.2855	653.0416	493.5417
Plate2 M4	3.1765	0.3832	408.4167	103.5000	7.0686	0.0002	581.5834	109.4583
Plate2 M5	35.2092	0.0052	3132.8335	87.7917	2.6236	0.0568	1137.7084	530.7917
Plate2 M6	2.3030	0.0120	1329.2083	577.0000	1.1591	0.4797	1339.2500	1157.9584
Plate2 M7	5.0857	0.0020	906.0417	192.7500	4.3270	0.0067	2837.0415	677.0000
Plate2 M8	7.2793	0.0001	6152.0415	796.0417	2.2431	0.0001	3782.5000	1675.4584
Plate2 M9	17.2228	0.0031	4544.1250	326.5000	0.3010	0.0115	4301.7080	14030.0840
Plate2 N1	4.1343	0.0000	10307.0000	2455.2915	1.2631	0.2369	10776.6250	8454.5625
Plate2 N10	1.3263	0.6703	686.9583	452.9375	1.3077	0.6078	565.2292	413.0000
Plate2 N11	1.8568	0.0948	1102.0000	662.0833	1.3383	0.2464	1112.6250	845.2916
Plate2 N12	1.7437	0.2378	651.0000	393.9167	1.9285	0.0057	1141.2084	575.7500
Plate2 N2	21.5294	no reps.	366.0000	17.0000				
Plate2 N3	20.6678	0.0257	1079.5416	40.5000	3.2917	0.0005	973.8958	306.3333
Plate2 N4	9.0207	0.0000	6306.3330	677.9583	2.5939	0.0000	4344.0420	1656.9167
Plate2 N5	1.6898	0.8873	118.8889	61.0000	1.7479	0.4941	141.6250	63.6250
Plate2 N6	2.0263	0.2779	431.4583	229.1458	1.8418	0.0977	666.2083	392.3333
Plate2 N7	1.7690	0.2671	550.2500	323.8125	1.2379	0.2858	1058.0416	847.2916
Plate2 N8	5.8597	0.0246	506.9375	124.9375	1.4533	0.3828	595.0000	395.7500
Plate2 N9	3.8460	0.0110	468.2917	133.3333	3.3328	0.0013	1230.8750	378.8333
Plate2 O1	1.5856	0.3563	3926.3750	2857.3333	2.7327	0.1270	1551.0625	663.7917
Plate2 O10	6.5473	0.0003	622.4584	120.6250	0.9937	0.9820	488.1250	482.7917
Plate2 O11	11.4404	0.0114	4355.3960	476.1250	2.2485	0.0454	2344.6458	1274.1666
Plate2 O12								
Plate2 O2	158.9457	0.0025	5958.8335	50.4792	135.6708	0.0001	3608.7917	33.2500
Plate2 O3	14.3575	0.0089	3128.6665	455.4167	3.5743	0.0000	1870.0833	502.8333
Plate2 O4	4.1395	0.0589	422.7708	93.4583	0.9017	0.8528	128.7083	133.6250
Plate2 O5	6.3505	0.0002	760.3333	119.6250	2.4348	0.3157	419.4792	248.5000

Plate2 O6	8.6408	0.0005	1487.8750	189.7500	3.5942	0.0000	2855.8333	772.7083
Plate2 O7	4.2414	0.0543	330.0417	90.1667	2.2835	0.2262	304.9583	152.6667
Plate2 O8	2.5656	0.3003	249.1250	112.2917	1.3587	0.3557	414.0000	299.6250
Plate2 O9					14.5500	no reps.	145.5000	10.0000
Plate2 P1	31.0412	0.0075	15561.3960	665.7083	12.7635	0.0000	6723.5000	463.0417
Plate2 P10	1.8727	0.5394	330.0625	169.6667	9.5466	0.0037	2627.7292	281.6458
Plate2 P11	1.6747	0.8970	141.5833	80.8333	11.6966	0.0000	560.5417	49.3125
Plate2 P12	1.7748	0.0890	1423.9584	830.7084	1.2981	0.1883	4719.9165	3164.6667
Plate2 P2	22.0000	no reps.	308.0000	14.0000				
Plate2 P3	10.3285	0.2976	631.2083	29.8750	2.6771	0.0636	605.1667	202.4583
Plate2 P4	52.2378	0.0003	17342.2500	381.5417	1.1822	0.3639	18298.2500	15358.0830
Plate2 P5	0.7782	0.6949	1040.3334	1350.8334	0.5959	0.0914	2298.3750	3701.8750
Plate2 P6	1.5096	0.2089	9643.7710	6512.0420	1.0057	0.9776	8907.5420	8928.8340
Plate2 P7	23.3989	0.0014	5699.4585	269.9583	2.1982	0.0003	1482.7917	683.5625
Plate2 P8	7.8525	0.0000	7241.2500	907.5000	4.2103	0.0000	4378.0835	1116.3959
Plate3 A1								
Plate3 A10	2.2251	0.4718	234.1250	111.4792	3.4481	0.0015	489.9375	143.0417
Plate3 A11	3.2673	0.6798	157.2500	47.6250	5.4417	0.0001	299.7083	54.1042
Plate3 A12	33.3500	no reps.	333.5000	10.0000				
Plate3 A13	0.1484	no reps.	84.0000	566.0000	42.7750	no reps.	427.7500	10.0000
Plate3 A14	4.7099	no reps.	381.5000	81.0000	6.2527	0.0923	207.0556	33.2222
Plate3 A15	1.7051	0.3362	435.7500	280.2917	0.9614	0.8913	396.0833	415.6875
Plate3 A16	42.4753	0.0000	1686.7084	39.0000	10.3841	0.0034	657.5416	52.3333
Plate3 A17								
Plate3 A18	1.0109	0.9817	1102.8750	1089.4999	1.0900	0.6728	1307.1875	1198.7083
Plate3 A19	2.2169	no reps.	150.7500	68.0000	6.3677	0.0000	766.7916	130.7917
Plate3 A2	1.3411	0.7564	273.0833	192.7500	1.9141	0.0440	294.2917	155.0000
Plate3 A20	1.7856	0.9327	167.1667	100.3333	7.4612	0.0021	877.2083	128.0833
Plate3 A21	1.1238	0.7845	3874.5415	3480.2500	0.6717	0.1493	2683.2500	4069.5833
Plate3 A22	1.0902	0.8493	4076.2917	3711.0415	0.7259	0.2189	2548.0000	3516.0000
Plate3 A23	0.9354	0.8974	4941.4580	5185.2500	0.6953	0.1784	4031.6667	5716.6250
Plate3 A24					4.4773	no reps.	98.5000	22.0000
Plate3 A3								
Plate3 A4								
Plate3 A5	6.2310	0.0002	641.2708	104.7917	1.3043	0.3073	612.1667	456.8333
Plate3 A6	1.5274	0.5593	307.5417	217.2917	1.5798	0.0763	497.3750	305.8333
Plate3 A7	2.5235	0.3665	277.8333	153.2500	3.6594	0.0000	489.7917	144.2500
Plate3 A8	2.8632	0.3683	202.2292	77.4792	1.1302	0.8131	115.9583	101.6250
Plate3 A9	3.5583	no reps.	427.0000	120.0000	0.6601	0.6283	98.9167	161.8333
Plate3 B1								
Plate3 B10	21.3106	0.0004	340.7083	17.7917	11.8555	0.0048	292.7083	27.8750
Plate3 B11	1.9034	0.3659	366.9167	213.4583	1.3621	0.3495	300.4167	263.5000
Plate3 B12								
Plate3 B13	2.8726	0.3854	300.1875	111.5625	14.3400	0.0000	640.6042	43.2917
Plate3 B14	1.3646	0.6141	393.9167	287.9167	1.7018	0.0366	455.7917	263.7083
Plate3 B15	1.9293	0.2346	389.0833	209.0833	1.9238	0.0307	333.2292	194.6667
Plate3 B16	2.5273	0.3088	288.9792	121.2917	1.3164	0.4605	193.3333	144.2917
Plate3 B17	6.9868	0.0012	661.5417	102.4167	3.6205	0.0024	456.8333	117.2917
Plate3 B18	4.0067	0.0935	318.0417	78.1250	3.5381	0.0001	447.6250	141.3750
Plate3 B19	1.8424	0.6266	226.6667	106.0833	3.9713	0.0000	609.1667	159.1667
Plate3 B2								
Plate3 B20	7.7756	0.2520	305.2500	38.7500	2.4199	0.0617	436.2083	166.7917
Plate3 B21	4.0200	0.0027	561.9167	144.2083	9.4405	0.0001	2196.4165	227.7500
Plate3 B22	1.9153	0.3166	317.1250	174.5833	1.1783	0.5236	471.2917	398.5417
Plate3 B23	1.0213	0.9915	162.6250	196.5000	3.3545	0.0015	279.5000	81.9583
Plate3 B24								
Plate3 B3					5.1000	no reps.	178.5000	35.0000
Plate3 B4	59.5933	0.0011	1396.2917	32.0833	1.6992	0.0083	2431.2083	1358.3750
Plate3 B5					4.9146	0.1485	111.4167	26.3333
Plate3 B6	3.0014	0.0000	20267.7500	6750.9165	0.9207	0.6969	14617.2080	15101.0410
Plate3 B7	1.9649	0.6919	342.7917	269.1667	1.3514	0.4940	600.9792	534.9166
Plate3 B8	1.2804	0.9407	152.4167	129.2500	1.3677	0.6507	281.1250	241.2500
Plate3 B9	1.7262	0.2527	577.5000	327.2917	2.0783	0.0061	511.2083	253.8125

Plate3 C1	3.3871	no reps.	105.0000	31.0000	3.2006	0.0811	208.5625	64.8750
Plate3 C10	1.7119	0.2086	903.0833	544.8333	6.7649	0.0000	862.1667	135.6667
Plate3 C11	8.4658	0.0000	2899.3125	343.4583	1.7373	0.1412	1377.5833	657.6667
Plate3 C12	31.8142	0.0000	3215.2083	100.0417	25.0173	0.0000	2422.8335	105.7500
Plate3 C13	1.5594	0.2328	1871.0833	1209.0834	0.9881	0.9588	1014.7917	993.0416
Plate3 C14	117.4930	0.0000	3736.0000	34.1250	2.2652	0.0315	2562.5417	1183.2500
Plate3 C15	54.4843	0.0009	3771.9583	82.5000	6.7145	0.0005	3315.1667	494.0833
Plate3 C16	7.4567	0.5269	144.0000	23.2500				
Plate3 C17	10.2884	0.2356	351.0000	36.7500	14.5235	0.0000	843.1249	60.2083
Plate3 C18	4.5652	no reps.	157.5000	34.5000	23.2049	0.0001	425.5417	21.5833
Plate3 C19					1.3951	0.4379	157.4375	118.1667
Plate3 C2	14.1000	no reps.	141.0000	10.0000	2.8950	0.0773	151.9722	48.9444
Plate3 C20	0.6861	0.8081	793.7500	1042.7084	0.7019	0.2357	1000.1250	1427.9583
Plate3 C21					4.5082	0.0084	158.2708	32.2708
Plate3 C22	17.2808	0.0002	2715.7500	169.1875	3.4707	0.0006	399.8333	127.5833
Plate3 C23	20.4271	0.0000	1395.1250	91.1667	17.9740	0.0196	1407.3334	106.5000
Plate3 C24	39.3140	0.0000	638.4167	17.0000	14.6052	0.0000	550.2916	39.2500
Plate3 C3	1.8063	0.2949	395.2083	227.5833	1.3901	0.1908	429.0000	297.5000
Plate3 C6	2.4322	0.2125	521.2084	208.0000	3.9129	0.0000	670.2917	182.5833
Plate3 C7	1.6033	0.6797	216.0208	135.9583	0.8684	0.6926	257.4583	301.7083
Plate3 C8	7.8751	0.0000	773.3333	98.9583	4.2738	0.0000	658.8333	177.1250
Plate3 C9	2.6852	0.2256	352.4583	136.1042	2.7662	0.1935	250.0833	99.9375
Plate3 D1	0.6443	0.7497	206.9167	312.4167	0.8070	0.4856	661.7500	727.5834
Plate3 D10	4.8819	0.2264	220.1042	85.3125	1.6383	0.6731	188.2917	108.2500
Plate3 D11	0.5528	0.9561	132.2500	192.6667	0.6063	0.5225	115.3333	162.0417
Plate3 D12	0.8462	0.9687	118.1667	180.5000	0.7897	0.7370	88.0000	106.3333
Plate3 D13	23.7064	0.0000	3869.7917	170.8542	8.6971	0.0000	1179.0833	134.9583
Plate3 D14	1.3713	0.6274	515.9792	461.6667	1.7041	0.0262	698.6041	442.5000
Plate3 D15								
Plate3 D16	1.3817	0.6601	317.4583	259.1250	0.9465	0.8648	301.1667	307.6250
Plate3 D17	2.0966	0.3313	334.3750	154.6250	3.1745	0.0001	493.8333	163.1667
Plate3 D18	2.4799	0.0144	931.1875	379.7292	8.6728	0.0000	6528.3335	752.4583
Plate3 D19	1.5621	0.7353	278.3750	179.6667	1.5852	0.0284	720.1666	448.6667
Plate3 D2	2.3784	0.6229	270.5208	158.0833	0.9956	0.9933	278.2292	299.4167
Plate3 D20	2.1889	0.4942	263.0000	124.2500	1.4277	0.3425	238.4167	173.6250
Plate3 D21	1.6610	0.4174	373.6042	229.8333	1.1519	0.5645	497.5417	434.1250
Plate3 D22	4.1482	0.4163	228.6250	57.2500	2.7079	0.0461	193.6250	66.5833
Plate3 D23	2.8528	0.4387	169.2500	58.1111	11.8351	0.0003	729.5833	63.0833
Plate3 D24	2.0499	0.5535	245.0000	118.5833	2.2788	0.0266	229.4583	96.4583
Plate3 D3								
Plate3 D4	0.5851	0.8831	536.3750	607.5833	0.4042	0.0428	618.9792	1462.2916
Plate3 D5								
Plate3 D6	1.4478	0.7996	302.6250	246.8333	0.9712	0.9278	336.4167	329.2917
Plate3 D7	0.8130	0.9434	118.3750	164.5625	1.3554	0.4289	197.5417	157.3750
Plate3 D8	2.1704	0.8723	265.5000	137.4375	2.0844	0.0358	274.2917	149.6875
Plate3 D9	1.9625	0.1257	933.6250	485.8333	0.9337	0.7976	631.0624	678.7916
Plate3 E1	4.5628	0.4701	265.7500	41.4583	3.3806	0.1790	208.0000	56.1250
Plate3 E10	1.1286	0.8379	505.0833	453.1250	1.7237	0.0187	797.1667	458.7708
Plate3 E11	7.0002	0.0083	876.0833	139.0833	3.6323	0.0149	843.5834	275.5417
Plate3 E12	1.0677	0.9659	217.0833	202.7083	0.9052	0.7129	649.7916	708.7916
Plate3 E13	0.1550	no reps.	53.0000	342.0000				
Plate3 E14	2.1290	0.4397	239.0417	114.8333	1.3700	0.4468	148.0625	104.2917
Plate3 E15	380.6030	0.0000	5201.7920	14.0833	131.2462	0.0026	3872.0000	42.6250
Plate3 E16	147.3549	0.0003	17086.4160	121.6667	63.8461	0.0003	9782.3545	172.6042
Plate3 E17	5.0339	0.0033	634.8958	119.8542	2.5451	0.0150	863.6667	353.3333
Plate3 E18	0.9862	0.9872	393.0833	452.8333	1.1184	0.6631	439.5417	417.2917
Plate3 E19	1.1505	0.8804	285.4792	252.1667	1.8800	0.0338	504.8333	299.2500
Plate3 E2	4.8602	0.0000	3146.4167	639.8750	2.1440	0.0458	3937.7083	1994.0416
Plate3 E20	7.9649	0.0000	2058.0625	290.9375	14.7463	0.0017	2433.9167	181.4167
Plate3 E21	1.7535	0.2962	444.7917	280.0000	1.5751	0.0411	813.7500	527.0417
Plate3 E22	181.3632	0.0005	16202.9180	114.5000	105.8009	0.0003	11735.5420	127.5833
Plate3 E23	3.0107	0.3003	371.8333	145.1667	1.8372	0.1196	198.1875	111.4167
Plate3 E24	2.1772	0.6283	181.2708	90.0625	1.1738	0.6729	206.9583	193.0417

