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An evaluation of the effects of over-production of ABA on whole plant water use, growth and productivity

by

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SUMMARY

Predicted climate change and increasing global populations suggest that water will become an increasingly scarce and valuable commodity. Breeding plants which produce equivalent yields with reduced water input is therefore vital to sustain and increase crop production in the future. The phytohormone, abscisic acid (ABA), is important in controlling plant responses to water stress. It may be possible to improve water use efficiency (WUE) by genetically modifying tomato and other species to maintain elevated levels of ABA under optimal (unstressed) conditions, thereby manipulating an intrinsic signalling mechanism which is known to mediate drought-induced alterations of stomatal behaviour.

ABA is synthesised *via* the oxidative cleavage of C_{40} epoxycarotenoid precursors, a reaction catalysed by the key enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED). Pure breeding transgenic tomato lines constitutively over-expressing *LeNCED1*, known as sp5 and sp12, both have elevated ABA concentrations, which reduce stomatal conductance under optimal (unstressed) growth conditions, thus conserving soil water during periods when corresponding wild type (WT) control plants were inefficient in its use. Under well-watered conditions, whole plant transpiration efficiency (TE_p) was significantly greater in both 'high ABA' lines (sp12 and sp5) than in WT plants.

The over-expression of *LeNCED1* was combined with over-expression of a gene (*LeBCH2*) encoding β-carotene hydroxylase (BCH), an enzyme acting earlier in the ABA biosynthetic pathway. These 'double transgene' lines (G28 and G29) consistently exhibited further improvements in ABA accumulation and TE_p relative to corresponding 'single transgene' parental lines. Lines G28 and G29 respectively exhibited 37 and 54 % improvements in TE_p relative to WT controls.

When evaluated as a potential 'high ABA' rootstock, it was found that the 'double transgene' G29 line did not provide a sufficiently strong root-sourced signal to affect the stomatal behaviour of scions. To increase ABA biosynthesis in the roots further, a programme designed to combine the over-expression of three ABA biosynthetic genes (*LeNCED1*; *LeBCH2*; *LePSY1*) was initiated with the objective of obtaining a rootstock which produced sufficient ABA to affect stomatal behaviour when grafted onto WT scions. Unfortunately, there was insufficient time to complete this work by the end of the period reported in this thesis, although the programme is ongoing.

ABBREVIATIONS

A	Assimilation rate
AAO	Abscisic aldehyde oxidase
ABA	Abscisic acid
ANOVA	Analysis of variance
AOG	ABA glucosyltransferase
AS-AO	ABA specific aldehyde oxidase
BCH	Beta-carotene hydroxylase (also known as CrtR-B)
bn	Billion
CaMV	Cauliflower mosaic virus
CAPRI	Controlled alternate partial root-zone drying
CCD	Carotenoid cleavage dioxygenase
cDNA	Complementary DNA
cf.	confer (consult)
CIRAS	Compound infra-red analyser system
cm	Centimeters
cv.	Cultivar(s)
d	Day
DMAPP	Dimethylallyl pyroposhophate
DNA	Deoxyribonucelic acid
DPA	Dihydrophaseic acid
DW	Dry weight
DXP	1-deoxy-D-xylulose-5-phosphate
DXR	1-deoxy-D-xylulose-5-phosphate reductoisomerase
DXS	1-deoxy-D-xylulose-5-phosphate synthase
et al.	et alia (and others)
e.g.	exempli gratia (for example)
FPP	Farnesyl pyrophosphate (farnesyl diphosphate)
FW	Fresh weight
g	Gram
GA	Gibberellic acid
GGPPS	Geranylgeranyl pyrophosphate synthase
GCR	G protein-coupled receptor
GPPS	Geranyl pyrophosphate synthase
g_s	Stomatal conductance
h	Hour
ha	Hectare
HCL	Hydrochloric acid
HPLC (PDA)	High performance liquid chromatography (photo diode array)
IAA	Indole-3-acetic acid
i.e.	id est (Latin, that is)
in prep.	In preparation
IPI	Isopentenyl pyrophosphate isomerase
IPP	Isopentenyl pyrophosphate (isopentenyl diphosphate)
Kg	Kilograms
l	Litre
LBA4404	Strain of *Agrobacterium tumefaciens*
LCY-B/E	Lycopene beta/epsilon-cyclase
LEA	Late embyrogenesis abundant
LHC	Light-harvesting complex

l.s.d.	Least significant diference (5% level)
mRNA	Messenger ribonucleic acid
MVA	mevalonic acid
μg	Micrograms
μl	Microlitres
m	Metre
mm	Millimetres
mg	Milligrams
ml	Millilitres
Mill.	Miller
min	Minute
Nz	Norflurazon
NCED	9-*cis*-epoxycarotenoid dioxygenase
NCEI	9-*cis*-epoxycarotenoid-forming isomerase
NPQ	Non-photochemical quenching
nptII	Neomycin phosphotransferase II
not	Notabilis (tomato ABA-deficient mutant allele)
NPK	Nitrogen/ phosphorus/ potassium
NSY	Neoxanthin synthase
ORF	Open reading frame
%	Percent
±	Plus or minus
P	Probability
PA	Phaseic acid
PAR	Photosynthetically active radiation
PCR	Polymerase chain reaction
PDS	Phytoene desaturase
pers. comm.	personal communication
pH	Hydrogen potential
PRD	Partial root-zone drying
PSII	Photosystem II
PSY	Phytoene synthase
Q-PCR	Quantitative real-time polymerase chain reaction
RDI	Regulated Deficit irrigation
RIA	Radio-immuno assay
RNA	Ribonucleic acid
S.E.	Standard error of the mean
SDW	Sterile distilled water
SSU	Small subunit
T_1	Generation resulting from selfing of T_0-generation individuals
T_2	Generation resulting from selfing of T_1-generation individuals
Tm2a	Tomato wildtype containing TMV resistance transgene
TMV	Tobacco mosaic virus
UK	United Kingdom
USA	United States of America
UV	Ultra-violet
VDE	Violaxanthin de-epoxidase
WT	Wild type
WUE	Water use efficiency
ZEP	Zeaxanthin epoxidase

1 INTRODUCTION

1.1 Water Use in Agriculture

Water deficit may be defined as occurring when the water content of a cell or tissue falls below that exhibited at its most hydrated state (Taiz and Zeiger, 2006). Plant water deficits may be induced by decreases in soil water availability which develop in the long-term or occur in response to short periods of drought. Plants may experience severe disruption of their physiological and metabolic processes in response to drought, and similar responses may be induced by other stress factors including extremes of temperature, light and nutrient supply (Verslues *et al*., 2006).

Global mean annual temperature at the end of the twentieth century was 0.7 ^{o}C higher than that recorded at the end of the nineteenth century, indicating a trend of climate change (Kalra, 2007). This, coupled with the decreased levels of precipitation that are expected to occur in some parts of the world as a result of global warming (Mannion, 1995), may result in increasingly frequent and widespread drought. Drought is currently one of the greatest limitations to the expansion of crop production from present-day agricultural areas (Chaves and Oliveira, 2004), and economically is the most damaging stress for crop production (Kramer, 1980; Boyer, 1982). The timing, duration and intensity of stress episodes are key in determining the effects of drought, and the ability of plants to cope with water stress differs greatly, with various species exhibiting a diverse range of drought resistance strategies (Schulze, 1986).

Of the world's available fresh water resource, approximately 70% is consumed by irrigated agriculture, a level that will be unsustainable in the future (Condon *et al*., 2004). The world's population is expected to increase by a further 2 bn people over the next three decades, increasing demands imposed on the world's water resources to meet domestic, industrial and environmental needs. There is currently increasing competition for water, with different regions and countries challenging for finite water resources (Ansink and Ruijs, 2008). Around 10% of agricultural irrigation is sourced from aquifers, often leading to underground water tables being unsustainably

exploited (Somerville and Briscoe, 2001). Future increases in global population will also be accompanied by rising demand for food, with the result that greater crop production will need to be achieved using smaller irrigation resources.