Plate3 E3	0.8018	0.8368	210.4583	276.1875	0.7427	0.3063	490.0000	637.0416
Plate3 E4	5.6910	0.3776	175.1111	28.0556	0.8000	no reps.	48.0000	60.0000
Plate3 E5	8.5239	0.0001	588.9584	69.5833	3.1134	0.0024	354.2708	120.3542
Plate3 E6	2.3971	0.0267	701.3750	292.9583	2.5296	0.0001	968.1667	401.5625
Plate3 E7	4.7319	0.0000	4636.2920	1015.1250	1.1227	0.5783	2924.1667	2652.4167
Plate3 E8	2.8429	0.0595	440.5000	179.8750	2.3964	0.0103	339.2917	156.1667
Plate3 E9	4.6486	0.0749	277.3125	55.3125	3.5365	0.0066	360.7500	125.7917
Plate3 F1					3.0923	no reps.	201.0000	65.0000
Plate3 F10	0.4360	0.6635	136.4583	300.0000	0.3175	0.0240	187.9167	581.4584
Plate3 F11	1.6155	0.7064	208.8333	160.7708	2.4403	0.0433	212.8542	96.3750
Plate3 F12	1.6766	0.4448	633.1250	525.6250	1.0055	0.9889	806.2500	860.8333
Plate3 F13								
Plate3 F14	1.3709	0.3810	1437.2083	1039.3750	1.5731	0.0132	1800.5834	1124.5000
Plate3 F15	1.1548	0.7504	942.7084	817.9583	2.2562	0.0000	1538.9166	692.9583
Plate3 F16	23.5263	0.0000	1266.5000	55.1042	4.1104	0.0332	590.5416	163.1667
Plate3 F17	8.4211	no reps.	240.0000	28.5000	0.4217	no reps.	91.5000	217.0000
Plate3 F18	1.4814	0.5760	301.0417	205.8750	1.7795	0.0302	410.0000	236.6667
Plate3 F19	2.4923	0.4419	273.5000	138.8333	1.1260	0.7502	229.8542	225.3958
Plate3 F2	3.6207	no reps.	157.5000	43.5000	0.0100	0.1892	-77.2500	207.0000
Plate3 F20								
Plate3 F21	4.7230	0.1017	320.0833	70.4167	2.3205	0.0032	414.0417	183.1250
Plate3 F22	1.4034	0.5823	406.0000	282.5833	1.2646	0.4233	349.6667	275.4167
Plate3 F23	1.5587	0.8144	176.5833	132.7500	2.5094	0.0169	244.1875	94.6875
Plate3 F24	8.1193	0.0006	4420.7500	616.7500	3.9572	0.0000	5067.1665	1272.3750
Plate3 F3	57.4074	0.0009	3402.1665	67.5000	27.4869	0.0001	1869.1250	69.1250
Plate3 F4	1.0453	no reps.	242.5000	232.0000	0.8454	0.6812	210.0000	225.4167
Plate3 F5	3.3535	0.5251	257.8333	69.8333	1.2330	0.6785	123.7917	95.2292
Plate3 F6	3.0592	0.6767	271.0000	163.3333	1.8738	0.2542	164.0625	73.3750
Plate3 F7	21.1750	no reps.	211.7500	10.0000	1.6925	0.7526	78.0000	52.1667
Plate3 F8	1.4788	0.7069	392.2083	287.0417	1.0412	0.9209	528.7500	464.8334
Plate3 F9	2.5635	0.0048	1212.1666	494.0416	1.2275	0.2588	1322.7500	1077.8750
Plate3 G1	6.2806	0.0036	456.3333	70.0833	1.6580	0.0941	585.5000	354.3125
Plate3 G10	1.1912	0.7255	1021.0000	909.9583	2.5646	0.0001	2067.1250	826.5834
Plate3 G11	4.3547	0.1670	320.3125	89.1875	18.5205	0.0063	1672.0000	104.7500
Plate3 G12	5.8934	0.4233	261.0000	49.0000	11.7187	0.0049	548.2500	43.0833
Plate3 G13	3.7123	0.3218	292.0000	83.0625	1.3097	0.4758	258.8333	172.8542
Plate3 G14	2.7865	0.4940	229.8125	77.6250	0.6347	0.5176	124.7500	186.6389
Plate3 G15	1.1960	0.7766	431.0833	362.2917	0.4352	0.0467	294.4583	689.7500
Plate3 G16	8.2956	0.0003	669.3125	90.0000	3.5395	0.0000	707.9584	197.5833
Plate3 G17	6.4216	0.0284	393.8750	49.9583	4.0632	0.0001	449.0625	116.2917
Plate3 G18	1.4127	0.5265	499.7917	368.1667	1.1599	0.5220	700.8333	593.7500
Plate3 G19					0.9127	0.9026	127.6875	122.1875
Plate3 G2	3.5905	0.5060	209.2500	56.8750	2.4229	0.1747	225.0000	113.0000
Plate3 G20	11.4486	0.0183	333.8750	30.3542	2.9119	0.0026	370.5000	133.2708
Plate3 G21	9.4000	no reps.	94.0000	10.0000				
Plate3 G22	4.7529	0.0004	714.8333	165.3333	6.4815	0.0058	1058.0000	178.0000
Plate3 G23	1.4589	0.8513	137.6667	92.9583	5.9482	0.0000	387.5833	59.1667
Plate3 G24	0.9942	0.9972	170.1250	175.2917	1.3678	0.3563	282.1875	211.1042
Plate3 G3	2.3216	0.6628	159.8750	72.2500	1.2511	0.6427	246.5417	180.5417
Plate3 G4	7.1528	0.1414	190.7500	27.3542	0.9616	0.9350	353.1667	299.6250
Plate3 G5	22.2500	no reps.	222.5000	10.0000	27.0000	no reps.	270.0000	10.0000
Plate3 G6	1.8061	0.3644	410.2083	239.0417	1.0526	0.8333	826.3750	798.2084
Plate3 G7					15.1664	0.0000	756.8333	47.1667
Plate3 G8	1.5664	0.6885	256.5625	174.4167	0.7651	0.4664	248.9583	355.9167
Plate3 G9					6.0083	0.1456	201.2500	33.5000
Plate3 H1	55.3391	0.0000	1435.5833	27.9583	3.3369	0.0051	1000.3542	339.0625
Plate3 H10	0.9000	0.8385	1652.5834	1820.3333	1.1860	0.3739	2052.0210	1769.0000
Plate3 H11	16.0912	0.0447	267.6250	14.1875	32.4165	0.0000	348.5625	10.0000
Plate3 H12					0.4123	0.8443	72.1250	110.1250
Plate3 H13	1.1392	0.9632	196.3333	174.0000	1.8379	0.4374	156.0000	85.6667
Plate3 H14	0.5565	no reps.	93.5000	168.0000	0.8158	0.8583	121.1250	139.6250
Plate3 H15					25.9091	no reps.	285.0000	11.0000
Plate3 H16	2.0907	0.4165	257.0625	133.5000	4.9488	0.0011	930.3750	196.0000

Plate3 H17	0.6091	0.8431	190.2083	262.0625	1.6534	0.0530	410.5417	252.0833
Plate3 H18	2.7962	0.0537	516.9583	199.3333	1.4551	0.1112	611.8750	422.6667
Plate3 H19	2.4877	0.0330	715.9375	286.9167	1.3165	0.1230	1516.4166	1137.7084
Plate3 H2					0.1062	no reps.	17.0000	160.0000
Plate3 H20	7.8556	no reps.	353.5000	45.0000	0.3159	no reps.	53.0000	167.7500
Plate3 H21	1.5552	0.8317	169.0833	112.8333	1.0501	0.9288	114.9167	113.1389
Plate3 H22	2.2492	0.4518	253.8333	125.3542	2.1369	0.1404	153.5417	75.1875
Plate3 H23					12.3427	0.0004	381.0000	25.8750
Plate3 H24	3.9080	0.3978	257.7500	67.0417	8.5824	0.0000	512.5833	63.4167
Plate3 H3	30.7105	0.0006	387.6250	10.8333	7.3517	0.0018	264.2917	34.4375
Plate3 H4	2.8001	0.3552	326.1667	234.8750	1.3445	0.5462	315.7917	319.0417
Plate3 H5	16.4872	0.0001	403.5833	26.5417	3.0246	0.0007	998.2917	344.5417
Plate3 H6	1.1627	0.8694	278.7292	254.0208	1.0878	0.7503	447.2500	422.7083
Plate3 H7	86.1358	0.0000	1829.2084	27.6250	98.7750	0.0003	1877.6667	22.5833
Plate3 H8	4.7144	0.0644	303.0833	109.8958	3.0528	0.0000	1611.2500	545.5833
Plate3 H9	53.3070	0.0000	1164.3750	24.7500	60.3666	0.0009	1748.5417	33.6250
Plate3 I1					2.2338	no reps.	86.0000	38.5000
Plate3 I10	1.0761	0.9768	186.6250	232.9375	0.7833	0.6105	151.9583	173.9167
Plate3 I11	1.3216	0.7304	300.7083	273.6667	1.6764	0.1130	369.9583	214.2083
Plate3 I12					6.5877	0.0112	120.7222	23.2778
Plate3 I13								
Plate3 I14	7.1076	0.0018	512.5209	73.5833	3.3539	0.0002	413.5833	122.5000
Plate3 I15	0.8270	0.7998	2420.5000	2902.3335	0.4656	0.0400	3124.2917	6954.9585
Plate3 I16	1.6654	0.2569	673.2084	449.6667	0.9864	0.9654	394.5625	394.1875
Plate3 I17	2.1058	0.0154	1882.1666	872.0000	0.7539	0.2736	1539.3750	2022.4584
Plate3 I18	1.5992	0.3976	511.3542	310.1875	1.0144	0.9504	620.0417	616.6667
Plate3 I19	2.4000	0.0759	516.4375	219.2917	0.7197	0.3028	381.0417	504.2500
Plate3 I2	0.4766	0.5691	432.3958	820.4167	0.9352	0.7570	1312.8125	1409.8542
Plate3 I20	19.6000	no reps.	196.0000	10.0000	24.8125	0.0043	955.9583	48.2500
Plate3 I21	18.5902	0.0020	575.9583	54.0000	25.2332	0.0010	4385.6665	194.2083
Plate3 I22	0.7743	0.7523	484.5833	601.5833	1.0620	0.8035	561.4167	543.5417
Plate3 I23	11.5897	0.0000	11830.2910	1105.3750	14.3223	0.0000	18160.5000	1253.7083
Plate3 I24	6.8186	0.0731	302.3750	65.3750	0.8965	0.7025	554.7500	568.1667
Plate3 I3	2.1512	0.1491	536.2917	256.0833	2.5387	0.0022	678.2500	252.8750
Plate3 I4								
Plate3 I5	20.0814	0.0000	1204.7083	59.7083	9.3783	0.0000	568.8751	60.0000
Plate3 I6	12.2317	0.0000	1664.2917	140.8333	4.2137	0.0000	907.2917	221.0417
Plate3 I7	1.8393	0.1687	516.2917	295.0417	0.7596	0.2964	993.7918	1336.2500
Plate3 I8	1.8629	0.7274	245.8125	141.6250	3.5646	0.0012	360.9583	94.2500
Plate3 I9	2.0009	0.3212	330.0208	184.9167	1.7178	0.0584	463.5000	269.7500
Plate3 J1	7.5239	0.0030	548.8541	75.6667	3.1453	0.0550	463.2500	145.3958
Plate3 J10	8.9879	0.0075	7528.3755	1063.4166	5.9036	0.0019	6218.3750	1119.7917
Plate3 J11	2.6590	0.8348	151.5833	99.0000	3.0860	0.2334	108.0625	41.8750
Plate3 J12	0.5438	0.4845	2184.8335	4074.0835	0.9225	0.7027	2742.5000	2944.1667
Plate3 J13	2.6270	0.0940	402.7500	157.8750	1.1755	0.7495	249.9791	206.7083
Plate3 J14								
Plate3 J15	1.5689	0.3316	685.0000	443.1042	1.4420	0.0521	1000.4167	689.1666
Plate3 J16	2.1740	0.4863	237.9583	100.7292	1.9909	0.0155	383.0833	188.2083
Plate3 J17					5.6857	0.0180	150.9792	23.7917
Plate3 J18	2.1277	0.3859	269.1250	119.8125	1.3105	0.2058	803.0416	602.2500
Plate3 J19	2.7821	0.0990	444.2500	180.9375	1.0575	0.8154	890.0000	799.3750
Plate3 J2								
Plate3 J20	7.2426	0.0586	320.6250	58.1250	2.8468	0.0128	351.5625	151.2708
Plate3 J21	1.3421	0.6800	377.0000	323.2917	1.2012	0.4491	478.5833	408.6250
Plate3 J22	1.9530	0.2965	375.6458	226.3958	0.9865	0.9600	455.0833	481.2500
Plate3 J23	3.1138	0.0813	434.2917	197.6250	3.3406	0.0004	364.5000	119.9167
Plate3 J24	61.1710	0.0008	7708.9585	146.8750	33.3957	0.0029	6240.3335	230.5417
Plate3 J3	2.4168	0.0239	824.2500	361.7083	1.2700	0.3007	3023.3750	2361.3335
Plate3 J4	0.8427	no reps.	75.0000	89.0000	1.3390	no reps.	79.0000	59.0000
Plate3 J5	55.4462	0.0010	2319.7083	47.6250	22.0413	0.0000	1171.2917	49.4167
Plate3 J6	6.1018	0.0037	2919.5417	552.5417	1.9605	0.0159	3252.8333	1672.5000
Plate3 J7	1.4910	0.4419	814.0833	577.5416	2.0234	0.0016	1707.6250	842.4583
Plate3 J8	29.4000	no reps.	294.0000	10.0000				

Plate3 J9								
Plate3 K1	1.1374	0.9632	141.4167	152.7500	1.9510	0.2485	136.7708	70.1667
Plate3 K10								
Plate3 K11	1.7691	0.2368	817.9583	466.8958	1.6174	0.0580	692.0555	428.1111
Plate3 K12	1.5726	0.6988	248.6250	166.0000	1.0514	0.8773	268.1250	249.5000
Plate3 K13	4.4305	0.0704	239.6250	61.0833	17.3152	0.0000	1401.2917	84.8333
Plate3 K14	11.9848	0.0000	948.8750	86.5417	5.0396	0.0000	541.2500	108.0000
Plate3 K15	6.0558	0.0000	8336.8340	1390.7500	1.8819	0.0517	8113.4580	4180.5835
Plate3 K16	151.5388	0.0008	6307.8750	47.2917	83.2393	0.0000	4582.5000	56.0000
Plate3 K17	2.8537	no reps.	234.0000	82.0000	10.5929	0.0000	571.9167	55.1250
Plate3 K18	4.0086	0.0027	564.3333	147.9583	2.6135	0.0004	647.3333	258.5417
Plate3 K19	10.7391	no reps.	247.0000	23.0000	4.2009	0.0082	156.0208	37.2917
Plate3 K2	1.6759	0.8196	117.7500	74.1945	1.5779	0.5230	175.8889	80.6667
Plate3 K20	6.8677	0.0004	578.4583	92.2708	10.4509	0.0001	2819.2083	290.8750
Plate3 K21	1.1419	0.9378	162.0417	162.4583	1.1615	0.7058	177.4167	153.2917
Plate3 K22	1.1914	0.7987	411.1250	373.2500	1.0631	0.8320	437.5208	452.2708
Plate3 K23	7.9753	0.0000	3204.0415	414.6667	3.9043	0.0147	1990.3333	419.5208
Plate3 K24	3.7921	0.3163	257.0000	63.1250	1.7161	0.3166	193.4167	122.8333
Plate3 K3	1.3986	0.6694	313.3750	239.5000	2.0974	0.0031	630.2084	292.4583
Plate3 K4	4.4640	0.5960	251.0000	45.1667	1.5176	0.5250	98.5556	60.4722
Plate3 K5	4.1842	no reps.	238.5000	57.0000	5.2669	0.0001	411.0000	74.3125
Plate3 K7					2.4696	0.3461	165.0000	64.3750
Plate3 K8	0.8521	0.8971	274.1042	293.1250	3.2189	0.0056	651.5208	220.2292
Plate3 K9					6.7609	no reps.	155.5000	23.0000
Plate3 L1	4.7507	0.1327	197.0833	55.5833	23.1307	0.0105	1272.1459	78.2917
Plate3 L10	0.9011	0.9624	113.5278	143.0833	0.9569	0.9461	132.1875	148.7917
Plate3 L11								
Plate3 L12	0.2426	0.8604	61.7500	249.3750	0.6100	0.7173	59.2917	88.3750
Plate3 L13	1.3734	0.5159	542.3750	393.7500	0.6107	0.2579	294.4375	355.8542
Plate3 L14	71.5494	0.0016	4543.6670	87.1667	27.7294	0.0004	3302.5833	127.2083
Plate3 L15	19.3500	no reps.	193.5000	10.0000	5.1750	no reps.	207.0000	40.0000
Plate3 L16	2.0250	no reps.	20.2500	10.0000	0.7604	0.9084	55.0000	21.5000
Plate3 L17	1.7194	0.1569	820.2084	481.9583	3.3835	0.0000	1268.2500	376.7500
Plate3 L18	0.9904	0.9910	289.2917	288.4583	1.4403	0.1459	447.7917	319.9167
Plate3 L19	1.6521	0.6997	194.3333	129.1875	2.4882	0.0601	211.5417	86.8333
Plate3 L2	2.1726	0.2065	659.3541	356.7917	0.9754	0.9348	366.1667	389.3125
Plate3 L20	11.3797	0.0001	652.1459	55.0000	4.1394	0.0000	489.9166	130.0417
Plate3 L21	0.8205	0.7777	632.5833	794.7083	0.6252	0.1209	874.1250	1394.2083
Plate3 L22	1.5602	0.6362	240.2917	163.1250	1.0895	0.7966	252.4167	241.4583
Plate3 L23					5.4167	no reps.	65.0000	12.0000
Plate3 L24	2.9925	0.5727	258.0000	83.2500	0.4744	no reps.	74.0000	156.0000
Plate3 L3								
Plate3 L4	31.5327	0.0029	1028.1666	45.7917	2.4597	0.0183	3422.6665	1385.1250
Plate3 L5	5.3668	0.0635	428.8333	127.2778	4.9995	0.0005	845.4584	175.1250
Plate3 L6	1.5434	0.5201	2656.3333	2002.0417	1.0130	0.9571	1417.7915	1414.0000
Plate3 L7	2.4986	0.1574	1053.0833	588.9584	0.8442	0.5026	1145.6875	1355.3542
Plate3 L8	2.0137	0.0194	3242.4583	1661.2083	0.9286	0.7301	4090.6250	4249.8335
Plate3 L9	4.7396	0.0000	1641.3334	383.0833	1.4860	0.0485	1054.9583	716.6250
Plate3 M1								
Plate3 M10	1.4028	0.7169	311.7083	219.9167	1.7336	0.0956	292.6458	172.6875
Plate3 M11	1.6036	0.6377	255.2292	222.7500	1.6940	0.0731	369.6667	232.4167
Plate3 M12	2.2695	0.2981	395.7500	177.0000	1.4618	0.3805	199.6875	153.5833
Plate3 M13	4.3036	0.2608	209.2083	48.6667	5.4267	0.0030	257.2292	47.3958
Plate3 M14	14.2009	0.0000	2432.8542	181.5833	2.3572	0.0001	1628.4584	703.1250
Plate3 M15	1.2116	0.8529	538.0833	377.1667	0.9973	0.9959	605.1250	710.0417
Plate3 M16					0.0500	no reps.	11.0000	220.0000
Plate3 M17	0.9459	0.9630	289.5208	255.2917	3.5105	0.0000	943.0208	276.3750
Plate3 M18	1.1154	0.8160	841.7084	755.2500	0.8738	0.6124	676.4792	775.8541
Plate3 M19	1.9543	0.2367	975.5417	567.5833	0.9407	0.7972	879.5000	955.0417
Plate3 M2	1.1545	0.8665	360.0000	287.0417	1.5582	0.2181	377.0417	257.2917
Plate3 M20	0.9596	0.9369	1131.8334	1210.5625	0.9219	0.7144	1355.2500	1460.6250
Plate3 M21	1.0184	0.9810	892.5000	872.6250	0.9339	0.7746	1073.6250	1165.3750
Plate3 M22	1.3261	0.5739	792.7083	687.1667	0.9673	0.8876	798.4584	833.4167

Plate3 M23	1.5719	0.1728	4162.3330	2687.7917	0.9274	0.7200	4695.2500	5192.2500
Plate3 M24					1.5602	0.7002	103.7500	64.2500
Plate3 M3	1.6023	0.2232	1147.4584	695.4167	0.9621	0.8706	901.8333	957.6667
Plate3 M4	4.2518	0.4965	149.9722	34.8611	1.1610	0.7708	99.9583	87.7917
Plate3 M5	1.6198	0.2757	552.3334	349.5417	1.2972	0.2296	705.8333	536.1666
Plate3 M6	3.7035	0.0001	1135.5834	314.0833	1.3609	0.0790	2285.6250	1697.6250
Plate3 M7	1.9617	0.2968	385.4583	188.0417	6.0783	0.0001	1696.1250	283.7917
Plate3 M8	1.1210	0.8217	645.9167	597.5417	1.0857	0.7365	576.1250	533.2500
Plate3 M9	0.6374	0.6986	331.3542	474.7083	1.4443	0.5019	395.8750	324.1667
Plate3 N1								
Plate3 N10	1.0898	0.9786	138.2083	198.7292	1.0992	0.9309	176.7917	191.7917
Plate3 N11	0.4807	no reps.	140.2500	291.7500	2.0693	0.2008	180.0625	98.1875
Plate3 N12	35.6250	no reps.	356.2500	10.0000	0.5477	0.7589	60.2917	77.5000
Plate3 N13	0.9810	0.9949	158.0625	165.1250	1.1718	0.7957	138.5556	119.4722
Plate3 N14	2.3999	0.1276	425.7083	215.5417	3.0422	0.0001	853.0000	293.7917
Plate3 N15	33.3246	0.0031	11115.9170	402.0833	15.1012	0.0000	7575.4375	512.4167
Plate3 N16	16.4468	0.0004	4089.7083	270.4583	10.2367	0.0001	3230.5835	315.8333
Plate3 N17	1.2548	0.7967	672.1666	632.5833	0.7976	0.5657	1062.5416	1416.9584
Plate3 N18					1.9039	0.5624	76.0000	54.5000
Plate3 N19	0.5592	0.6635	196.4583	338.0833	0.8518	0.5046	1101.0417	1315.4166
Plate3 N2	0.5468	0.9973	149.2500	110.0000	11.1048	0.0069	172.8333	17.3333
Plate3 N20	184.9704	0.0000	2514.4375	15.8750	124.6800	0.0001	2174.7917	19.6667
Plate3 N21	45.9328	0.0000	3979.1665	85.9583	26.0593	0.0001	3232.2500	127.3750
Plate3 N22	1.4173	0.5301	512.5000	362.2917	0.9558	0.8622	512.6250	533.2500
Plate3 N23								
Plate3 N24	1.5258	0.7468	173.1667	124.4722	1.7851	0.1181	265.5833	142.5000
Plate3 N3	5.8521	0.0050	483.4167	79.2083	5.0663	0.0004	419.1667	92.5417
Plate3 N4	2.5417	0.3360	223.0417	86.0417	0.9537	0.8848	327.6458	321.7917
Plate3 N5	16.6808	0.0022	7919.5000	552.5417	6.3134	0.0000	4052.6250	625.1250
Plate3 N6	1.6908	0.1050	3028.9165	1810.0000	2.9444	0.0000	5209.5000	1771.8750
Plate3 N7	178.6183	0.0003	4576.3330	30.5417	69.8429	0.0050	3183.8335	75.7083
Plate3 N8	2.6559	0.6501	539.0417	258.7917	0.5560	0.0951	1061.0416	1885.5833
Plate3 N9	2.3714	no reps.	249.0000	105.0000	1.8523	0.2639	161.4375	77.8125
Plate3 O1	0.4373	0.6566	192.7083	393.5833	0.8689	0.7499	312.4167	344.9167
Plate3 O10	1.9165	0.7947	160.4375	97.7500	1.9273	0.2679	223.0000	104.0000
Plate3 O11	5.9600	0.0000	2562.0210	442.4167	2.0851	0.0052	2070.2085	1153.9166
Plate3 O12								
Plate3 O13	1.7385	no reps.	113.0000	65.0000	3.9132	0.0045	265.3333	73.7917
Plate3 O14	2.0623	0.6054	232.7778	126.7222	1.9623	0.3943	142.9167	94.7500
Plate3 O15	3.4307	0.0000	1748.5833	563.7916	2.0221	0.0306	1300.9167	640.9166
Plate3 O16	0.3772	0.4748	548.2083	1360.4166	0.5820	0.0920	860.2500	1418.4583
Plate3 O17	73.4687	0.0000	2852.5000	38.4167	50.4887	0.0000	2067.8750	40.1667
Plate3 O18	0.7928	0.8049	284.6875	342.7292	0.7559	0.4109	310.8333	392.6667
Plate3 O19					1.5628	0.4928	165.5625	108.2500
Plate3 O2	4.3291	no reps.	85.5000	19.7500	2.0132	0.3403	169.2778	61.9444
Plate3 O20	2.8507	0.0609	398.5000	143.0000	1.1038	0.6866	708.1250	633.9166
Plate3 O21	3.7385	0.6050	176.3333	50.8333	1.2246	0.6716	145.0000	124.0000
Plate3 O22	1.4083	0.9229	156.7500	101.7500	0.9513	0.9245	136.5833	144.3333
Plate3 O23	3.1657	0.5806	183.3750	58.2500	4.6202	0.1173	169.8333	42.7500
Plate3 O24	18.8161	0.0000	1362.5416	71.5000	5.9908	0.0000	1340.4167	246.1458
Plate3 O4	1.8917	0.7317	155.3542	100.7292	1.2738	0.6509	158.0625	119.8750
Plate3 O5	2.0461	0.3573	327.5000	158.2500	2.0563	0.0287	381.9167	210.9167
Plate3 O6	1.2038	0.7663	506.2708	410.2500	1.2114	0.4171	661.2916	544.5000
Plate3 O7	2.0305	0.3832	377.0000	180.4583	1.5917	0.1307	548.8542	351.8333
Plate3 O8	4.1514	0.0002	910.4167	211.2917	12.8633	0.0018	2889.2290	253.2917
Plate3 O9	56.7459	0.0003	1458.0417	29.4167	38.9921	0.0001	787.7084	20.6667
Plate3 P1	2.1954	0.7586	117.8750	84.2500	1.5738	0.6384	167.7500	133.0278
Plate3 P10	1.3724	0.4534	3451.8750	2704.3333	0.8439	0.4633	3040.2917	3574.6250
Plate3 P11	0.4996	0.6785	214.0833	458.7083	0.7532	0.4728	225.7083	302.1667
Plate3 P12					6.8600	no reps.	85.7500	12.5000
Plate3 P13	0.8828	0.8338	1673.5000	1873.3750	0.7086	0.2124	1245.7500	1690.8334
Plate3 P14	0.8010	0.7314	1184.3334	1491.4583	0.8098	0.4033	1308.2291	1596.1667
Plate3 P15	22.4445	0.0002	561.7500	25.4167	25.7065	0.0005	503.1459	17.0417