As scarcity of water imposes huge restrictions on crop yield great emphasis should therefore be placed on the development of methods which improve the water use efficiency (WUE) of both rain-fed and irrigated crops (Anderson *et al*., 1999; Hamdy *et al.*, 2003; Parry *et al.*, 2005). At the whole plant level, WUE can be defined as the ratio of the quantity of biomass produced to the quantity of water transpired (Jones, 2004).

Three key processes can be exploited in breeding programmes to improve crop yield in water limited environments (Condon *et al*., 2004). The first is the acquisition of more of the available water by crops, rather than allowing this valuable resource to be wasted by evaporation from the soil surface or drainage beyond the root zone. Extending rooting depth might increase the amount of soil water available for uptake by the roots, while inclusion of species with deeper roots in cropping rotations may provide 'biopores' for use by subsequent crops (Hamblin and Hamblin, 1985).

A second approach is to improve the WUE of arable crops through direct selection of genotypes with increased WUE at the individual leaf level (Condon *et al*., 2004); this has been a breeding objective for many years (Fisher, 1981). Condon *et al*. (2004) suggested two possible routes to achieve this: the first involves a reduction in the leaf to air vapour pressure gradient which drives transpiration, e.g. by growing crops in cooler, more humid environments, while the second involves increasing the gradient driving the diffusion of CO_2 into the stomatal cavities which can be achieved by decreasing stomatal conductance or by increasing the rate of uptake of CO_2 by photosynthesis. The development of isotopic carbon discrimination techniques to measure WUE instantaneously (Hall *et al*., 1994) dispelled the previous belief that there was no genetic variation within individual species (Fischer, 1981). Species with the C_4 pathway of photosynthesis have a higher WUE than C_3 species (Taiz and Zeiger, 2006), and measurements of carbon isotope discrimination in C_3 species (Farquar *et al*., 1989) provide a powerful tool for breeding plants with improved WUE (Condon *et al*., 2004).

A third approach is to partition more of the achieved biomass to the harvested product, and this again represents a long-standing breeding objective. The green revolution, involving new semi-dwarf cultivars of crops such as wheat and rice with increased harvest indices (HI), has resulted in the production of varieties suited to high inputs of fertilisers and irrigation. Although supplemental irrigation may benefit yields and WUE in water-limited environments (Oweis *et al*., 2000; Turner, 2004), many regions of the world rely on rain-fed farming for food production. Irrigation can sometimes cause problems of salinisation, disrupting plant water status by inflicting conditions of salt stress (Verslues *et al*., 2006). Passioura (1977) suggested a framework for the consideration of factors affecting yield in water-limited environments, where the yield is the product of total water use, WUE and harvest index. Total water use can be reduced by several simple actions, such as controlling the presence of weeds to increase the quantity of water available for transpiration by crops.

It is well known that a major effect of limited soil water availability is reduced carbon fixation resulting from decreases in stomatal aperture. As water use before anthesis is important in determining the number of grains set in wheat, water stress may also directly affect grain yield (French and Schultz, 1984). Agriculture depends to a large extent on the success of plant reproduction, which is obviously necessary to sustain the next generation. However, most importantly, reproductive products like grain, fruit and nuts provide the bulk of the world's food supply (Boyer and Westgate, 2004). In the USA, where over 75% of the arable land is devoted to crops with valuable reproductive structures, Boyer (1982) showed that average yield was only 18% of the maximum recorded. These crops are therefore not realising their full yield potential, and the availability of water during the growing season is thought to be a major contributor to this problem (Boyer, 1982).

1.1.1 Development of Novel Irrigation techniques

Irrigation scheduling has traditionally aimed to achieve an optimal water supply for maximal crop productivity (Jones, 2004). The increasing cost and scarcity of irrigation resources has more recently led to the development of techniques designed to minimise water use. Simple adjustments to irrigation methods, for

example, the application of water *via* trickle irrigation, have allowed more accurate application of irrigation requirements and this increases irrigation efficiency: the proportion of irrigation water that is delivered to the root zone, or that is transpired by the crop. However, more novel methods of irrigation have been used in attempts to meet the more exacting requirements of increasing plant WUE, whilst maintaining the quantity and quality of crop production.

Plants respond to changes in their immediate environment and perceive and adapt to soil drying before water deficits develop in the shoots (Richards *et al*., 1991). This may involve modification of growth and functioning, sometimes to the detriment of crop yield (Boyle *et al*., 1991). The growth, development and yield of crops are highly sensitive to reduced soil water availability (Boyer, 1982). Mild water deficits cause partial closing of stomata (Davies *et al*., 1978; Turner, 1997) and this increases WUE because of the non-linear relationship between stomatal aperture and rate of carbon assimilation (Section 5.1.2.2). It is well known that plants grown on land subject to periodic soil drying exhibit a higher WUE (Bacon, 2004). By exploiting long-distance signalling mechanisms, plants can be manipulated to improve WUE or reduce unnecessary vegetative growth, whilst maintaining fruit production/yield with reduced supply of water (Davies and Hartung, 2004).

1.1.1.1 Regulated Deficit Irrigation

Deliberate withholding of water can be used as a management strategy to manipulate water use, and is often referred to as regulated deficit irrigation (RDI). RDI involves removal of irrigation for specific time periods, or to coincide with specific developmental stages during a crop cycle (Boland *et al*., 1993; Algre *et al*., 1999). This can be utilised in many situations where water is in short supply, or in fruit production where excessive vegetative vigour can restrict fruit development or predispose plants to diseases. It is well known that fruit growth is often favoured over vegetative growth when assimilate/water supplies are limiting (Chalmers *et al*., 1986). The technique can sometimes improve the quality of fruit crops, for example, the size of grape berries (McCarthy, 1997), and has also been shown to improve WUE (Goodwin *et al*., 1992). In other cases, the use of RDI may have negative consequences, such as reductions in fruit yield (Matthews and Anderson, 1998) or fruit quality (Matthews *et al*., 1990). The technique ideally requires constant

monitoring of plant and soil water status, which can cause it to be costly in terms of both time and financial input.

1.1.1.2 Partial Root Drying Irrigation

The possibility of stimulating the response of plants to water deficit in a controlled and sustained way with a view to improving WUE and/or manipulating vegetative vigour has resulted in the development of the novel partial root drying (PRD) irrigation technique. The system is based on pioneering research involving split-root systems, whereby the root system is simultaneously exposed to both wet and dry soil (Blackman and Davies, 1985). One half of the root zone is irrigated whilst the remainder is left to dry, with irrigation being switched regularly from one side of the root system to the other to allow roots in the previously dry soil to experience a period of recovery whilst maintaining the supply of root signals by drying the other half of the root-zone (Loveys *et al*., 2000). PRD irrigation has also been referred to as controlled alternate partial root-zone irrigation (CAPRI) (Kang and Zhang, 2004).