Plate3 P16	1.9901	0.3163	311.0417	160.9583	1.4158	0.2759	292.1250	195.6667
Plate3 P17	4.6808	0.0000	1136.8750	251.9583	1.5798	0.0149	1140.0834	696.6250
Plate3 P18	11.6089	0.0000	1166.5416	106.0417	5.4438	0.0016	887.0000	168.5833
Plate3 P19	1.3013	0.7568	258.8750	224.5000	1.0509	0.8662	334.6667	307.6250
Plate3 P2	17.3500	no reps.	173.5000	10.0000	4.4936	0.0457	192.7500	49.0000
Plate3 P20	2.4658	0.0233	694.7917	298.5833	2.7188	0.0003	636.3333	234.2083
Plate3 P21	6.8027	0.0000	4508.2085	700.2500	3.1476	0.0000	2627.8335	838.2083
Plate3 P22	11.5241	0.0000	1647.0416	145.4583	6.3660	0.0000	1018.0417	174.3333
Plate3 P23	1.1796	0.8093	392.7500	348.1250	1.3679	0.2329	580.0000	410.0833
Plate3 P24	1.4479	0.4559	622.0833	446.8333	1.4553	0.0706	766.4167	530.0833
Plate3 P3	38.8000	no reps.	388.0000	10.0000	14.2886	0.2105	348.5000	24.2500
Plate3 P4	0.4056	0.6344	174.5833	371.5417	0.5595	0.1356	343.8542	569.8125
Plate3 P5	1.7839	0.0926	1432.7084	779.9583	2.7357	0.0000	2417.5417	883.5834
Plate3 P6	18.3863	0.0027	7116.0415	457.2500	9.3341	0.0000	6495.5000	688.9167
Plate3 P7					26.7500	no reps.	267.5000	10.0000
Plate3 P8					9.4000	no reps.	94.0000	10.0000
Plate3 P9	1.2702	0.8012	286.0417	268.6667	1.0420	0.9070	229.2083	240.8750
Plate4 A1	0.5313	no reps.	398.5000	750.0000	1.1964	no reps.	67.0000	56.0000
Plate4 A10	1.8780	0.8707	157.5000	78.2500	0.0631	0.2761	33.5000	164.7500
Plate4 A11					0.4158	0.5509	163.0000	205.0000
Plate4 A12	3.2105	0.5404	610.1666	146.5000	1.3205	0.6000	1149.7500	1073.2500
Plate4 A13	0.2495	no reps.	67.0000	268.5000				
Plate4 A14								
Plate4 A15	12.1765	no reps.	310.5000	25.5000	4.3238	no reps.	227.0000	52.5000
Plate4 A16					2.9383	no reps.	119.0000	40.5000
Plate4 A17	0.7514	0.7990	247.0000	310.2917	0.9507	0.8750	297.5417	315.2083
Plate4 A18	3.0323	no reps.	141.0000	46.5000	2.5422	0.2349	258.0000	131.5833
Plate4 A19	0.1291	0.9225	27.5000	234.7500	0.5744	0.6195	56.5000	98.5000
Plate4 A2	7.5755	no reps.	401.5000	53.0000				
Plate4 A20	2.9167	no reps.	105.0000	36.0000	1.2416	0.8752	131.0000	71.5000
Plate4 A21	2.5621	0.5455	224.6250	77.1250	1.0353	0.9222	261.6667	241.2500
Plate4 A22	3.0054	0.0505	694.1042	288.8958	0.4162	0.1499	967.7083	2062.9792
Plate4 A23	15.9020	no reps.	405.5000	25.5000	1.1098	no reps.	237.5000	214.0000
Plate4 A24								
Plate4 A3	3.0458	no reps.	365.5000	120.0000	1.2821	no reps.	50.0000	39.0000
Plate4 A4								
Plate4 A5	2.2718	0.0122	1548.9584	691.3750	1.0568	0.7958	1179.2500	1130.3333
Plate4 A6	1.2005	0.8511	355.3542	321.1250	0.9859	0.9681	398.0000	415.4375
Plate4 A7	5.8537	no reps.	360.0000	61.5000	1.4264	0.5713	185.4167	129.4167
Plate4 A8	2.4268	0.7825	163.3333	64.6667	1.6296	0.4026	226.3333	104.4167
Plate4 A9	1.9964	0.3455	346.8958	201.6875	1.5223	0.1827	360.5834	239.3125
Plate4 B1								
Plate4 B10	2.6994	0.2500	463.5417	181.9792	1.3795	0.3507	347.1250	265.9375
Plate4 B11	13.3542	0.4249	243.5000	15.5000	18.3759	0.0000	851.7916	42.3750
Plate4 B12	4.0243	0.1717	424.2500	87.5000	13.8889	0.0001	1669.6041	142.6042
Plate4 B13	10.0500	no reps.	100.5000	10.0000	4.1260	0.1398	121.2500	30.5000
Plate4 B14	13.2176	0.0000	1384.5416	108.1458	10.3604	0.0031	1837.0209	165.0417
Plate4 B15	9.3000	no reps.	93.0000	10.0000				
Plate4 B16								
Plate4 B17								
Plate4 B18					1.4667	no reps.	88.0000	60.0000
Plate4 B19	7.2212	0.2998	377.7500	87.2500	2.4286	0.1172	310.8750	143.5000
Plate4 B2	1.7530	0.8036	165.8750	109.3750	1.5193	0.5509	149.7500	107.3750
Plate4 B20	3.1304	no reps.	72.0000	23.0000	1.1336	0.8879	1002.5833	543.5000
Plate4 B21	11.4198	0.0027	352.9792	29.1250	2.9483	0.0030	466.2292	158.8542
Plate4 B22	10.9015	0.0002	571.5833	50.1667	2.3723	0.0209	510.1875	241.4792
Plate4 B23	14.1571	0.0009	462.5417	31.5417	2.5655	0.0503	277.0625	114.0417
Plate4 B24	19.7308	0.0000	614.2083	36.9167	2.9065	0.0001	666.9166	233.6250
Plate4 B3	3.1395	0.3462	323.7500	98.3750	1.7231	0.2755	635.9375	404.5625
Plate4 B4								
Plate4 B5								
Plate4 B6					1.3677	0.7423	88.5000	65.0000
Plate4 B7	7.1014	no reps.	245.0000	34.5000	1.1906	0.6475	212.8333	196.5000

Plate4 B8	19.1000	no reps.	191.0000	10.0000	2.3279	no reps.	142.0000	61.0000
Plate4 B9					1.6793	0.5372	111.0000	58.0625
Plate4 C1	16.3928	0.0883	623.0000	31.7500	1.4222	0.5763	125.6250	111.2500
Plate4 C10								
Plate4 C11					1.9522	no reps.	112.2500	57.5000
Plate4 C12					2.9545	no reps.	65.0000	22.0000
Plate4 C13	0.5585	0.8934	80.6250	148.1250	1.1655	0.8002	136.8125	108.3750
Plate4 C14								
Plate4 C15								
Plate4 C16								
Plate4 C17								
Plate4 C18								
Plate4 C19								
Plate4 C2	2.2597	no reps.	87.0000	38.5000				
Plate4 C20	1.1815	0.8650	388.4792	306.1250	0.7967	0.5386	294.5833	312.2500
Plate4 C21								
Plate4 C22								
Plate4 C23	2.0851	0.8130	113.3333	55.1667	1.1971	0.7814	113.6250	109.2917
Plate4 C24	0.1571	0.6881	53.0833	341.3333	0.0924	0.0020	83.1250	972.6666
Plate4 C3	6.9048	no reps.	145.0000	21.0000				
Plate4 C4								
Plate4 C5	5.2400	no reps.	393.0000	75.0000				
Plate4 C6								
Plate4 C7					1.5969	0.6321	98.0000	55.8750
Plate4 C8	0.3099	0.7545	80.7083	278.1042	0.3830	0.0506	175.6667	436.1250
Plate4 C9								
Plate4 D1	17.0668	0.0001	2673.2917	162.9167	3.1917	0.0296	1407.1666	453.7083
Plate4 D10	0.6419	0.9210	88.2500	145.1250	0.7799	0.6654	139.1111	185.2500
Plate4 D11								
Plate4 D12	2.4089	0.6278	195.0625	75.6875	2.8054	0.0611	176.5278	59.9444
Plate4 D14	7.4190	0.5994	260.3333	36.8333	3.5610	no reps.	612.5000	172.0000
Plate4 D15								
Plate4 D16	18.2937	0.0000	980.0626	44.9583	2.6181	0.0043	704.6250	278.5833
Plate4 D17	0.6833	0.7476	284.9375	374.0417	0.6354	0.2599	267.5417	413.4375
Plate4 D18	94.2580	0.0000	3115.1667	32.7917	89.0021	0.0001	3354.9167	39.1667
Plate4 D19	0.5297	0.6352	335.2917	600.9166	0.6739	0.2068	523.7084	767.9167
Plate4 D2					0.7603	no reps.	55.5000	73.0000
Plate4 D20					8.1376	no reps.	443.5000	54.5000
Plate4 D21	2.7704	0.0056	922.0834	334.7083	0.8679	0.6678	339.2083	372.8750
Plate4 D22	7.9674	0.4169	223.5625	24.6250	2.9615	0.1299	240.3750	80.9375
Plate4 D23								
Plate4 D24								
Plate4 D3	20.3000	no reps.	203.0000	10.0000				
Plate4 D4								
Plate4 D5					9.6939	no reps.	237.5000	24.5000
Plate4 D6	1.2132	no reps.	82.5000	68.0000	3.5023	0.0207	239.3611	66.7222
Plate4 D7	1.6145	0.8751	147.0000	131.7500	2.6431	0.0664	233.3333	78.6111
Plate4 D8					0.2538	0.7169	95.5000	98.7500
Plate4 D9					0.9605	0.9635	178.0000	174.5000
Plate4 E1								
Plate4 E10	2.0637	no reps.	186.2500	90.2500	1.4402	0.3562	180.6250	134.5625
Plate4 E11								
Plate4 E12					0.4906	0.3771	230.2500	415.8750
Plate4 E13								
Plate4 E14								
Plate4 E15								
Plate4 E16	6.3371	no reps.	282.0000	44.5000	0.4534	no reps.	92.5000	204.0000
Plate4 E17	3.5318	0.5451	229.8333	62.7500	0.6549	0.7427	78.6250	116.8750
Plate4 E18					0.0605	no reps.	44.0000	727.0000
Plate4 E19								
Plate4 E2	3.2958	no reps.	234.0000	71.0000				
Plate4 E20					0.1627	no reps.	41.5000	255.0000
Plate4 E21	5.5089	no reps.	232.7500	42.2500	7.0858	0.0019	223.3889	32.3889

Plate4 E22	7.1524	0.0385	802.3333	120.8333	0.8108	0.6543	711.7777	737.7222
Plate4 E23	11.1176	no reps.	283.5000	25.5000				
Plate4 E24					1.3821	no reps.	85.0000	61.5000
Plate4 E3	23.5652	no reps.	271.0000	11.5000	0.2778	no reps.	55.0000	198.0000
Plate4 E4					0.2397	no reps.	29.0000	121.0000
Plate4 E5	1.8209	0.0813	1584.8958	883.2500	1.3225	0.1497	1313.1458	933.7917
Plate4 E6					7.4458	no reps.	309.0000	41.5000
Plate4 E7	37.6000	no reps.	376.0000	10.0000	1.1358	0.8199	141.6875	117.9167
Plate4 E8								
Plate4 E9								
Plate4 F1					1.5418	no reps.	405.5000	263.0000
Plate4 F10					23.9318	no reps.	526.5000	22.0000
Plate4 F11								
Plate4 F12					8.3448	no reps.	242.0000	29.0000
Plate4 F13								
Plate4 F14	17.3813	0.0000	3304.9165	195.3333	2.0816	0.0303	2819.5415	1390.5000
Plate4 F15	3.4737	0.6744	204.2500	46.5000				
Plate4 F16	8.8378	no reps.	163.5000	18.5000				
Plate4 F17								
Plate4 F18					5.1077	no reps.	166.0000	32.5000
Plate4 F19	130.8784	0.0000	4813.2710	30.4792	58.7906	0.0000	5605.6455	134.5417
Plate4 F2	1.7269	0.6512	211.5833	129.9583	0.5801	0.2007	281.4375	476.9375
Plate4 F20					1.9079	0.5943	128.0000	61.0000
Plate4 F21	2.0844	0.7367	167.7222	79.3889	1.9717	0.2024	120.2708	57.7500
Plate4 F22								
Plate4 F23								
Plate4 F24	13.9763	0.0000	12024.2920	889.9168	10.7818	0.0000	23383.6250	2055.2502
Plate4 F3	0.9874	0.9930	236.9792	225.8750	0.5861	0.3696	117.6667	213.0208
Plate4 F4								
Plate4 F5								
Plate4 F6	1.9953	0.3990	300.5000	158.2500	1.0753	0.8550	178.2500	183.3958
Plate4 F7					0.3673	0.3050	296.6667	715.6667
Plate4 F8					1.0100	no reps.	454.0000	449.5000
Plate4 F9	1.6082	0.8251	136.0000	85.1667	0.9996	0.9992	180.9583	205.5417
Plate4 G1	1.8039	0.7968	224.6250	148.2500	0.6231	0.7310	83.0000	150.7500
Plate4 G10	0.7324	no reps.	91.0000	124.2500	2.4590	0.1285	411.0000	171.5833
Plate4 G11					7.4064	no reps.	633.2500	85.5000
Plate4 G12					3.7907	no reps.	163.0000	43.0000
Plate4 G13								
Plate4 G14	3.1657	0.4078	320.7500	77.2500	0.7498	0.6711	171.7500	200.1875
Plate4 G15								
Plate4 G16								
Plate4 G17								
Plate4 G18	7.1138	0.5942	127.7500	20.2500	68.2000	no reps.	682.0000	10.0000
Plate4 G19					0.2900	no reps.	67.0000	231.0000
Plate4 G2								
Plate4 G20								
Plate4 G21								
Plate4 G22								
Plate4 G23								
Plate4 G24	4.0356	0.7921	80.0000	21.0000	4.3684	no reps.	124.5000	28.5000
Plate4 G3	1.9002	0.3448	539.7083	269.3958	0.6434	0.3062	531.6875	629.5833
Plate4 G4					0.5908	0.7030	96.5000	244.2500
Plate4 G5	3.1767	0.0707	554.5834	211.5833	0.6675	0.5441	136.2292	166.0208
Plate4 G6	8.2000	no reps.	184.5000	22.5000	1.3056	no reps.	47.0000	36.0000
Plate4 G7					8.2000	no reps.	82.0000	10.0000
Plate4 G8					2.3699	no reps.	173.0000	73.0000
Plate4 G9					1.1294	no reps.	48.0000	42.5000
Plate4 H1					19.0714	no reps.	267.0000	14.0000
Plate4 H10								
Plate4 H11								
Plate4 H12								
Plate4 H13	31.1453	0.0000	644.7500	22.1667	2.2958	0.1193	243.8958	118.4583

Plate4 H14	39.0229	0.0229	4273.1875	49.6875	10.0368	0.0014	330.7292	17.5417
Plate4 H15								
Plate4 H16					0.0928	no reps.	18.0000	194.0000
Plate4 H17	5.9320	0.0111	419.5000	66.1250	2.8550	0.0935	209.1944	71.1944
Plate4 H18	8.8780	0.0631	301.9583	28.7083	15.5900	0.0006	235.5833	14.3333
Plate4 H19	60.1699	0.0009	5457.0830	104.2083	3.4900	0.0000	3623.4167	1043.2916
Plate4 H2	2.6769	no reps.	196.7500	73.5000	2.9352	0.0745	361.5000	156.6667
Plate4 H20	8.5525	0.0000	1517.8333	174.9583	8.6828	0.0004	1086.9166	145.5000
Plate4 H21	2.2996	0.0537	797.6250	340.0833	1.1023	0.7418	469.5417	420.8750
Plate4 H22	1.8432	0.7877	304.1667	156.8333	0.7707	0.8114	103.3333	168.0000
Plate4 H23								
Plate4 H24	7.4667	no reps.	112.0000	15.0000				
Plate4 H3	18.3000	no reps.	183.0000	10.0000				
Plate4 H4								
Plate4 H5	1.1628	0.8993	382.3750	295.7083	0.9426	0.8886	195.2917	220.2917
Plate4 H6	1.9240	0.6993	193.4167	98.6667	4.7157	0.0036	343.5417	70.2917
Plate4 H7	6.8453	0.0001	606.9583	92.9583	11.8933	0.0004	2742.8333	232.7083
Plate4 H8								
Plate4 H9	2.1747	0.5499	252.0625	123.7500	0.5880	0.3837	129.8750	213.2917
Plate4 I1								
Plate4 I10	1.8126	0.7905	338.1250	172.1250	0.9428	0.9123	267.5000	246.6250
Plate4 I11								
Plate4 I12	6.2432	0.5652	263.5000	77.2500	2.5436	0.1089	338.0000	147.6250
Plate4 I13								
Plate4 I14								
Plate4 I15	24.0059	0.1111	474.2778	11.3889	9.4380	0.0387	205.8333	23.5833
Plate4 I16	5.0505	no reps.	500.0000	99.0000	4.8640	0.1530	245.2500	49.5000
Plate4 I17	2.2072	no reps.	245.0000	111.0000	1.5087	0.7122	143.7500	110.7500
Plate4 I18					0.1873	0.2739	40.8333	370.6667
Plate4 I19								
Plate4 I2								
Plate4 I20					12.6458	no reps.	303.5000	24.0000
Plate4 I21								
Plate4 I22					4.5014	0.2280	254.0000	42.0000
Plate4 I23								
Plate4 I24					4.2057	no reps.	184.0000	43.7500
Plate4 I3	4.2222	0.5460	240.0000	54.0000	0.9041	0.9367	103.5000	127.5000
Plate4 I4								
Plate4 I5								
Plate4 I6								
Plate4 I7								
Plate4 I8	2.0909	no reps.	115.0000	55.0000	2.4544	0.4130	137.5000	55.3750
Plate4 I9					26.0125	0.0396	271.0000	10.0000
Plate4 J1								
Plate4 J10	1.3043	0.9568	95.2500	72.0000	1.3172	0.7019	290.3333	251.2222
Plate4 J11	0.6878	no reps.	76.0000	110.5000				
Plate4 J12	3.5351	0.0002	4120.5420	1207.0000	1.2697	0.2667	3343.5835	2667.8750
Plate4 J13	28.6861	0.0000	745.6041	26.3958	4.0724	0.0078	502.5833	156.5417
Plate4 J14	8.3626	0.0016	409.6250	47.6250	4.0408	0.0181	345.6667	83.1667
Plate4 J15								
Plate4 J16	6.0278	no reps.	108.5000	18.0000				
Plate4 J17								
Plate4 J18	7.9315	0.0282	475.5000	59.9375	0.9789	0.9657	245.1667	260.6250
Plate4 J19	63.6739	0.0000	3309.7500	53.2083	2.4074	0.0215	2411.8335	1007.0834
Plate4 J2	8.1352	0.0011	847.4584	96.6250	1.1648	0.4894	1664.2917	1442.7500
Plate4 J20	0.2043	no reps.	48.0000	235.0000	1.3380	0.7681	132.0000	89.6667
Plate4 J21								
Plate4 J22	13.5102	no reps.	662.0000	49.0000	2.6522	no reps.	61.0000	23.0000
Plate4 J23	4.3988	0.0569	315.7500	103.4792	4.3084	0.0045	210.5417	45.2917
Plate4 J24	22.6000	no reps.	226.0000	10.0000				
Plate4 J3								
Plate4 J4								
Plate4 J5								