The physiological studies mentioned in the previous paragraph used plants with split root systems to demonstrate that stomatal responses to soil drying may occur in the absence of any change in leaf turgor or water potential (Blackman and Davies, 1985). Another key observation was that the production and expansion of the leaves of apple trees could be restricted by applying a PRD treatment (Gowing *et al*., 1990). Loveys (1991) suggested that it might be possible to exploit long-distance chemical signalling to regulate the vegetative growth of grapevines, while Dry and Loveys (1998) demonstrated that the PRD technique could be used to reduce grapevine vigour with no yield penalty in terms of wine production; WUE was also improved. The reduction in canopy density caused by PRD irrigation improved light penetration to the grape berries, partly by reducing lateral shoot development and thereby improving fruit quality (Dry *et al*., 1996). The precise physiological reasons why PRD did not adversely affect the fruit yield of grapevines is yet to be confirmed, although it has been suggested that the restricted hydraulic linkage between fruit and vegetative plant parts is important (Davies *et al*., 1998). The use of PRD potentially allows the water requirements of plants to be met by supplying just half of the normal water requirement without significantly affecting shoot water status (Dry and Loveys, 1999), although the reported WUE values for plants irrigated by PRD varies

widely between species. Thus, an increase in WUE of over 50% was observed in pepper and potato plants irrigated *via* PRD (Dorji *et al*., 2005; Liu *et al*., 2006), whereas there was no significant increase in the WUE of PRD tomato plants relative to fully irrigated controls (Zegbe-Domínguez *et al*., 2006).

One theory underpinning the use of PRD is that the signals induced by water stress originate from the drying roots, whilst the water supplied from the irrigated part of the root zone prevents the development of severe water deficits. It has been reported that PRD increases xylem sap ABA concentration and pH (Stoll *et al*., 2000), both of which are believed to be potentially important components of the signal generated by roots when they encounter dry soil (*cf.* Section 1.2.3.1). PRD irrigation may reduce vegetative growth and this can be beneficial in optimising the yield of crops such as grapevines which suffer yield losses resulting from excessive vegetative growth, which can be expected to reduce light penetration to the fruit and be accompanied by excessive transpiration, as in raspberry (Grant *et al*., 2004) and cotton (Tang *et al*., 2005).

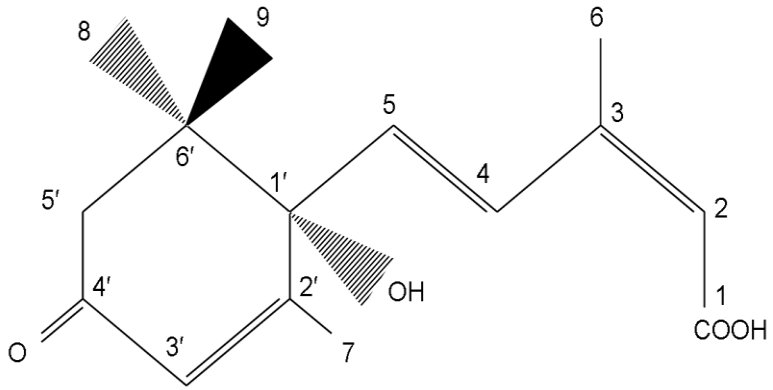
The root-to-shoot ratio of biomass partitioning often increases when plants are water stressed, providing access to a larger soil volume (Munns and Sharp, 1993). The PRD system could initiate the transfer of biomass from vegetative growth of the shoot to increase the density of the root system, potentially improving access of roots to water and essential minerals located deeper within the soil profile (Mingo *et al*., 2004; Kirda *et al*., 2005). The PRD system has advantages over RDI as shoot and fruit water status are more likely to be sustained despite a reduction in irrigation applied (Mingo *et al*., 2003), thus minimising the negative implications for yield observed in plants subjected to RDI (Section 1.1.1.1). The PRD technique is also simpler to implement than RDI as it simply requires adaptation of pre-existing irrigation systems (Loveys *et al*., 2000). Numerous studies of the effects of PRD on yield and water use in a range of crop species indicate that it may be possible to improve plant WUE by manipulating the intrinsic signalling mechanisms that plants normally use to respond to water stress. The phytohormone abscisic acid (ABA) has often been implicated in the control of such responses.

1.2 Abscisic Acid

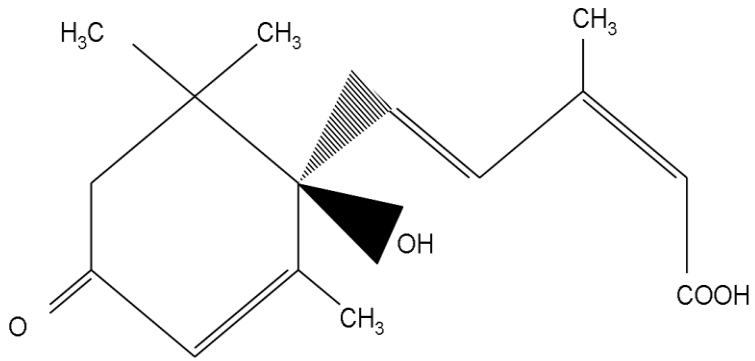
1.2.1 Discovery of ABA

During the early 1960s, researchers were investigating inhibitory compounds that appeared to be associated with seed and bud dormancy. In the UK, Wareing *et al*. (1964) isolated a compound referred to as "dormin" from *Acer pseudoplatanus*; this was relatively abundant in early autumn, when the trees were entering dormancy. A compound isolated in the USA and initially named "abscisin II" was present at high levels prior to the abscission of cotton fruit (Ohkuma *et al*., 1963). These two compounds were revealed to be chemically identical shortly after their discovery (Cornforth *et al*., 1965). The names "dormin" and abscisin II" were subsequently dropped and the substance was renamed abscisic acid, now regarded as one of the major plant growth hormones (Addicott *et al*., 1968).

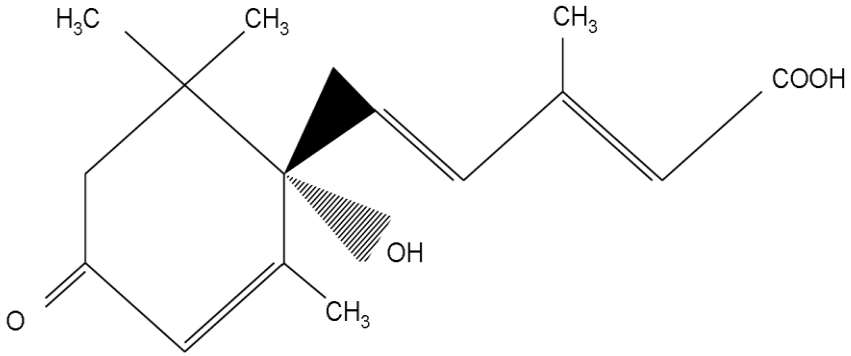
ABA is ubiquitous in all vascular plant tissues (Milborrow, 1967), as well as some non-vascular plants (Addicott and Lyon, 1969; Knight *et al*., 1995). It is a 15 carbon, sesqueterpenoid and naturally occurs as the (+) 2-*cis*, 4-*trans* ABA isomer (Addicott, 1983). The asymmetric carbon in position 1 means that ABA can occur as either of two optical isomers and, when it is chemically synthesised, it occurs as a racemic mixture of these two S (+) and R (-) enantiomers (Fig. 1.1). During the rapid physiological response of stomatal closure, the S (+) enantiomer is the only active form, although both appear to be biologically active in longer term responses, for example, during seed maturation (Taiz and Zeiger, 2006). The naturally occurring S (+) 2-*cis*, 4-*trans* ABA can easily be converted to the biologically inactive S (+) 2-*trans*, 4-*trans* form, as groups around the bond between C2 and C3 in the side chain can readily adopt either the 2–*cis* or 2–*trans* configuration. This conversion from active 2–*cis* to inactive 2–*trans* geometric isomers occurs when ABA is exposed to UV radiation (Mousseron-Canet *et al*., 1968).



(S)-*cis*-ABA (naturally occurring active form)



(R)-*cis*-ABA (inactive in stomatal closure)



(S)-2-*trans*-ABA (inactive, but interconvertible with active [*cis*] form)

Figure 1.1. Chemical structure of the S (+) and R (-) optical isomers of 2-*cis*-ABA, and the S (+)-2-*trans* geometrical isomer of ABA. The numbers in the diagram of (S)-*cis*-ABA identify the carbon atoms in the ring and carboxylic acid sidechain (after Taiz and Zeiger, 2006).

1.2.2 Functions of ABA

1.2.2.1 The role of ABA in seed development and seed dormancy

ABA has been implicated in the regulation of several essential processes during seed development, including accumulation of nutritive reserves, induction/ maintenance of seed dormancy, acquisition of desiccation tolerance and prevention of precocious germination. Control of these physiological processes by ABA depends on active hormone levels, which can be modulated *via* synthesis from precursors and the rates of catabolism or translocation from/to other tissues (Nambara and Marion-Poll, 2003). Reciprocal crosses using wild type (WT) and ABA-deficient mutants revealed that ABA in the seed is mainly maternally-sourced during seed development, whilst induction/maintenance of seed dormancy is more closely correlated with embryonically synthesised ABA (Karssen *et al*., 1983). Maternal tissues can also supply the embryo with ABA precursors, which can potentially be utilised for ABA biosynthesis (Jones and Brenner, 1987). During the period between late seed maturation and germination, ABA catabolism is also important in the regulation of seed ABA content (Okamoto *et al*., 2006).

ABA can accumulate in all seed tissues, either through relocation from the maternal plant *via* the phloem (Hoad, 1995) or synthesis in the seed itself (Karssen, 1983). Maternal ABA is responsible for the first peak of ABA during seed development and seed coat (testa) maturation (Frey *et al*., 2004) and has been reported to help prevent precocious germination in mid-embryogenesis (Raz *et al*., 2001). The fact that ABA levels are high during mid-to-late seed development is consistent with the view that ABA helps to maintain developing embryos in an embryonic state (Taiz and Zeiger, 2006). If an immature embryo is removed from the seed midway through development, it will germinate precociously; however, when exogenous ABA is applied to isolated young embryos, premature germination can be prevented (Rock and Quatrano, 1995). Genetic studies of various different *viviparous* (*Vp*) mutants in grain crops also support the theory that ABA is important for the correct timing of germination. Mutant *Vp* seeds typically do not pass through the normal quiescent and/or dormant developmental state; for example, the *Vp* mutants in maize germinate on the cob whilst still attached to the plant and many of these mutants

have subsequently been found to be either ABA-deficient or ABA-insensitive (Robertson, 1955; Tan *et al*., 1997).

The ABA content of seeds during early embryogenesis is typically low, gradually increasing during the maturation phase, when storage proteins and lipids accumulate and seed volume and dry weight reach their maximum (Hopkins, 1999). This accumulation correlates with the acquisition of desiccation tolerance and ABA is thought to regulate this process (reviewed by Leung and Giraudat, 1998). At this point, gibberellic acid and auxin levels decrease (Kermode, 2005). During the acquisition of desiccation tolerance, seeds lose around 90% of their original water content (Ingram and Bartels, 1996). As this loss of water occurs, ABA promotes the synthesis of proteins that are thought to promote desiccation tolerance, called late-embryogenesis-abundant (LEA) proteins (Delseny *et al*., 2001). The increase in mRNA levels for these genes correlates with high ABA levels in the seed (Rock and Quatrano, 1995).

During seed development, the expression levels of key ABA biosynthetic genes increases. The accumulation of ABA in seed is thought to be regulated by the expression of members of the NCED gene family (Nambara and Marion-Poll, 2005). Over-expression of *LeNCED1* increased ABA concentrations in transgenic tomato seed, substantially increasing dormancy in this normally non-dormant, domesticated species (Thompson *et al*., 2000; see later for a more detailed description of this work), and high levels of *HvNCED1* transcripts have been detected in dormant barley embryos (Millar *et al*., 2006). The expression of *ZEP*, a gene located earlier in the ABA biosynthetic pathway than the *NCED* genes, is also increased during seed development (Audran *et al*., 1998). However, regulation of steps downstream of that catalysed by the *ZEP* enzyme is the major determinant of ABA accumulation in seeds (Frey *et al*., 2006). After the induction of desiccation tolerance, ABA concentration declines rapidly in all seed tissues (Ackerson, 1984), with expression levels of *ZEP* and some of the *NCED* genes also falling at this time (Lefebvre *et al*., 2006). This marks the production of fully developed, mature, dehydrated seed (Taiz and Zeiger, 2006).

Germination is the transition between the resting seed and the time of visible radicle emergence (Bewley and Black 1994). It occurs partly as a result of the chemical weakening of the endosperm by hydrolytic enzymes, the production of which is at least partly controlled by the embryo. Seed dormancy can be an adaptive trait that may sometimes improve the survival of the next generation by optimising the distribution of germination over time (Kermode, 2005), and can be described as a block to germination of an intact viable seed under otherwise favourable conditions (Hilhorst, 1995; Bewley, 1997). Dormant seeds do not germinate and during this phase they are incapable of producing the enzymes that degrade cell walls (Groot *et al*., 1988). Dormancy can be described as either primary or secondary (Finch-Savage and Leubner-Metzger, 2006). The former is induced by ABA during seed maturation on the mother plant (Hilhorst, 1995), while the onset of secondary dormancy is correlated with a second peak of ABA, synthesised within the embryo of fully imbibed seeds (Karssen, 1983; Groot *et al*., 1991; Finkestein *et al*., 2002). ABA-deficient mutants of various species have been used to demonstrate the importance of ABA in the induction of dormancy, with ABA-deficiency consistently being associated with at least some reduction in seed dormancy in mature seed. Seed of the ABA-deficient tomato mutant *sitiens* germinate slightly more quickly than WT seed and have also been reported to exhibit some degree of increased viviparous germination in over-ripe fruit (Groot and Karesson, 1992). In seed of the strongly dormant *Nicotiana plumbaginifolia*, the ABA-deficient (*aba1*) mutation confers rapid germination relative to the highly dormant fresh seed of WT (Leydecker *et al*., 1995).

Inhibitors of carotenoid synthesis have been used to demonstrate that continuous synthesis of endogenous ABA is required for the maintenance of seed dormancy (e.g. Cadman *et al*., 2006). The strong dormancy of freshly harvested *N. plumbagifolia* seed was released when fluridone, a well-known herbicidal inhibitor of carotenoid biosynthesis (and therefore inhibitor of ABA biosynthesis) was applied (Grapin *et al*., 2000). Genetic manipulation of the biosynthetic pathway also demonstrated the role of ABA in seed dormancy. Induced expression of *PuNCED1* in transgenic tobacco delayed germination in imbibed seed (Qin and Zeevaart, 2002). Antisense down-regulation of *NpZEP* reduced ABA biosynthesis, resulting in rapid germination of freshly harvested transgenic seed from this strongly dormant

species (Frey *et al*., 1995). When the same ABA biosynthetic gene (*ZEP*) was over-expressed, this resulted in transgenic seed with a slight additional delay in germination relative to freshly harvested WT seed (Frey *et al*., 1995).

The release of seed dormancy has been hypothesised to involve a shift from a high to low ABA:GA ratio, *via* increased GA synthesis and ABA catabolism/degradation (Ali-Rachedi *et al.*, 2004; Cadman *et al*., 2006). The ABA content of a seed is at its lowest preceding germination and the process commences with the uptake of water by the dry seed i.e. imbibition (Finch-Savage and Leubner-Metzger, 2006). Phaseic acid and dihydrophaseic acid, both major products of ABA catabolism, have been reported to increase in concentration in correlation with the level of imbibition in barley seeds (Jacobsen *et al*., 2002) and *Arabidopsis* (Kushiro *et al*., 2004).