Plate4 J6								
Plate4 J7	1.1632	0.9498	236.5000	225.8750	0.9572	0.9318	598.2500	549.0000
Plate4 J8					0.6889	no reps.	62.0000	90.0000
Plate4 J9								
Plate4 K1	0.0989	no reps.	41.0000	414.5000				
Plate4 K10	0.7688	0.9329	127.8333	169.0000	1.3776	0.5672	153.1458	118.8958
Plate4 K11	0.3905	0.6552	182.1458	458.4375	0.4071	0.1471	284.9375	772.2708
Plate4 K12	2.5455	no reps.	154.0000	60.5000	0.8782	0.8645	206.3333	229.3333
Plate4 K13								
Plate4 K14								
Plate4 K15	17.7000	no reps.	177.0000	10.0000	12.2343	0.0004	273.0208	21.2292
Plate4 K16								
Plate4 K17					25.3000	no reps.	253.0000	10.0000
Plate4 K18								
Plate4 K19								
Plate4 K2								
Plate4 K20					21.6000	no reps.	216.0000	10.0000
Plate4 K21					8.8000	no reps.	132.0000	15.0000
Plate4 K22					6.1000	no reps.	61.0000	10.0000
Plate4 K23					2.5895	no reps.	123.0000	47.5000
Plate4 K24					3.6000	no reps.	126.0000	35.0000
Plate4 K3	6.6304	0.0098	745.9375	91.3542	2.1178	0.1516	426.3750	203.7500
Plate4 K4								
Plate4 K5	21.2059	no reps.	360.5000	17.0000				
Plate4 K6	7.7647	no reps.	132.0000	17.0000	1.7951	0.6400	113.2500	59.5000
Plate4 K7								
Plate4 K8	2.4730	no reps.	183.0000	74.0000				
Plate4 K9								
Plate4 L1					1.5211	no reps.	54.0000	35.5000
Plate4 L10					5.6486	0.0204	240.3889	38.0556
Plate4 L11								
Plate4 L12								
Plate4 L13					8.1957	no reps.	188.5000	23.0000
Plate4 L14	7.7616	0.3745	125.5417	16.5833	3.0334	0.0341	390.6667	82.8333
Plate4 L15	20.7500	no reps.	207.5000	10.0000	14.1000	no reps.	141.0000	10.0000
Plate4 L16	3.8199	0.3749	204.4375	51.9583	2.0037	0.5575	171.2500	91.5000
Plate4 L17	1.8040	0.3846	445.9167	230.1667	1.0351	0.9254	262.5000	286.5000
Plate4 L18	132.9153	0.0017	2563.6250	19.2500	1.2074	0.6948	876.8541	457.8750
Plate4 L19	27.4797	0.0000	2023.3333	70.4583	8.0377	0.0002	2017.8334	259.0417
Plate4 L2	2.7778	no reps.	200.0000	72.0000	2.6197	0.3117	292.2500	136.2500
Plate4 L20	1.4988	0.9442	112.5000	92.6667	1.7225	0.4638	188.5000	112.0000
Plate4 L21	18.5507	0.0000	2021.1250	106.6667	0.5075	0.0542	1639.5834	2999.3750
Plate4 L22	12.8690	0.1943	340.8750	24.0625	19.1222	0.0435	279.5000	15.5000
Plate4 L23								
Plate4 L24								
Plate4 L3	6.3824	no reps.	217.0000	34.0000				
Plate4 L4	2.9539	0.6327	389.1667	125.5000	4.2367	0.2249	200.6250	44.8750
Plate4 L5					1.9556	no reps.	352.0000	180.0000
Plate4 L6	1.5105	no reps.	108.0000	71.5000				
Plate4 L7	3.2391	0.3272	239.7500	81.4375	1.3572	0.5592	168.5833	122.8611
Plate4 L8	8.6905	no reps.	365.0000	42.0000				
Plate4 L9								
Plate4 M1								
Plate4 M10	1.0259	no reps.	119.0000	116.0000				
Plate4 M11								
Plate4 M12								
Plate4 M13								
Plate4 M14								
Plate4 M15					7.7414	no reps.	224.5000	29.0000
Plate4 M16					5.2667	no reps.	79.0000	15.0000
Plate4 M17								
Plate4 M18	0.6049	0.7062	1101.2500	1750.2917	1.5075	0.0621	1151.5208	816.2709
Plate4 M19								

Plate4 M2								
Plate4 M20								
Plate4 M21								
Plate4 M22								
Plate4 M23								
Plate4 M24	2.4929	no reps.	263.0000	105.5000	0.5128	0.5128	89.2500	196.2500
Plate4 M3					0.0100	no reps.	-12.0000	337.0000
Plate4 M4								
Plate4 M5								
Plate4 M6	3.4242	no reps.	226.0000	66.0000				
Plate4 M7								
Plate4 M8								
Plate4 M9	1.4278	no reps.	128.5000	90.0000				
Plate4 N1								
Plate4 N10					0.7797	no reps.	46.0000	59.0000
Plate4 N11								
Plate4 N12					3.1545	0.3428	171.0000	51.5000
Plate4 N13					0.9931	0.9956	95.2500	96.2500
Plate4 N14	35.7537	0.0000	2996.7915	81.3750	1.9782	0.0001	2417.7917	1211.9166
Plate4 N15	5.4196	0.0525	393.2708	65.3750	3.3254	0.0005	480.8958	143.3958
Plate4 N16								
Plate4 N17								
Plate4 N18	20.2326	0.0201	842.8889	30.5000	1.9285	0.0748	11946.6875	6304.2500
Plate4 N19	13.2067	0.0015	4223.8125	364.0625	1.4280	0.2724	3571.0000	2471.7083
Plate4 N2	0.3840	0.5813	266.9583	702.6250	2.0301	0.0435	251.6667	121.1250
Plate4 N20	40.8816	no reps.	1553.5000	38.0000				
Plate4 N21	27.5271	0.0013	681.0417	26.0833	2.7318	0.1033	105.6389	40.3611
Plate4 N22	32.5061	0.0000	1767.2917	57.6250	2.6735	0.0020	1582.7084	647.1875
Plate4 N23					19.1000	no reps.	191.0000	10.0000
Plate4 N24	3.7525	0.6366	278.9167	68.9167	2.3744	0.1662	1526.1458	738.5208
Plate4 N3	4.9075	0.0000	1871.5416	387.3750	1.2990	0.2595	942.1251	788.7916
Plate4 N4	1.8584	0.8137	170.3333	92.1667	1.1786	0.8973	91.0000	79.5000
Plate4 N5					0.8759	no reps.	254.0000	290.0000
Plate4 N6	2.7778	no reps.	125.0000	45.0000				
Plate4 N7	6.3175	0.0133	3012.7500	681.1250	1.4391	0.2145	4742.3750	3079.6250
Plate4 N8	3.3977	no reps.	149.5000	44.0000	0.2369	0.4119	106.2500	237.7500
Plate4 N9	1.3028	no reps.	92.5000	71.0000				
Plate4 O1								
Plate4 O10	0.3544	0.5464	309.3750	843.7083	0.4299	0.0385	753.3750	1747.8333
Plate4 O11	0.0100	no reps.	0.0000	120.0000				
Plate4 O12					1.3428	0.7013	138.7500	99.2500
Plate4 O13					1.3250	no reps.	53.0000	40.0000
Plate4 O14	2.4368	0.7346	241.0000	88.2500	1.5029	0.5966	121.2500	79.9167
Plate4 O15	8.9265	no reps.	303.5000	34.0000				
Plate4 O16	19.1000	no reps.	191.0000	10.0000				
Plate4 O17	1.2683	0.9552	184.7500	118.0000	0.7004	0.7431	170.6667	164.6667
Plate4 O18								
Plate4 O19								
Plate4 O2								
Plate4 O20					18.0000	no reps.	180.0000	10.0000
Plate4 O21								
Plate4 O22								
Plate4 O23					3.5982	0.2366	272.5000	69.2500
Plate4 O24								
Plate4 O3	4.6895	0.2080	378.0417	68.3958	3.7518	0.1836	156.6667	32.8333
Plate4 O4								
Plate4 O5								
Plate4 O6								
Plate4 O7								
Plate4 O8	19.3805	0.0025	381.2292	22.6458	6.8646	0.0043	227.5417	31.2083
Plate4 O9								
Plate4 P1	8.7143	no reps.	305.0000	35.0000	15.7000	no reps.	314.0000	20.0000
Plate4 P10								

Plate4 P11								
Plate4 P12								
Plate4 P13	7.3067	no reps.	274.0000	37.5000	2.0095	0.4596	153.5000	66.3333
Plate4 P14	33.8000	no reps.	338.0000	10.0000	5.1671	no reps.	502.5000	97.2500
Plate4 P15	3.7396	no reps.	179.5000	48.0000				
Plate4 P16					17.6000	no reps.	352.0000	20.0000
Plate4 P17								
Plate4 P18					11.4713	0.0287	219.5000	23.1667
Plate4 P19	3.0345	0.4891	276.4167	86.7500	0.7220	0.3691	274.6458	391.7917
Plate4 P2	1.3628	0.9126	119.6250	81.4375	1.8703	0.4769	175.2500	69.1250
Plate4 P20					2.2267	no reps.	525.5000	236.0000
Plate4 P21	21.4365	0.1794	348.6250	13.5000	1.8124	0.7293	73.2500	84.0000
Plate4 P22	8.1788	0.1308	316.8125	33.8750	1.2774	0.8049	101.1667	76.8333
Plate4 P23								
Plate4 P24	0.7109	0.9522	518.6666	475.3333	0.3441	0.4124	144.1667	793.3334
Plate4 P3	4.3281	no reps.	277.0000	64.0000				
Plate4 P4	1.2691	0.8337	223.2083	175.5000	0.7826	0.5939	168.9167	204.8750
Plate4 P5					1.5517	0.5502	360.0000	217.7500
Plate4 P6	5.6562	0.0005	672.9167	145.0000	2.0920	0.0044	687.8750	356.5833
Plate4 P7	1.4761	0.6757	238.3333	169.4167	1.0997	0.8146	211.0417	194.8958
Plate4 P8					0.2112	no reps.	34.0000	161.0000
Plate4 P9					0.7487	no reps.	147.5000	197.0000
Plate5 A1								
Plate5 A10	5.9176	0.2383	324.1250	49.6875	2.2434	0.1780	284.6667	140.2500
Plate5 A11								
Plate5 A12								
Plate5 A13								
Plate5 A14								
Plate5 A15	4.2022	0.1529	454.9583	95.3333	5.5477	0.0003	334.5000	55.2917
Plate5 A16	6.6802	no reps.	219.3333	32.8333	3.0665	0.0807	206.5625	92.0625
Plate5 A17	37.3000	no reps.	373.0000	10.0000				
Plate5 A18	11.2521	0.0000	1313.2916	131.0625	2.0784	0.0015	1132.5416	527.9583
Plate5 A19	18.6521	0.0007	668.1250	36.0417	2.6779	0.0104	561.6666	267.4167
Plate5 A2					22.6000	no reps.	452.0000	20.0000
Plate5 A20	23.0788	0.0000	1243.5000	51.0417	2.3787	0.0073	815.4584	329.9583
Plate5 A21	5.3038	0.1127	437.7500	64.6667	4.0427	0.0649	442.5417	119.3125
Plate5 A22	5.2542	0.0141	2123.9375	407.8125	1.8422	0.1150	2877.3750	1651.2500
Plate5 A23	3.1880	0.6084	174.3750	56.8750	1.6935	no reps.	157.5000	93.0000
Plate5 A24	6.8108	no reps.	504.0000	74.0000	1.1938	no reps.	95.5000	80.0000
Plate5 A3	36.7155	0.0000	2098.8750	54.6458	4.0656	0.0120	1314.5625	531.0834
Plate5 A4								
Plate5 A5	51.4500	0.0000	1705.1666	34.0000	2.4862	0.0028	1173.4166	465.5833
Plate5 A6								
Plate5 A7	3.3428	0.7717	114.3750	38.1250	1.8002	0.4309	179.2500	86.0000
Plate5 A8	14.1990	0.0253	267.5555	19.6667	3.9925	0.1722	164.7500	39.0000
Plate5 A9	2.6715	0.7903	202.7500	61.0000	19.5000	no reps.	195.0000	10.0000
Plate5 B1								
Plate5 B10								
Plate5 B11	15.2098	0.0491	196.0833	12.7778	1.6234	0.4974	121.8750	78.0000
Plate5 B12	2.2946	0.7676	189.6250	75.3750	3.5372	0.0078	323.3333	95.7292
Plate5 B13								
Plate5 B14	13.2677	0.0000	3768.5625	278.9583	3.7283	0.0247	1856.6667	413.5417
Plate5 B15								
Plate5 B16								
Plate5 B17								
Plate5 B18								
Plate5 B19	11.4994	0.0000	1180.8126	105.0417	4.1851	0.0072	1076.0834	291.1667
Plate5 B2	7.7229	no reps.	320.5000	41.5000	2.2549	0.5470	94.5000	42.0000
Plate5 B20								
Plate5 B21								
Plate5 B22								
Plate5 B23								
Plate5 B24	9.9017	0.4857	187.5000	22.5000	2.5297	0.0806	346.7500	139.0000

Plate5 B3	15.8750	no reps.	635.0000	40.0000				
Plate5 B4								
Plate5 B5								
Plate5 B6					1.3066	no reps.	317.5000	243.0000
Plate5 B7	3.8582	0.0039	989.6875	282.2292	1.1452	0.6743	1032.0834	933.6250
Plate5 B8	3.3183	0.1228	1369.2500	420.2500	0.8998	0.8115	795.2500	964.1250
Plate5 B9	6.0951	0.2804	183.5625	29.7708	0.3246	0.0831	151.8333	470.1250
Plate5 C1	18.0106	0.0000	760.1250	43.1667	2.1138	0.0230	459.6250	217.1250
Plate5 C10								
Plate5 C11	4.4369	0.5255	226.3125	46.9375	0.2145	0.3044	86.3750	399.8750
Plate5 C12	44.0456	0.0000	1031.9166	24.7500	1.2302	0.6003	426.5417	346.1667
Plate5 C13	3.9070	no reps.	210.0000	53.7500	6.1994	0.1315	126.0833	17.4167
Plate5 C14	3.2107	0.6756	181.2500	52.3333	1.1883	0.8357	121.1111	73.2222
Plate5 C15								
Plate5 C16	11.3605	no reps.	977.0000	86.0000				
Plate5 C17					1.9783	no reps.	91.0000	46.0000
Plate5 C18	23.7083	no reps.	284.5000	12.0000				
Plate5 C19								
Plate5 C2	23.9054	0.0010	530.3958	19.4167	3.0487	0.0014	489.7708	174.8125
Plate5 C20								
Plate5 C21	4.4795	0.5826	211.0000	44.5000				
Plate5 C23	9.2564	0.0035	430.4792	47.0208	2.5061	0.2053	168.2500	59.0000
Plate5 C24	1.1446	no reps.	95.0000	83.0000	0.5228	0.4505	80.8333	144.0000
Plate5 C3								
Plate5 C4								
Plate5 C5					11.5238	no reps.	242.0000	21.0000
Plate5 C6					28.1000	no reps.	281.0000	10.0000
Plate5 C7	9.9813	0.4803	155.7500	15.2500	8.4437	0.0418	85.4375	10.0000
Plate5 C8	29.0219	0.0000	772.6250	25.5417	5.7463	0.0000	442.2083	79.0417
Plate5 C9								
Plate5 D1	15.8065	no reps.	245.0000	15.5000	6.1447	0.0336	235.8333	37.9167
Plate5 D10	20.7396	0.0003	617.7500	27.5000	2.8637	0.0129	641.4375	254.5000
Plate5 D11								
Plate5 D12	5.0493	0.4794	139.4167	28.3333	4.5934	no reps.	209.0000	45.5000
Plate5 D13								
Plate5 D14					0.8113	0.8762	84.7500	153.2500
Plate5 D15					2.0578	no reps.	240.2500	116.7500
Plate5 D16	17.2695	0.0004	440.5000	30.5833	3.5192	0.0223	190.7917	53.6042
Plate5 D17								
Plate5 D18	19.7000	no reps.	197.0000	10.0000	2.8457	no reps.	230.5000	81.0000
Plate5 D19								
Plate5 D2	1.6130	0.9300	104.2500	60.2500	30.4000	no reps.	304.0000	10.0000
Plate5 D20								
Plate5 D21					38.7000	no reps.	387.0000	10.0000
Plate5 D22					5.1216	no reps.	189.5000	37.0000
Plate5 D23								
Plate5 D24								
Plate5 D3	34.0002	0.0781	1650.2500	37.5000	9.9777	0.0718	721.0000	62.7500
Plate5 D4					19.7000	no reps.	197.0000	10.0000
Plate5 D5	36.3325	0.0000	542.2083	15.3750	17.2462	0.0025	498.7708	26.1042
Plate5 D6	13.2210	0.0000	838.9999	65.4583	11.3981	0.0029	1427.6041	156.3125
Plate5 D7	68.0003	0.0006	3619.6250	55.9583	37.3084	0.0000	2755.6250	75.7500
Plate5 D8	9.4553	0.1485	205.0208	20.5625	0.1822	0.0174	141.0417	717.0417
Plate5 D9	8.9002	0.0201	356.5000	40.6250	0.2478	0.0303	89.1042	453.7917
Plate5 E1	4.7838	0.6020	124.3333	25.3333	7.4074	0.1974	179.1250	25.5000
Plate5 E10	11.0417	0.0090	677.3125	70.4375	9.1728	0.0003	927.1250	92.1250
Plate5 E11								
Plate5 E12					8.2021	0.0556	125.0833	16.5000
Plate5 E13	7.1173	0.5955	239.0000	31.0000				
Plate5 E14	0.6825	0.7982	183.8333	271.0625	2.2912	0.1859	186.0625	78.3750
Plate5 E15	19.6097	0.0000	1235.6250	56.3333	5.7839	0.0006	588.4375	87.2292
Plate5 E16	15.5551	0.0010	756.8889	49.6667	0.9426	0.9402	159.8750	193.6875
Plate5 E17	14.4750	0.0002	947.4167	79.1667	2.8875	0.0572	569.3959	163.9583

Plate5 E18	6.5499	0.2988	278.6250	43.7500	2.9803	0.1476	212.3750	63.0000
Plate5 E19	12.1258	0.0532	316.6875	27.7500	1.3743	0.4428	337.8750	240.8750
Plate5 E2								
Plate5 E20	13.1406	0.0072	604.9584	41.0833	3.0112	0.0158	289.3750	89.7500
Plate5 E21	5.1863	0.1948	240.8958	45.6458	3.5250	0.1054	142.7500	48.8750
Plate5 E22	8.8724	0.0001	1735.8750	204.5417	2.3304	0.0091	1369.8750	639.1459
Plate5 E23	8.1981	0.3421	241.4375	30.6875	2.4025	0.4310	80.5000	35.3333
Plate5 E24	7.2558	no reps.	312.0000	43.0000				
Plate5 E3								
Plate5 E4	12.8422	0.1162	194.6944	15.8333	18.1636	0.0170	241.0000	13.5000
Plate5 E5	11.2542	0.4953	205.7500	17.0000	2.4609	0.1417	136.2708	65.7083
Plate5 E6	1.7497	0.8419	246.5000	141.0000	0.5179	0.4355	132.3056	342.3055
Plate5 E7	22.0374	0.0105	755.6459	28.3333	3.4343	0.0404	1095.6041	348.1042
Plate5 E8	6.8294	0.0622	339.0208	40.2292	3.4565	0.0112	146.2708	41.4375
Plate5 E9					0.3609	no reps.	61.0000	169.0000
Plate5 F1	3.6937	0.5898	146.1250	49.5000	2.1726	0.5558	92.5000	42.0000
Plate5 F10	10.1565	0.0000	1994.0000	206.0417	1.9070	0.0421	1896.0625	1105.0209
Plate5 F11								
Plate5 F12	11.8234	0.0001	487.6667	51.9167	2.8456	0.0003	555.8125	211.3750
Plate5 F13								
Plate5 F14	10.1904	0.0025	643.4166	54.3333	6.0673	0.0001	397.3750	65.3333
Plate5 F15								
Plate5 F16								
Plate5 F17								
Plate5 F18								
Plate5 F2	6.8114	0.0005	1521.2917	233.7083	2.5642	0.0002	1361.7083	530.7917
Plate5 F20					0.3023	no reps.	107.0000	354.0000
Plate5 F21	3.2916	0.2556	307.7917	83.3750	2.1991	0.0571	234.1250	114.0000
Plate5 F22	5.6885	0.0016	1238.6876	192.1250	2.4202	0.0042	1018.0625	391.6250
Plate5 F23								
Plate5 F24	4.9116	0.0174	491.7500	103.2708	2.2230	0.0235	513.9583	247.9375
Plate5 F3	0.1511	no reps.	27.5000	182.0000				
Plate5 F4	9.4673	0.5638	166.4167	16.3333	3.8904	0.1506	212.1667	56.6667
Plate5 F5								
Plate5 F6	1.8051	0.7925	310.5000	175.2500	2.1563	0.1009	788.6250	393.7500
Plate5 F7	9.0772	0.0089	596.1459	49.8333	8.6825	0.0000	955.9168	105.5833
Plate5 F8	13.1129	no reps.	406.5000	31.0000	2.0326	0.2986	720.0000	354.0000
Plate5 F9	42.6243	0.0000	1060.7500	25.4583	3.2476	0.0128	1089.8333	364.0417
Plate5 G1	3.8979	0.2698	211.8750	57.0208	7.2339	0.0009	344.1042	47.3958
Plate5 G10	12.7451	0.1889	408.8333	28.6667	20.5878	0.0001	340.2917	15.6250
Plate5 G11	14.8884	0.0326	324.2708	17.2708	4.2923	0.1010	524.2084	125.9167
Plate5 G12	7.0318	0.0430	282.9792	40.0833	8.9077	0.0045	378.5208	37.5000
Plate5 G13								
Plate5 G14	0.5464	0.7316	225.8333	398.6667	1.0505	0.9274	188.9375	173.9167
Plate5 G15					3.8390	0.2981	166.0000	45.5000
Plate5 G16								
Plate5 G17					12.4304	0.1016	164.5000	13.7500
Plate5 G18	6.8000	no reps.	68.0000	10.0000				
Plate5 G19	8.8767	0.1680	250.4792	27.5000	2.9282	0.0970	931.7917	440.7500
Plate5 G2								
Plate5 G20	8.2500	no reps.	165.0000	20.0000	3.8571	no reps.	135.0000	35.0000
Plate5 G21	7.2805	no reps.	298.5000	41.0000				
Plate5 G22	16.5691	0.0000	1994.0000	124.5833	2.5678	0.0006	661.2916	264.7917
Plate5 G23	5.6357	0.1205	540.2708	73.9375	2.9954	0.0231	459.1042	143.7500
Plate5 G24	35.5433	0.0000	2683.7500	73.7083	1.1727	0.4304	4968.4795	4455.7915
Plate5 G3	3.4571	no reps.	121.0000	35.0000	12.4219	0.0101	152.7500	10.7500
Plate5 G4					0.7881	no reps.	46.5000	59.0000
Plate5 G5	3.5569	0.7425	163.5000	40.0000	0.6428	0.4169	377.7083	517.5000
Plate5 G6					1.7694	0.5189	134.4167	72.5000
Plate5 G7	22.9000	no reps.	229.0000	10.0000				
Plate5 G8	25.5449	0.0000	700.3334	29.0208	2.3329	0.1865	467.4167	205.3333
Plate5 G9					6.7805	0.1279	213.5000	32.2500
Plate5 H1	5.7650	0.2625	294.5833	46.2778	6.8702	0.0029	351.5625	44.9167

Plate5 H10	13.5885	0.0017	322.5417	23.4583	2.0219	0.1656	276.8542	148.8542
Plate5 H11					0.8969	0.9153	141.3750	101.7500
Plate5 H12	24.1854	0.0011	428.1250	16.4583	1.7884	0.3392	255.8125	148.3958
Plate5 H13	12.6916	0.0001	714.6042	58.8333	6.5525	0.1637	310.5833	44.7778
Plate5 H14	2.4884	no reps.	107.0000	43.0000	2.7809	no reps.	1485.0000	534.0000
Plate5 H15					1.2897	no reps.	300.5000	233.0000
Plate5 H16								
Plate5 H17								
Plate5 H18	25.1486	0.0000	1022.1251	39.4583	19.5716	0.0000	855.2500	43.9375
Plate5 H19	3.1580	no reps.	293.1667	92.8333	3.5904	0.0249	229.4583	63.7708
Plate5 H2	3.1967	0.5918	221.3750	54.3750	5.1083	0.0386	208.3750	45.7500
Plate5 H20					9.7312	0.1224	212.0000	26.5000
Plate5 H21	8.3085	0.0125	334.7500	39.0625	2.6727	0.0812	203.3750	80.7500
Plate5 H22	17.5575	0.0007	601.2084	35.7500	1.5256	0.0682	1150.9375	793.7292
Plate5 H23	5.5475	0.3394	266.6667	47.3333	4.1687	no reps.	173.0000	41.5000
Plate5 H24					0.1139	0.4420	26.5000	173.0000
Plate5 H3					0.0100	no reps.	7.0000	6500.0000
Plate5 H4	25.7582	0.5117	470.5000	11.0000	13.8842	0.0242	247.5000	18.0000
Plate5 H5	19.1500	no reps.	191.5000	10.0000	0.9012	0.8972	116.4167	135.9167
Plate5 H7	5.1602	0.3029	320.7500	66.0000	2.3286	0.1959	187.7500	77.0000
Plate5 H8	4.9126	no reps.	253.0000	51.5000	32.8000	no reps.	328.0000	10.0000
Plate5 H9	8.0877	0.0014	527.6250	62.8333	1.2513	0.3509	1319.1459	1061.8125
Plate5 I1								
Plate5 I10	10.8711	0.0000	2882.5000	284.9167	2.8771	0.0001	3413.7500	1168.6667
Plate5 I11	3.7408	0.6332	272.5000	49.6667	1.0324	0.9486	670.0000	639.8750
Plate5 I12	34.0000	no reps.	340.0000	10.0000	2.1377	0.5193	236.5000	75.7500
Plate5 I13								
Plate5 I14	22.1288	0.0001	436.1042	20.4167	5.0100	0.0284	134.8333	25.7500
Plate5 I15	14.0594	0.0000	1714.9375	116.2500	2.2783	0.0138	1077.3333	501.6250
Plate5 I16	9.7271	0.0002	559.0208	59.4167	1.8988	0.0896	261.1667	133.4583
Plate5 I17					2.8040	0.3067	178.0000	58.6250
Plate5 I18	5.5592	0.0086	536.6875	100.3958	1.3554	0.2893	580.9583	507.8750
Plate5 I19	31.2000	no reps.	312.0000	10.0000	2.9947	0.3335	84.1667	33.8333
Plate5 I2								
Plate5 I20	1.2036	0.8370	251.2917	243.7083	1.5881	0.1121	358.7500	224.6250
Plate5 I21	11.7514	0.0000	482.7917	41.3750	1.9360	0.0018	1457.7916	797.2917
Plate5 I22	24.7462	0.0000	3003.7915	123.2917	1.0701	0.8033	5724.7915	5545.1250
Plate5 I23	11.2664	0.0211	731.9375	46.6458	2.5005	0.1368	300.5000	78.3750
Plate5 I24								
Plate5 I3	6.4516	no reps.	100.0000	15.5000	2.4161	0.2617	158.4375	68.3750
Plate5 I4								
Plate5 I5	3.8667	no reps.	232.0000	60.0000	0.6319	0.2176	358.8125	526.1042
Plate5 I6	5.4589	0.0046	442.6667	95.5208	0.7532	0.2648	2064.2083	2706.5417
Plate5 I7					2.8575	0.0780	167.8125	53.4167
Plate5 I8	7.9648	0.1968	236.1667	31.4167	3.5756	0.0019	334.0000	111.2083
Plate5 I9					4.6198	0.0362	204.0000	48.9444
Plate5 J1								
Plate5 J10	1.8809	0.2525	617.1250	351.9375	1.3605	0.1678	674.1667	507.9583
Plate5 J11								
Plate5 J12	5.0878	0.0000	23737.6680	4806.2920	1.5741	0.0743	43668.8500	27871.2290
Plate5 J13	9.4223	0.0002	491.6875	56.3333	5.4548	0.0014	419.0833	79.8333
Plate5 J14	1.2336	0.6755	2270.6665	1785.9584	1.9191	0.3673	1942.9166	1333.8750
Plate5 J15	7.6339	0.0000	889.9166	112.0833	1.5259	0.1965	339.1667	222.8750
Plate5 J16	3.3787	0.5217	156.8333	45.6667	4.4076	0.1724	200.5000	46.7500
Plate5 J17	4.8727	no reps.	268.0000	55.0000				
Plate5 J18	15.3996	0.0976	369.9167	25.5833	2.3921	0.4894	87.8750	49.3750
Plate5 J19	0.9563	0.9939	121.7500	132.2500	1.5600	0.6232	158.5000	84.1667
Plate5 J2	5.1136	0.0000	3980.2917	738.7084	1.3813	0.0825	5110.0415	3720.5833
Plate5 J20					0.9659	0.9752	197.7500	290.7500
Plate5 J21	4.3787	0.0177	1417.0835	324.2292	3.2074	0.0019	524.6666	167.0625
Plate5 J22	5.9482	0.0395	325.2500	51.3333	2.9905	0.0218	196.7917	67.0417
Plate5 J23								
Plate5 J24	3.6029	0.0035	570.1667	175.3333	2.0134	0.0011	798.0834	407.1667