1.2.2.2 ABA and growth regulation

Whilst the function of ABA in seed development, desiccation and germination is relatively well understood, its effect on various aspects of plant growth provides some controversy. For many years, partly because of the observation that an increase in ABA accumulation in response to water stress coincided with reduced growth rates, it was generally accepted that ABA acts as an inhibitor of growth (Quarrie and Jones, 1977). In a number of experiments involving exogenous applications of ABA, reductions in leaf and shoot growth were reported (Zhang and Davies, 1990; Davies, 1995 and papers cited therein). However, the extent to which exogenous ABA treatments of unstressed plants reproduce the role of increased endogenous ABA in response to water stress is uncertain and, in some cases, has been shown to be unrepresentative (Sharp and LeNoble, 2002). Studies of endogenous control of growth by ABA include split root experiments showed that plants with half of their root system growing in dry soil typically had lower leaf growth rates than plants with all of their root system in wet soil, but this effect could be reversed when the roots in contact with dry soil were severed (Gowing *et al*., 1991), suggesting a root-to-shoot signal that reduced growth and whose identity could be ABA. Also, studies of ABA-deficient mutants at low soil water content appeared to suggest that accumulation of ABA inhibits shoot growth. For example, seedlings of the maize *vp5* mutant were reported to have significantly greater growth rates than WT controls when grown at low soil water content (Saab *et al*., 1990).

When a similar experiment was carried out over a longer period, this effect appeared to be confined to the early stages of seedling growth, and the growth rate of WT plants later exceeded that of the ABA-deficient mutant (Sharp and LeNoble, 2002).

Studies of the interaction between ABA and ethylene have provided some re-evaluation of the role of ABA in inhibiting/promoting growth by suggesting that an important function of ABA may be to restrict ethylene biosynthesis following exposure to stress (Sharp and LeNoble, 2002). ABA and ethylene appear to have an antagonistic relationship. The leaves of the ABA-deficient tomato mutant *flacca* (*flc*) produce two-fold more ethylene than WT controls (Tal *et al*., 1979) even when grown at higher shoot water status than WT plants (Sharp *et al*., 2000). ABA-deficient mutants typically exhibit morphological symptoms characteristic of excess ethylene, including responses resembling leaf epinasty and increased adventitious rooting (e.g. Tal, 1966). Under well-watered conditions, shoot growth was greater in WT plants than in ABA-deficient *vp5* mutants (Saab *et al*., 1990). Although this reduction in growth of *vp5* plants could potentially have been directly due to excessive water loss, the stunted growth phenotype of both ABA-deficient maize and tomato plants was maintained when grown at high humidity, perhaps due to excessive ethylene production, which is inhibited by normal WT levels of endogenous ABA (Sharp *et al*., 2000). This suggested that ABA is not a growth inhibitor, but instead promotes shoot growth by suppressing excess ethylene production associated with water stress (Sharp *et al*., 2002).

When exogenous ABA was applied *via* a foliar spray to the ABA-deficient tomato mutant *flacca* and *Arabidopsis* mutant *aba2-1*, ethylene evolution in leaves decreased and leaf area was increased (Sharp *et al*., 2000; LeNoble *et al*., 2004). Consistent with this observation, when *flc* scions were grafted onto WT rootstocks (*flc*/WT), the increased root-synthesised ABA was associated with decreased ethylene evolution in the *flc* scions relative to *flc* self-grafts (*flc/flc*) (Dodd *et al*., 2009). Interestingly, even though the addition of the WT rootstock normalised ethylene evolution in *flc*/WT plants, leaf area was still only 75% of equivalent WT/WT self-grafts, suggesting shoot-synthesised ABA may promote normal leaf growth (Dodd *et al.*, 2009). In support of this conclusion, experiments using the double ABA-deficient and ethylene insensitive *Arabidopsis* mutant (*aba2-1* and

etr1-1) showed that these plants had leaf areas intermediate between WT and *aba2-1* plants (LeNoble *et al*., 2004), supporting the hypothesis that one function of ABA is to act as a direct (ethylene-independent) promoter of leaf area. The exact mechanism of this effect is still uncertain, but this line of thought is contrary to previously accepted views of ABA as a growth inhibitor.

1.2.2.3 ABA and the response to abiotic stress

Plants have the ability to modify many aspects of their biochemistry, physiology, growth and development to cope with variations in water supply. Many studies have shown that abscisic acid (ABA) is of central importance in these responses, regulating plant water status, at least in part, *via* its ability to influence stomatal behaviour (Zeevaart and Creelman, 1988).

1.2.2.3.1 Root-to-shoot signaling

ABA is an important component of the complex mechanisms which enable plants to co-ordinate water supplies with demand and thereby optimise productivity in response to both short and long term environmental fluctuations (Passioura, 2002). Plants have the ability to perceive a lack of soil moisture available to the roots and relay this information to the shoot, a process often described as root-to-shoot communication. Chemical and hydraulic signals may be integrated to initiate the adaptive responses of the shoot when roots encounter dry soil (Davies *et al*., 1994; Comstock, 2002).

Although it was initially thought that a decline in leaf water status was a key signal in allowing plants to respond to water stress, it was later shown that leaf water potential and turgor may remain unchanged even though water-conserving responses have been induced (reviewed by Davies and Zhang, 1991). It has long been known that rapid stomatal closure precedes ABA accumulation in the shoot (Beardsell and Cohen, 1975), and such changes in stomatal behaviour in the absence of a decline in leaf water status can be closely correlated with declining soil water availability (Davies *et al*., 2005). Previous workers have used several methods to demonstrate this, including maintaining leaf water status as soil dries using pressure balancing techniques (Gollan *et al.*, 1986) or splitting roots between wet and dry soil (Stoll *et*

al., 2000; Mingo *et al.*, 2003). These techniques highlight the importance of root-to-shoot signalling in the absence of changes in leaf water status.

The concentration of endogenous ABA in a tissue at any particular time is modulated by the rates of ABA biosynthesis and catabolism (Nambara and Marion-Poll, 2005), and can also be elevated by release of ABA from an inactive glucose conjugate (Lee *et al.*, 2006). Almost all plant cells can synthesise ABA (Cutler and Krochko, 1999), which may be subsequently transported around the plant through the xylem and phloem. The concentration of ABA in the xylem stream has been proposed to act as one possible measure of soil water availability, allowing plants to regulate their physiology, growth and development accordingly (Zhang and Davis, 1991). As soil drying occurs, roots experience water stress and respond by initiating changes in physiology, metabolism and gene expression (Griffiths and Bray, 1996). Xylem sap ABA concentration increases as soil water potential decreases (Davies and Zhang, 1991), an effect which may be associated with water conservation responses in the shoot, possibly including stomatal closure.

Roots synthesise ABA in response to water stress (Hartung and Davies, 1991), predominantly in the root tips (Zhang and Tardeiu, 1996). A 5- to 6-fold increase in root ABA concentration was detected in water stressed root tips of pea (*Pisum sativum*) and *Commelina communis* (Zhang and Davies, 1987). More substantial ABA synthesis may occur in the leaves themselves as a result of changes in shoot water status induced by dry soil or atmospheric conditions (Wright, 1977; Wilkinson, 2004). Soil drying has also been reported to reduce ABA catabolism by root cells (Liang *et al.*, 1997). The capacity of ABA to provide a root-to-soot signal was demonstrated by incubating the roots of intact plants in solutions containing various concentrations of ABA, which caused ABA to accumulate in leaves and stomatal closure (Zhang and Davies, 1987). Although ABA concentration in the roots may show a correlation with soil moisture levels (Zhang and Davies, 1989), the precise role of root-synthesised ABA in long-distance signalling remains unclear (Sauter *et al.*, 2001). Although some of the ABA detected in roots and the xylem sap may have been synthesised in root tissues (Thompson *et al.*, 2007b), some may have been released from glucose-conjugated forms within the root (Sauter and Hartung, 2000), some may have been synthesised in the shoot and imported to the

roots through the phloem (Jiang *et al.*, 2004), and some could have been taken up from soil water in free or conjugated forms (reviewed by Hartung *et al*., 2002).