Plate5 J3	9.9070	no reps.	213.0000	21.5000				
Plate5 J4	8.0120	no reps.	665.0000	83.0000				
Plate5 J5					9.0098	0.1694	127.5000	13.2500
Plate5 J6	9.2378	0.4285	188.0000	24.0000	3.3554	0.0422	190.5556	52.8333
Plate5 J7	15.0786	0.0009	1056.3541	93.0833	3.4185	0.0000	1013.3749	290.7917
Plate5 J8	5.4153	0.6447	739.2500	151.2500	11.5828	0.2185	5498.0000	1029.2500
Plate5 J9								
Plate5 K1	10.6429	no reps.	298.0000	28.0000				
Plate5 K10	42.9322	0.0000	857.4166	20.6667	2.6734	0.0002	1078.1667	420.7083
Plate5 K11	1.5423	0.7614	246.1250	156.6250	1.2496	0.5913	211.5000	193.0833
Plate5 K12	17.9059	0.0001	480.0625	27.1667	2.2341	0.0282	1645.5834	729.8750
Plate5 K13								
Plate5 K14	7.4840	0.0000	3827.5415	513.9167	2.9243	0.0001	1359.8750	491.0833
Plate5 K15	39.3373	0.0001	5567.2085	143.3333	2.5744	0.0526	3728.5417	1509.5416
Plate5 K16								
Plate5 K17	13.7757	0.2230	305.0000	29.0000	2.8897	0.2356	172.4167	45.6667
Plate5 K18	2.8011	no reps.	246.5000	88.0000	2.2030	0.2940	118.3889	52.4444
Plate5 K19					1.6113	0.5415	118.9167	73.0833
Plate5 K2	19.5674	0.0000	679.3750	30.7500	3.9942	0.0303	563.8958	158.6250
Plate5 K20	4.2288	0.6243	222.0000	46.7500	4.8336	0.1546	236.5000	49.5000
Plate5 K21	6.8369	0.2191	243.3333	37.5000	1.6458	0.3604	168.3750	119.3750
Plate5 K22	12.6226	0.0002	464.6250	37.3750	2.2795	0.0002	1292.2083	584.3750
Plate5 K23	5.4980	0.1962	224.5625	41.5625	0.7704	0.6333	1132.6667	2886.6665
Plate5 K24					2.5540	0.1314	181.0278	65.8611
Plate5 K3	20.1347	0.1996	291.0000	15.0000				
Plate5 K4					22.1818	no reps.	244.0000	11.0000
Plate5 K5								
Plate5 K6	10.9798	0.3660	168.2500	14.6250	8.4551	0.0630	202.0000	23.7500
Plate5 K7	3.7452	0.5492	165.6250	51.7500	3.7656	0.0442	178.1250	52.6250
Plate5 K8	9.9084	0.1351	331.6667	40.5000	1.1182	0.8649	134.6667	117.6667
Plate5 K9	29.9050	0.0028	382.5625	12.6250	1.1339	0.8839	216.5417	120.4792
Plate5 L1	10.0563	0.0022	480.7916	43.9167	2.4195	0.1430	720.1459	302.6667
Plate5 L10	9.3927	0.0000	634.0834	67.5000	2.8497	0.0101	241.7083	74.9375
Plate5 L11					4.4721	0.2833	130.2500	29.0000
Plate5 L12	5.6125	0.0821	362.9583	58.2083	1.8265	0.0350	801.6250	487.3750
Plate5 L13	2.9382	0.4712	245.3125	88.1875	0.8238	0.7575	117.2778	152.8056
Plate5 L14	1.6859	0.9196	91.2500	61.2500	2.1766	0.3756	242.2500	195.4167
Plate5 L15	1.0171	0.9970	123.5000	169.5000	1.4577	0.5862	209.0000	122.0000
Plate5 L16	3.8117	0.6051	275.7500	123.7500	2.1223	0.2485	139.7778	70.8611
Plate5 L17	2.5993	0.2767	248.5000	94.6042	3.9075	0.0032	333.2708	73.6250
Plate5 L18	26.1624	0.0000	1840.5833	68.5833	1.1747	0.4436	2380.3750	2063.1250
Plate5 L19	29.1622	0.0000	2368.3750	88.9583	0.9264	0.7323	2933.2915	2973.1250
Plate5 L2	2.2667	0.6658	133.2500	90.2500	0.7413	0.5888	184.5833	205.3958
Plate5 L20	2.6297	0.4270	206.3958	84.0833	2.0302	0.1502	210.0208	105.0833
Plate5 L21								
Plate5 L22	4.8391	no reps.	210.5000	43.5000				
Plate5 L23								
Plate5 L24								
Plate5 L3					0.3986	0.5237	115.5000	221.5000
Plate5 L4	6.7886	0.0008	531.2916	73.8542	10.6621	0.0053	961.8124	106.6250
Plate5 L5								
Plate5 L6	12.7775	0.0001	590.8750	41.7500	1.7980	0.0019	1611.2916	891.7916
Plate5 L7	11.1263	0.0000	729.8750	73.6250	1.5388	0.0251	1721.6667	1119.0000
Plate5 L8	1.8864	0.6502	169.7708	93.0000	0.6558	0.2749	248.5000	369.6250
Plate5 L9					1.6207	0.2583	164.3125	88.6250
Plate5 M1								
Plate5 M10	4.0706	0.0003	1575.1667	412.5417	1.4370	0.2972	1464.1667	1243.1250
Plate5 M11								
Plate5 M12								
Plate5 M13					11.4105	0.1157	177.5000	16.0000
Plate5 M14	13.4446	0.4472	213.0000	14.5000	4.6481	0.0449	190.8125	38.1875
Plate5 M15	13.6675	0.0003	2485.8750	211.7917	2.3541	0.0123	373.2917	155.1875
Plate5 M16	7.1903	0.0038	451.1875	59.2083	4.7891	0.0015	411.8542	92.7500

Plate5 M17					0.0100	no reps.	-29.5000	187.5000
Plate5 M18	4.2547	no reps.	338.2500	79.5000	3.1569	0.1237	279.8333	102.3333
Plate5 M19	10.4876	0.3284	230.1250	23.7500	2.4617	0.0011	753.6667	305.7292
Plate5 M2	11.0263	0.3264	182.7500	16.1250				
Plate5 M20	14.1326	0.0000	2374.4167	168.6667	2.2385	0.0012	942.9791	431.2500
Plate5 M21	1.8654	no reps.	97.0000	52.0000	0.6152	0.7277	73.0000	138.5000
Plate5 M22	18.2375	0.0002	8656.2705	549.0833	2.1455	0.0251	11866.5840	5570.0000
Plate5 M23								
Plate5 M24	9.2094	0.1395	233.8333	30.2500	3.3835	0.1272	145.6250	42.7500
Plate5 M3	3.5011	0.4902	162.6389	49.7500	1.2094	0.8583	245.0000	280.7500
Plate5 M4	6.4870	0.2732	351.1667	48.3333	0.3170	0.1619	62.0833	221.5000
Plate5 M5								
Plate5 M6	132.3183	0.0000	4598.5835	35.8333	61.7823	0.0013	3775.3335	68.8333
Plate5 M7	78.3218	0.0000	1580.2500	21.0000	64.1632	0.0000	1442.3750	22.7083
Plate5 M8	4.0794	0.5372	200.7500	49.0833	2.0649	0.0547	225.0833	105.0833
Plate5 M9	3.9946	0.0083	543.4791	151.3958	1.2316	0.4836	515.5833	440.4583
Plate5 N1					2.0388	no reps.	105.0000	51.5000
Plate5 N10					0.1122	no reps.	17.0000	151.5000
Plate5 N11	2.9273	0.4136	176.4583	58.8750	2.2556	0.0600	355.3333	152.0417
Plate5 N12	5.9450	0.0000	16961.2500	2881.0417	1.7427	0.0072	37687.1250	21388.0410
Plate5 N13	7.3163	0.0004	787.3750	101.3750	3.7188	0.0002	384.0417	96.0833
Plate5 N14	1.1061	0.8747	1145.5416	837.0000	1.5043	0.0939	1231.8333	825.3334
Plate5 N15								
Plate5 N16	6.6296	no reps.	179.0000	27.0000				
Plate5 N17	2.0216	no reps.	117.2500	58.0000	1.1724	0.8090	115.1250	109.4583
Plate5 N18	8.3830	0.1499	297.5000	35.5000	3.8920	0.0915	213.0000	53.6667
Plate5 N19	2.4773	0.3663	212.9167	89.3333	1.0088	0.9813	225.1042	194.8333
Plate5 N2	4.0580	0.0055	628.3959	155.3958	3.2577	0.0008	1613.5417	470.2500
Plate5 N20	1.6770	0.9077	92.6667	104.3333	0.9101	0.9292	301.6667	444.7500
Plate5 N21	1.6319	0.7640	146.7917	88.7083	1.8681	0.1630	189.1667	99.7083
Plate5 N22								
Plate5 N23								
Plate5 N24	3.9428	0.0002	856.5833	245.4583	2.0061	0.0004	1097.5000	553.0416
Plate5 N3	17.6769	0.0000	1001.2083	57.0417	3.5531	0.0539	543.6875	173.7917
Plate5 N4	11.3884	0.0002	453.5833	40.3750	13.1704	0.0066	737.1666	57.5417
Plate5 N5								
Plate5 N6	5.1188	0.6537	453.0000	52.7500	8.9119	0.1515	165.5000	18.2500
Plate5 N7	4.6363	0.0024	1002.3333	250.3542	3.2114	0.0001	968.8750	313.2083
Plate5 N8	5.6221	0.3565	532.5833	79.5833	3.9415	0.0301	622.9375	174.0625
Plate5 N9	1.2246	0.7255	495.2083	414.7083	3.0808	0.0001	724.3750	259.9792
Plate5 O1	8.7944	0.3502	142.0625	17.0000				
Plate5 O10	6.8455	0.0114	309.0000	45.4583	2.4569	0.6088	111.2500	72.6667
Plate5 O11					2.6129	no reps.	6622.5000	2534.5000
Plate5 O12								
Plate5 O13	56.5102	0.0001	3812.5000	69.7500	2.7084	0.0927	2023.6666	798.6666
Plate5 O14	4.7089	0.1374	245.0556	54.7222	2.6125	0.0384	168.5000	62.8333
Plate5 O15	24.7976	0.0000	1164.7709	48.2292	6.4799	0.0000	453.1458	74.1667
Plate5 O16	1.6629	no reps.	148.0000	89.0000				
Plate5 O17	15.4678	0.0000	732.6666	47.6250	5.2658	0.0003	427.9583	70.1250
Plate5 O18	8.7924	0.0048	309.1042	38.3750	10.8321	0.0000	770.2500	72.4167
Plate5 O19	6.5928	0.1702	200.8750	32.2917	8.7088	0.0000	512.0000	62.0417
Plate5 O2	12.7287	0.0000	590.2500	45.5417	1.6930	0.0086	1199.2916	697.3750
Plate5 O20	14.1172	0.0001	2472.5000	175.1667	2.1420	0.0014	1110.0417	509.8750
Plate5 O21	6.6156	0.0427	392.0417	51.8125	1.5668	0.5050	236.7083	141.6667
Plate5 O22	4.1980	0.3066	200.6667	43.8333	2.9682	0.0361	179.8958	70.2083
Plate5 O23					1.9034	0.5167	2453.0000	614.5000
Plate5 O24					7.8846	no reps.	102.5000	13.0000
Plate5 O3	1.9139	0.4131	370.4167	212.6250	0.3398	0.0349	190.4167	552.0000
Plate5 O4	1.9620	0.8430	225.3333	89.5000	0.2748	0.0994	111.9792	398.8958
Plate5 O5	3.7549	0.0334	433.9792	119.2083	0.1400	0.0051	148.1250	928.7708
Plate5 O6	6.0478	0.0033	425.2917	70.0833	1.0771	0.8746	549.5417	481.0417
Plate5 O7	14.5610	0.0062	351.0417	23.9167	1.4857	0.0671	730.1667	471.7083
Plate5 O8	6.6819	0.0034	462.4792	68.0625	2.0233	0.4675	130.0000	58.0000

Plate5 O9	5.3584	0.3543	185.8125	36.1250	1.7859	0.2500	143.1042	74.2708
Plate5 P1	29.1331	0.0000	1729.1250	60.8333	3.1294	0.0045	988.2709	285.6042
Plate5 P10	3.5097	0.5994	176.7500	65.5000	3.4821	0.1721	173.7500	46.5000
Plate5 P11					9.8571	no reps.	138.0000	14.0000
Plate5 P12	37.8247	0.0000	1618.5000	42.8333	1.8330	0.3317	751.0833	344.9583
Plate5 P13	9.2595	0.0000	1524.7083	180.1667	1.4271	0.4095	385.5833	230.2917
Plate5 P14	11.0239	0.0000	1681.3125	160.2292	2.9600	0.0006	2526.6875	1180.3125
Plate5 P15	3.4631	0.4560	263.9583	75.3125	2.3119	0.0908	356.9375	202.5625
Plate5 P16	7.5146	0.0069	2605.5835	331.7500	2.9224	0.0115	1234.7916	425.1875
Plate5 P17	2.9610	0.6472	203.0000	62.1250	4.8507	0.0042	272.2083	52.0417
Plate5 P18	21.6845	0.0000	1619.5416	86.0417	1.1011	0.6451	2639.7915	2463.2500
Plate5 P19	30.6417	0.0000	2227.2500	79.5000	0.9926	0.9721	3636.0208	3528.3750
Plate5 P2	1.4180	0.9267	182.0000	100.3333	1.8131	0.3274	395.8750	341.3125
Plate5 P20	1.4972	no reps.	132.5000	88.5000	1.1370	no reps.	166.0000	146.0000
Plate5 P21								
Plate5 P22	5.4458	no reps.	226.0000	41.5000				
Plate5 P23								
Plate5 P24								
Plate5 P3	6.7446	no reps.	620.5000	92.0000	0.0127	no reps.	29.0000	2283.5000
Plate5 P4								
Plate5 P5								
Plate5 P6								
Plate5 P7					9.2308	no reps.	120.0000	13.0000
Plate5 P8	9.3406	0.0015	487.5833	45.5833	3.7834	0.0646	253.1667	76.3889
Plate5 P9	8.6677	0.0004	575.7084	65.0000	2.6783	0.0081	266.7917	107.0833
PLC1	2.4951	0.0018	1430.7917	588.7916	1.6804	0.0032	1531.9167	922.5417
PLD(1)	2.4974	0.0009	8654.7920	3411.5000	1.3135	0.1513	7376.7085	5609.9585
PLD(3)	2.6974	0.0136	1510.9584	629.7084	1.0205	0.9563	1328.2083	1138.4166
PR21	0.3227	0.5789	272.3542	778.2500	0.9177	0.8648	255.6250	300.7292
RD21a	3.9395	0.0000	2451.2085	647.8750	2.4001	0.0093	1448.1666	493.3125
RNS1	7.5360	0.0000	12726.2500	1773.1250	3.7700	0.0003	5171.0835	1360.5416
RNS2	1.6249	0.6028	729.6667	425.3542	1.2028	0.6853	597.4791	463.5833
SAG12	66.1236	0.0004	8822.4710	164.4250	34.3507	0.0000	5831.3750	184.5875
SAG12h	593.2599	0.0002	18030.4550	34.6308	35.2898	0.1762	8711.5900	308.2855
SAG12n	498.0351	0.0003	29530.0400	67.2313	268.9586	0.0026	18554.5760	80.1319
SAG13	2.8373	0.0002	1890.6250	660.1666	1.8036	0.0011	2036.3124	1118.5833
SAG14	6.8509	0.0193	986.3750	186.3333	0.7656	0.4015	743.5625	934.9792
SAG15	3.7384	0.0002	1210.1666	350.7500	1.4300	0.0767	1999.7291	1380.2916
SAG18	1.1900	0.7178	1152.1666	893.2500	1.4388	0.0454	1546.2084	1060.4166
SAG21	6.8071	0.0000	7896.3745	1263.1666	1.1567	0.4850	6407.2500	5442.3330
SAG27	1.4835	0.5027	452.2917	308.9583	2.5029	0.0217	452.9375	190.0833
Sen4	4.5494	0.0001	1282.6250	284.5833	1.9396	0.0691	545.1250	287.5625
Sen5	0.7072	0.5993	4141.3330	5902.6250	1.1072	0.6487	1771.3334	1649.0000
SRG3	2.3034	0.0234	877.3333	362.0417	2.1795	0.0043	593.9375	268.3542
STP4	2.7650	0.1962	290.1667	110.0000	1.2044	0.7507	326.1667	272.8333
TFR	3.3359	0.0628	346.5417	105.2500	2.7636	0.0098	342.5000	124.5000
TUBh	0.1735	0.1487	370.8828	2169.6797	1.6771	0.5882	354.2031	412.5000
TUBn	0.1999	0.1055	798.2990	3965.1543	1.2018	0.7337	750.5313	920.1173
UBIQ	1.6340	0.1372	6797.4580	4050.1665	1.2618	0.2578	7239.6665	5831.1665

Appendix 6

A6 Raw data for RT-PCR

The following tables summarise the raw data obtained for each of the replicates of the RT-PCR experiments.