Zhang and Davies (1989) reported that xylem sap ABA initially appeared to originate from roots encountering drying soil, but was later also derived from older leaves. In an alternative experimental approach to investigate this question, ABA was removed from xylem sap collected from drought-stressed plants; when the sap was subsequently fed to unstressed, detached leaves, the antitranspirant effect of the sap had been lost (Zhang and Davies, 1991). However, in similar experiments by other researchers, an antitranspirant effect was maintained even when ABA had apparently been removed. Grafting experiments using WT and ABA-deficient scion/rootstock combinations have provided an ambiguous picture of the effect of root-sourced ABA on stomatal behaviour (also discussed in Chapter 8) as some experiments suggest that shoot-derived ABA is important for the control of stomatal aperture (Holbrook *et al*., 2002), whilst others imply that root-sourced ABA has a role in controlling stomatal conductance (e.g. Tal *et al*., 1970). It has recently been suggested that ABA biosynthesis in the roots is not involved in determining shoot responses to drought, and it is instead a hydraulic signal that communicates the occurrence of soil drying from root to shoot (Christmann *et al*., 2007). In support of this view, the use of ABA-dependent reporter gene activation to show localised ABA concentrations suggests that ABA accumulation induced by water-stress occurs first in the vascular tissue of the shoots, with root and guard cell accumulation occurring later (Christmann *et al*., 2005). This lack of early ABA accumulation in the roots may reflect either the rapid export of ABA from the roots or the presence of alternative long-distance signals (Taiz and Zeiger, 2006).

As well as a hydraulic signal, several other methods of root-to-shoot communication have been proposed, including changes in xylem sap pH (Schurr *et al*., 1992; Wilkinson and Davies, 1997) or the occurrence of a non-ABA chemical signal (Dodd *et al*., 2005). Alkalisation of xylem sap pH has been reported to occur in response to soil drying (Wilkinson and Davies, 1997) and may be correlated with stomatal closure (Hartung and Radin, 1989; Gollan *et al.*, 1992; Wilkinson *et al.*, 1998). Alkalisation of xylem sap was proposed as a root-sourced soil drying signal (Schurr *et al.*, 1992; Slovik & Hartung, 1992) capable of providing an early

indication of soil water deficits (Wilkinson and Davies, 1997; Wilkinson *et al.*, 1998; Jia and Davies, 2007). This change in pH apparently maximises the quantity of ABA loaded into the xylem in the roots and has been suggested to promote more efficient loading into the xylem sap from the ABA stores located in parenchyma cells in the stem (Hartung *et al*., 2002). Stomatal closure in response to xylem-supplied buffers has been demonstrated to be ABA-dependent as stomatal aperture did not decrease when detached leaves of the ABA-deficient tomato mutant *flacca* were supplied with a pH 7 buffer, a treatment which caused stomatal closure in WT leaves (Wilkinson *et al.*, 1998). Redistribution of ABA in the leaf can lower xylem sap ABA concentration, especially when the xylem sap is acidic. ABA can redistribute into the cytoplasm/plastids of mesophyll cells according to the anion trap concept (Slovic *et al*., 1995). The effect of pH on the availability of ABA for action on the guard cells is described more fully in Section 1.2.3.2.

The glucose ester of ABA (ABA-GE) is often present at higher concentrations than ABA in the soil solution (Sauter and Hartung, 2000). When ABA-GE is synthesised in the cytoplasm of the cortex of plant tissues, its release across the plasma membrane of the adjacent parenchyma cells into the xylem is rate-limiting because of the low permeability of plasma membranes to this highly polar compound (Baier *et al*., 1990). Sauter and Hartung (2002) reported that, when ABA concentration in the xylem sap is similar to water stressed plants, ABA is distributed to the stem tissue in a process which occurs within 10-20 minutes (Hartung *et al*., 2002). Therefore, xylem sap ABA concentration does not remain constant during its transport over relatively long distances (Jiang and Hartung, 2008). This is not the case for the concentration of ABA-GE, whose concentration remains constant due to its inability to permeate surrounding plasma membranes (Sauter and Hartung, 2002). Increased concentrations of glucose esters of ABA, PA and DPA have been found in the xylem sap of drought stressed sunflower and rice plants (Bano *et al*., 1994). ABA-GE has been proposed as a candidate for a hormonal long distance stress signal (Jiang and Hartung, 2008). Although the relative importance of the various proposed signalling mechanisms remains unclear, it is likely that more than one mechanism may interact to produce the complex signal that is conveyed from roots to shoots in response to soil drying (Tardieu and Davies, 1993).

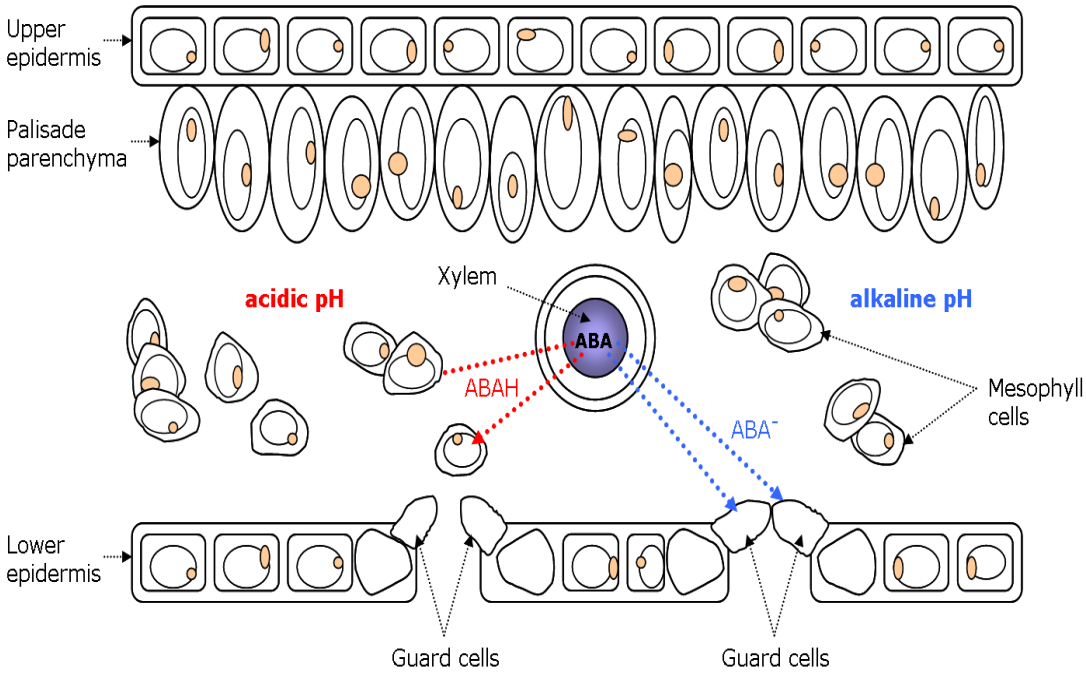


Figure 1.2. Schematic representation of the redistribution of ABA according the anion trap concept (after Taiz and Zeiger, 2006).