A6.1 Raw data for *puv* primers

stage	rep 1	bkgnd	rep 2	bkgnd	rep 3	bkgnd	-RT	bkgnd
-2	4311.37	58.71	3615.42	56.62	4863.46	64.01	552.45	46.31
-1	4054.57	60.40	3927.10	58.74	4452.09	62.66	1090.50	45.76
0	5054.90	62.02	4714.74	59.85	5758.55	62.30	427.85	45.79
1	3880.97	62.21	3556.12	60.22	3983.14	61.16	677.40	45.87
2	2730.73	61.77	2732.94	60.14	3278.55	60.63	732.87	46.63
3	3078.90	60.48	2405.29	58.78	2879.61	58.53	410.40	45.87
4	46.55	58.43	78.46	57.10	52.63	56.79	1370.27	46.66
5	0.00	59.18	0.00	58.01	883.61	57.29	1472.14	48.58

Table A6.1.1 Raw data for *puv* band intensity and background for three replicates of each developmental stage, as well as for a –RT control for each developmental stage, as obtained from GeneSnap.

	(Spot - bkgnd) - (-RT - bkgnd)			As % of maximum					
stage	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3		Mean	SD
-2	3746.52	3052.67	4293.32	81.25	71.44	80.79		77.83	5.54
-1	2949.43	2823.62	3344.70	63.97	66.08	62.94		64.33	1.60
0	4610.82	4272.83	5314.19	100.00	100.00	100.00		100.00	0.00
1	3187.22	2864.37	3290.45	69.12	67.04	61.92		66.03	3.71
2	1982.72	1986.56	2531.68	43.00	46.49	47.64		45.71	2.42
3	2653.88	1981.98	2456.55	57.56	46.39	46.23		50.06	6.50
4	0.00	21.37	0.00	0.00	0.50	0.00		0.17	0.29
5	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00

Table A6.1.2 Derived data for *puv* primers. The background values were subtracted from the band intensity values and the background corrected –RT control values were subtracted from the background corrected band values. The intensity of each band as a percentage of the brightest band in the series was calculated, so that values for the same clone could be compared between gels and between images. The mean and standard deviation were calculated to be plotted and presented in section 7.3.

A6.2 Raw data for *SAG12* primers

stage	rep 1	bkgnd	-RT	bkgnd
-2	1831.58	65.46	461.47	73.69
-1	3867.36	68.12	416.86	70.75
0	3060.88	70.90	201.60	69.48
1	13812.60	79.91	220.94	69.41
2	15496.40	91.09	235.73	69.43
3	16934.90	97.04	292.93	68.45
4	15881.30	96.34	255.10	68.19
5	16295.00	89.51	161.31	69.01

Table A6.2.1 Raw data for *SAG12* (32 cycles) band intensity and background for one replicate of each developmental stage, as well as for a –RT control for each developmental stage, as obtained from GeneSnap.

stage	(Spot - bkgnd) - (-RT - bkgnd)	As % of maximum
-2	1378.34	8.30
-1	3453.12	20.79
0	2857.86	17.20
1	13581.17	81.75
2	15239.01	91.73
3	16613.39	100.00
4	15598.05	93.89
5	16113.19	96.99

Table A6.2.2 Derived data for *SAG12* primers (32 cycles). The background values were subtracted from the band intensity values and the background corrected –RT control values were subtracted from the background corrected band values. The intensity of each band as a percentage of the brightest band in the series was calculated, so that values for the same clone could be compared between gels and between images.

stage	rep 1	bkgnd	rep 2	bkgnd	rep 3	bkgnd	-RT 1	bkgnd	-RT 2&3	bkgnd
-2	91.73	41.91	9.62	57.20	71.46	44.42	84.06	44.76	88.61	61.57
-1	140.05	42.59	171.75	58.01	106.54	44.61	115.66	44.73	123.70	61.77
0	91.24	43.67	107.30	59.77	79.12	44.30	67.31	44.74	96.07	61.24
1	113.67	44.40	131.88	61.44	153.61	43.93	95.30	44.72	134.95	61.33
2	2154.14	46.61	4270.24	64.71	2929.95	43.86	101.52	45.09	122.25	61.50
3	3371.74	48.07	6753.38	66.80	5037.07	44.76	121.85	44.36	180.53	61.16
4	1850.72	46.79	3593.69	64.29	3179.29	45.28	112.11	43.72	135.50	59.75
5	510.64	44.84	913.26	61.48	783.27	44.75	51.62	43.63	66.61	59.52

Table A6.2.3 Raw data for *SAG12* (27 cycles) band intensity and background for three replicates of each developmental stage, as well as for two –RT controls for each developmental stage, as obtained from GeneSnap.

	(Spot - bkgnd) - (-RT - bkgnd)			As % of maximum					
stage	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3		Mean	SD
-2	10.53	0.00	0.00	0.32	0.00	0.00		0.11	0.19
-1	26.53	192.71	0.00	0.82	2.82	0.00		1.21	1.45
0	25.00	98.85	0.00	0.77	1.45	0.00		0.74	0.72
1	18.69	160.67	36.06	0.58	2.35	0.74		1.22	0.98
2	2051.09	4282.70	2825.33	63.18	62.77	57.98		61.31	2.89
3	3246.18	6822.75	4872.94	100.00	100.00	100.00		100.00	0.00
4	1735.55	3621.18	3058.26	53.46	53.08	62.76		56.43	5.48
5	457.80	874.77	731.43	14.10	12.82	15.01		13.98	1.10

Table A6.2.4 Derived data for *SAG12* primers (27 cycles). The background values were subtracted from the band intensity values and the background corrected –RT control values were subtracted from the background corrected band values. The intensity of each band as a percentage of the brightest band in the series was calculated, so that values for the same clone could be compared between gels and between images. The mean and standard deviation were calculated to be plotted and presented in section 7.3.

A6.3 Raw data for *LP H9* primers

stage	rep 1	bkgnd	-RT	bkgnd
-2	15788.90	76.52	2679.52	73.24
-1	17417.70	92.81	8996.29	75.14
0	16003.20	94.37	5220.50	73.38
1	16218.00	94.30	5980.25	72.50
2	16190.20	96.62	5699.36	71.28
3	15202.80	94.37	2924.18	69.63
4	15962.30	92.12	5315.54	70.42
5	15597.30	84.37	7899.20	69.10

Table A6.3.1 Raw data for *LP H9* (32 cycles) band intensity and background for one replicate of each developmental stage, as well as for a –RT control for each developmental stage, as obtained from GeneSnap.

stage	(Spot - bkgnd) - (-RT - bkgnd)	As % of maximum
-2	13106.09	100.00
-1	8403.74	64.12
0	10761.71	82.11
1	10215.94	77.95
2	10465.49	79.85
3	12253.88	93.50
4	10625.06	81.07
5	7682.83	58.62

Table A6.3.2 Derived data for *LP H9* primers (32 cycles). The background values were subtracted from the band intensity values and the background corrected –RT control values were subtracted from the background corrected band values. The intensity of each band as a percentage of the brightest band in the series was calculated, so that values for the same clone could be compared between gels and between images.

stage	rep 1	bkgnd	rep 2	bkgnd	rep 3	bkgnd	-RT 1	bkgnd	-RT 2&3	bkgnd
-2	3669.69	46.21	7755.82	63.28	6123.59	57.29	215.94	45.93	497.03	65.32
-1	4821.14	50.57	10125.30	71.83	9091.05	56.79	526.59	46.87	1457.00	67.17
0	2361.82	51.14	4858.23	73.77	3536.67	58.53	193.12	46.65	400.78	66.29
1	4854.25	51.42	10126.00	75.03	8281.68	61.16	215.76	46.31	450.45	65.55
2	3234.81	51.26	6677.54	73.73	5965.99	60.63	242.82	46.30	553.34	65.53
3	2755.00	50.38	5593.69	71.96	3832.52	62.66	249.00	46.11	433.45	65.03
4	1829.81	49.05	3649.90	69.35	3389.25	62.30	324.96	45.93	606.69	64.52
5	960.75	47.22	2024.92	65.52	1703.43	64.01	385.72	45.65	685.29	63.71

Table A6.3.3 Raw data for *LP H9* (27 cycles) band intensity and background for three replicates of each developmental stage, as well as for two –RT controls for each developmental stage, as obtained from GeneSnap.

	(Spot - bkgnd) - (-RT - bkgnd)			As % of maximum					
stage	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3		Mean	SD
-2	3453.47	7260.83	5634.59	74.53	75.12	71.91		73.85	1.71
-1	4290.85	8663.64	7644.43	92.61	89.63	97.56		93.27	4.01
0	2164.22	4449.97	3143.65	46.71	46.04	40.12		44.29	3.63
1	4633.38	9666.07	7835.62	100.00	100.00	100.00		100.00	0.00
2	2987.03	6116.00	5417.55	64.47	63.27	69.14		65.63	3.10
3	2501.73	5153.31	3401.44	53.99	53.31	43.41		50.24	5.92
4	1501.73	3038.38	2784.78	32.41	31.43	35.54		33.13	2.15
5	573.46	1337.83	1017.85	12.38	13.84	12.99		13.07	0.74

Table A6.3.4 Derived data for *LP H9* primers (27 cycles). The background values were subtracted from the band intensity values and the background corrected –RT control values were subtracted from the background corrected band values. The intensity of each band as a percentage of the brightest band in the series was calculated, so that values for the same clone could be compared between gels and between images. The mean and standard deviation were calculated to be plotted and presented in section 7.3.

A6.4 Raw data for *P1 F4* primers

stage	rep 1	bkgnd	rep 2	bkgnd	rep 3	bkgnd	-RT	bkgnd
-2	1264.52	66.11	950.05	41.91	1094.70	63.28	634.59	75.94
-1	3538.48	72.78	2378.14	42.59	2247.98	65.52	1050.91	76.08
0	8381.86	80.43	4655.50	44.84	5403.29	69.35	241.65	74.74
1	13186.50	86.98	7677.16	44.40	10598.76	71.83	1124.65	73.96
2	11220.10	88.96	6642.54	46.79	10177.15	71.96	1209.50	74.42
3	12977.80	93.39	7324.41	46.61	9792.37	73.77	601.21	73.86
4	13422.50	95.32	7764.59	48.07	10567.73	75.03	1035.51	74.33
5	12900.40	85.03	8385.02	43.67	10120.71	73.73	3026.90	72.33

Table A6.4.1 Raw data for *P1 F4* band intensity and background for three replicates of each developmental stage, as well as for a –RT control for each developmental stage, as obtained from GeneSnap.

	(Spot - bkgnd) - (-RT - bkgnd)			As % of maximum					
stage	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3		Mean	SD
-2	639.76	349.49	472.76	5.17	4.96	3.12		4.42	1.13
-1	2490.87	1360.72	1207.64	20.14	12.67	31.52		21.44	9.49
0	8134.52	4443.75	5167.04	65.78	54.21	71.22		63.74	8.69
1	12048.83	6582.07	9476.24	97.44	99.42	94.36		97.07	2.55
2	9996.06	5460.67	8970.11	80.84	94.11	87.32		87.42	6.64
3	12357.06	6750.45	9191.24	99.93	96.43	97.71		98.02	1.77
4	12366.00	6755.33	9531.52	100.00	100.00	100.00		100.00	0.00
5	9860.79	5386.78	7092.40	79.74	74.41	72.58		75.58	3.72

Table A6.4.2 Derived data for *P1 F4* primers. The background values were subtracted from the band intensity values and the background corrected –RT control values were subtracted from the background corrected band values. The intensity of each band as a percentage of the brightest band in the series was calculated, so that values for the same clone could be compared between gels and between images. The mean and standard deviation were calculated to be plotted and presented in section 7.3.

A6.5 Raw data for tubulin primers

stage	rep 1	bkgnd	rep 2	bkgnd	rep 3	bkgnd	-RT 2	bkgnd	-RT 3	bkgnd
-2	2776.71	55.84	6651.16	44.48	17116.50	71.31	650.41	44.76	1988.47	73.15
-1	4261.47	57.60	8592.50	48.99	18623.70	79.83	2265.66	45.28	7672.92	74.59
0	6693.68	57.91	9365.28	49.72	19094.60	81.80	606.73	44.42	1780.03	73.74
1	3838.84	56.92	6978.79	48.22	16907.90	78.92	1366.03	44.61	4313.53	73.65
2	1757.46	55.84	4728.86	46.71	14074.90	76.51	2049.72	44.75	5950.38	74.06
3	1714.80	55.36	3736.90	45.60	11246.90	74.33	869.81	44.30	2670.06	71.94
4	495.72	53.64	309.98	43.64	815.53	71.47	2121.00	43.93	6206.42	71.17
5	575.65	52.78	7.21	43.53	144.72	70.57	2962.11	43.86	8739.93	71.04

Table A6.5.1 Raw data for tubulin band intensity and background for three replicates of each developmental stage, as well as for a –RT control for each developmental stage, as obtained from GeneSnap.

	(Spot - bkgnd) - (-RT - bkgnd)			As % of maximum					
stage	rep 1	rep 2	rep 3	rep 1	rep 2	rep 3		Mean	SD
-2	2720.87	6001.03	15129.87	41.00	68.56	87.42		65.66	23.35
-1	4203.87	6323.13	10945.55	63.35	72.24	63.25		66.28	5.16
0	6635.77	8753.25	17306.51	100.00	100.00	100.00		100.00	0.00
1	3781.92	5609.16	12589.10	56.99	64.08	72.74		64.61	7.89
2	1701.62	2677.18	8122.07	25.64	30.58	46.93		34.39	11.14
3	1659.44	2865.79	8574.46	25.01	32.74	49.54		35.76	12.55
4	442.08	0.00	0.00	6.66	0.00	0.00		2.22	3.85
5	522.87	0.00	0.00	7.88	0.00	0.00		2.63	4.55

Table A6.5.2 Derived data for tubulin primers. The background values were subtracted from the band intensity values and the background corrected –RT control values were subtracted from the background corrected band values. The intensity of each band as a percentage of the brightest band in the series was calculated, so that values for the same clone could be compared between gels and between images. The mean and standard deviation were calculated to be plotted and presented in section 7.3.