1.2.2.3.2 The Anion Trap

As described in Section 1.2.2.3.1, the increase in xylem sap ABA concentration with decreasing soil water potential may act as part of the root-to-shoot signal (Davies and Zhang, 1991). The majority of ABA synthesis in plants occurs in the shoot in response to several physiological triggers, including reductions in turgor (Pierce and Raschke, 1980) or cell volume (Ackerson and Radin, 1983). The early stages of the ABA biosynthetic pathway occur in the plastids, but the final steps, i.e. conversion of xanthoxin to ABA via ABA-aldehyde, takes place in the cytosol. The distribution of ABA (including both that synthesised in the leaves and that reaching the leaves through the xylem) between cells and the free space (apoplast) is determined according to pH gradients and follows 'the anion trap concept' (Slovic *et al.*, 1995). This represents an important regulatory mechanism in the control of stomatal aperture and therefore plant water status (Wilkinson and Davies, 2002).

ABA is a weak acid (pKa = 4.8) and its distribution within the cellular compartments of leaves is strongly affected by apoplastic pH (Slovic *et al.*, 1995), as ABA uptake into cells and sub-cellular compartments occurs by diffusion in accordance with pH gradients (Fig. 1.2). Under well watered conditions, xylem sap

and apoplastic pH is relatively acidic (between pH 5 and 6), and this may mean that ABA arriving in or synthesised by leaves does not reach the sites of action on the guard cells. It may instead be taken up into the alkaline compartments of the leaf (symplast or phloem) (Sauter *et al.*, 2001), a process likely to restrict access to ABA by the guard cells under non-stressed conditions (Jia and Davies, 2007). During photosynthesis, protons are pumped into the thylakoids through the thylakoid membranes, causing the stroma of the chloroplasts to become more alkaline. The resultant pH gradient means that undissociated ABA (ABAH) in the more acidic compartments loses a proton upon entering the alkaline stroma (Hopkins, 1995). It is then trapped as anionic ABA (ABA^-) which has low membrane permeability (Kaiser and Hartung, 1981). When photosynthesis stops during periods of darkness, the pH of the stroma falls, resulting in conversion of ABA^- to ABAH, which can then be released from the chloroplasts. This mechanism is also potentially involved in the light-dark control of stomatal conductance (Kaiser and Hartung, 1981).

During drought, apoplastic pH increases which favours the dissociation of ABAH to produce anionic ABA (ABA^-), reducing sequestration of ABA within mesophyll cells and their chloroplasts. ABA arriving in the leaf is therefore likely to remain within the apoplast for longer, meaning that more ABA can gain access to the guard cells, potentially causing stomatal closure (Hartung 1983). The process of relocating ABA into the alkaline phloem also appears to be restricted by stress, further increasing apoplastic ABA levels. The action of ABA on guard cells is discussed in Section 1.2.2.3.3.

1.2.2.3.3 ABA and stomatal aperture

Stomata regulate the diffusive conductance of leaves, thereby influencing the rate of water loss and carbon gain (Taiz and Zeiger, 2006). Observations that ABA concentration increases in water stressed leaves (Wright and Hiron, 1973) and that exogenously applied ABA induces stomatal closure (Cummings and Sondheimer, 1973) provided the first indication of the potential importance of ABA in the mechanisms that regulate stomatal behaviour. Subsequent laboratory and field experiments have demonstrated a correlation between xylem sap ABA concentration and the degree of stomatal opening (e.g. Zhang and Davies, 1989; Tardieu, 1992; Jia and Zhang, 1999). ABA can affect guard cells directly, inducing osmotic efflux and

hence turgor loss, resulting in stomatal closure (Zhang and Davies, 1990; Blatt *et al*., 2000; Ng *et al*., 2001).

Stomata are microscopic pores enclosed by a pair of guard cells, and stomatal conductance is regulated by the osmotic shrinking and swelling of these cells. Drought may induce changes in apoplastic pH which cause extracellular accumulation of ABA, previously trapped in its anionic form (Section 1.2.2.3.2), thereby facilitating its delivery to the guard cells (Wilkinson and Davies, 2002). Stomatal opening and closure are facilitated by the movement of K^+, H^+ and Cl^- and the metabolism, movement and synthesis of organic ions, especially malate (MacRobbie, 1988; Talbott and Zeiger, 1998). High solute concentrations in the cytosol and vacuoles lead to endosmosis, thereby increasing guard cell volume and turgor, leading to stomatal opening, whilst the loss of solutes from guard cells reduces cell volume and turgor, resulting in guard cell shrinkage and stomatal closure (Pei and Kuchitsu, 2005).

The exact signalling mechanisms responsible for stomatal opening and closure have been the focus of much research: it has been demonstrated that increased cystolic Ca^{2+} concentration, achieved *via* influx across the plasma membrane and release from cytosolic stores, is important in mediating the action of ABA on guard cells (Allen *et al*., 2000; Murata *et al*., 2001; Pei *et al*., 2000). Accumulation of Ca^{2+} in the cytosol of guard cells inhibits H^+ ATPase and activates anion channels in the plasma membrane, causing the loss of Cl^- ions and membrane depolarisation, which subsequently induces outward-rectifying K^+ channels. Nitric oxide (NO) accumulation in guard cells has also been shown to be involved in ABA-dependent stomatal closure (Garcia-Mata *et al*., 2003; Desican *et al*., 2004; Sokolovski *et al*., 2005) as the guard cells of *Arabidopsis* produce NO in response to exogenous application of ABA. In a variety of plant species, application of NO donors induces stomatal closure (Neill *et al*., 2002), whilst ABA-induced stomatal closure is decreased following the application of NO scavengers. Recent research has also implicated other signalling components and roles of relevant ion channels in mediating the action of ABA on guard cells. It is now believed that modulation of stomatal aperture is associated with a complex series of cellular biochemical events (Acharya and Assman, 2009), including the production of reactive oxygen species

(ROS) (Pei *et al*., 2000; Zhang *et al*., 2001), elevation of cytosolic pH (Irving *et al*., 1992), protein phosphorylation/dephosphorylation (Li *et al*., 2000; Merlot *et al*., 2001; Mustilli *et al*., 2002), and reorganisation of the cytoskeleton (Hwang and Lee, 2001), in addition to those mentioned previously.

These mechanisms influence ion transport in the apoplast surrounding guard cells, thus reducing guard cell turgor and causing stomatal closure (Assmann, 1993). As mentioned previously, the reduction in guard cell turgor required for stomatal closure is facilitated by a large-scale efflux of K+ and other anions (Taiz and Zeiger, 2006). Opening of the calcium-activated, slow (S-type) anion channels in the plasma membrane has been shown to be promoted by ABA (Schroeder *et al*., 2001). The rapid (R-type) anion channels are also activated in the presence of ABA (Raschke *et al*., 2003). The apoplast surrounding guard cells is regarded as the most important compartment for the action of ABA on stomata as it is the only compartment from which ABA can enter guard cells. In response to water stress, ABA concentration in the guard cell apoplast may increase by up to 30-fold (Zhang and Outlaw, 2001; Outlaw, 2003).

1.2.3 The ABA biosynthesis pathway

As with many other hormones, plant responses to ABA depend on its concentration and on the sensitivity of the tissue involved (Taiz and Zeiger, 2006). The concentration of active ABA may fluctuate according to developmental stage or environmental conditions and depends on the processes of biosynthesis, catabolism, compartmentation and transport. ABA is a sesquiterpeniod (C_{15}), which is synthesised in most plant tissues, including those of roots and leaves (Cornish and Zeevaart, 1985; Zhang and Davies, 1987; Christmann *et al.*, 2005) *via* two distinct biosynthetic pathways. Phytopathogenic fungi are thought to synthesise ABA *via* a pathway which utilises the C_{15} compound farnesyl pyrophosphate (FPP) (Bennet *et al*., 1981). As this pathway involves conversion of the C_{15} FPP precursors to the C_{15} ABA compound, it has been termed the "direct pathway" (Zeevaart and Creelman, 1988). A similar "direct pathway" was initially believed to operate in tissues of higher plants but it is now widely accepted that ABA is synthesised by an "indirect pathway" involving the oxidative cleavage of xanthophyll (C_{40} oxygenated carotenoid) precursors, via C_{15} intermediates (Zeevaart and Creelman, 1988).

The "indirect pathway" was first suggested when it was noted that there were structural similarities between ABA and the end groups of some carotenoids, making these compounds ideal candidates to be precursors for ABA (Ohkuma *et al*., 1965). Experiments in which ^{14}C-labelled xanthoxin was supplied to tomato and bean plants demonstrated that this C_{15} aldehyde can be converted to ABA and its catabolites, suggesting that xanthoxin was a potential intermediate in ABA biosynthesis (Taylor and Burden, 1972). It was subsequently established that ABA was synthesised from stored xanthophyll precursors such as zeaxanthin, violaxanthin and neoxanthin isomers, and was not synthesised directly by successive oxidative steps at the C_{15} level. This was established using labelled (heavy) oxygen to confirm that only one atom of ^{18}O is incorporated into the carboxyl group of the ABA molecule (Creelman and Zeevaart, 1984). The indirect route of ABA synthesis has also been confirmed by characterisation of ABA-deficient mutants in which the C_{40} level of the pathway is affected (Duckham *et al*., 1991; Rock and Zeevaart, 1991; Schwartz *et al*., 1997; Seo and Koshiba, 2002). Genes encoding the catalytic enzymes have now been cloned for most steps in this pathway, or their function confirmed using ABA-deficient mutants blocked at a specific step (North *et al*., 2007). The earlier part of the ABA biosynthetic pathway occurs in chloroplasts and other plastids (Liotenberg *et al*., 1999; Taylor *et al*., 2000). The biosynthesis of ABA can conveniently be divided into four sections: the plastidial/MEP pathway, α and β xanthophyll biosynthesis branches, xanthophyll formation/cleavage and the dedicated C_{15} phase, in which ABA is formed.

1.2.3.1 The Plastidial/MEP Pathway to lycopene

The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway occurs in the plastids, during which the colourless carotenoid phytoene is formed (Fig. 1.3). An important initial step in the MEP pathway is that catalysed by the thiamine pyrophosphate (TPP) dependent enzyme, 1-deoxy-D-xylulose 5-phosphate synthase (DXS); this step involves condensation of glyceraldehyde 3-phosphate and pyruvate into 1-deoxy-D-xylulose 5-phosphate (DXP) (Sprenger *et al*., 1997). The *Arabidopsis* mutant that is defective for DXS (*cloroplastos alterados* or *cla 1)* is incapable of producing carotenoid/xanthophyll compounds and therefore exhibits an albino phenotype (Lois *et al*., 1998), but this phenotype can be reversed by application of DXP (the product of DXS) (Mandel *et al*., 1996; Estévez *et al*., 2001), thus allowing

normal carotenoid production to be restored. When expression of *CLA 1* was upregulated in transgenic *Arabidopsis* plants, the production of chlorophyll, carotenoids and ABA were all increased (Estévez *et al*., 2001). Similarly, tomato plants over-expressing *DXS1* exhibited a 1.6-fold increase in carotenoid concentration (Enfissi *et al*., 2005). These deficiency and upregulation experiments demonstrate that the step catalysed by DXS is likely to a key rate-limiting step at an early stage in the pathway (Taylor *et al*., 2005).

Conversion of DXP to 2-C-methyl-D-erythritol 4-phosphate (MEP) occurs next in a reduction reaction catalysed by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR). In contrast with DXS, over-expression studies involving DXR encoding constructs indicated that the step catalysed by the DXR enzyme is not rate-limiting in ripening tomato fruit (Rodríguez-Concepción *et al*., 2001). DXP is a substrate for thiamine and pyridoxine formation and conversion of DXP to MEP is considered the first committed step of the MEP pathway (Carretero-Paulet *et al*., 2002). The next reaction involves synthesis of 1-hydroxy-2-methyl-2(E)-butenyl-4-pyrophosphate (HMBPP), the ultimate precursor of IPP in the plastidial pathway of isoprenoid synthesis. This occurs *via* a series of four reactions, leading to the formation of the universal isoprene intermediates, IPP and DMAPP (reviewed by Rodríguez-Concepción and Boronat, 2002). In the final step in this phase of the pathway, the enzyme hydroxymethylbutenyl diphosphate (HMBPP) reductase (HDR) catalyses the formation of both isopentyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) (Arigoni *et al.*, 1997; Lichtenthaler *et al.*, 1997; Hoeffler *et al.*, 2002). It has been reported that post-transcriptional silencing of an *HDR* gene in tobacco resulted in a loss of pigmentation in the leaves (Page *et al*., 2004), suggesting that HDR may be rate-limiting for carotenoid production. Isomerisation of IPP to DMAPP by isopentenyl pyrophosphate isomerase (IPI), providing pools of the two main C_5 'building blocks' of terpenoid biosynthesis, marks the end of the MEP section of the pathway.

Four of the C_5 units are used to assemble the 20-carbon compound, geranylgeranyl pyrophosphate, which is a precursor used in the biosynthesis of many compounds including gibberellins, phytol and tocopherols, causing potential competition between various different branches of the terpenoid biosynthetic pathways (Taylor *et al*., 2005). GGPP is formed through a series of condensation reactions between IPP and DMAPP to

form C_{10} geranyl diphosphate (GPP), C_{15} farnesyl diphosphate (FPP) and finally C_{20} GGPP (Cunningham and Gantt, 1998), all catalysed by the enzyme geranylgeranyl pyrophosphate synthase (GGPS). In *Arabidopsis*, the GGPS encoding gene family contains 12 genes, five of which were characterised by Okada *et al*. (2000) and identified as being expressed in different sub-cellular locations (*GGPS1*, *GGPS2*, *GGPS3*, *GGPS4* and *GGPS6*). Two genes in this family *GGPPS1* (gene product targeted to photosynthetic tissue) and *GGPPS3* (gene product targeted to root tissue) encode proteins with transit peptides directing them to the plastids and both were shown to form GGPP from IPP derived from the MEP pathway (Okada *et al*., 2000). The step catalysed by GGPPS is likely to be of key importance in both MEP (plastidial) and mevalonate (cystolic) pathways (Taylor *et al*., 2005).

The colourless C_{40} compound phytoene is formed by the head-to-head condensation of two C_{20} GGPP molecules and is the first committed step in carotenoid biosynthesis (Cunningham and Gantt, 1998; Hirschberg, 2001). This key reaction marks the beginning of the C_{40} level of the pathway and is catalysed by phytoene synthase (PSY) (Bartley *et al*., 1992). This important regulatory step in carotenoid synthesis has been manipulated to increase carotenoid accumulation in many species, most notably to genetically engineer 'golden rice' to have enhanced nutritional quality (Ye *et al*., 2000). In tomato fruit, expression levels of *PSY* were found to be closely correlated with carotenoid content during ripening (Giuliano *et al*., 1993). Antisense expression of a *PSY* gene in tobacco plants reduced both the carotenoid and chlorophyll content of the leaves (Busch *et al*., 2002). The *PSY1* gene in tomato encodes the enzyme responsible for catalysing the conversion of GGPP to phytoene in chromoplasts (Bramley *et al*., 1992), and antisense expression in transgenic tomato plants resulted in the formation of pale coloured leaves, yellow ripening fruits (Maunders *et al*., 1987), substantially reduced total carotenoid concentrations and undetectable levels of lycopene (Bird *et al*., 1991). Constitutive over-expression of *PSY1* led to significantly increased carotenoid levels in all plant tissues (Fray *et al*., 1995). Constructs designed to over-express in a seed-specific manner resulted in substantially increased carotenoid and xanthophyll concentrations and ABA levels were elevated sufficiently to cause increased seed dormancy (Lindgren *et al*., 2003).

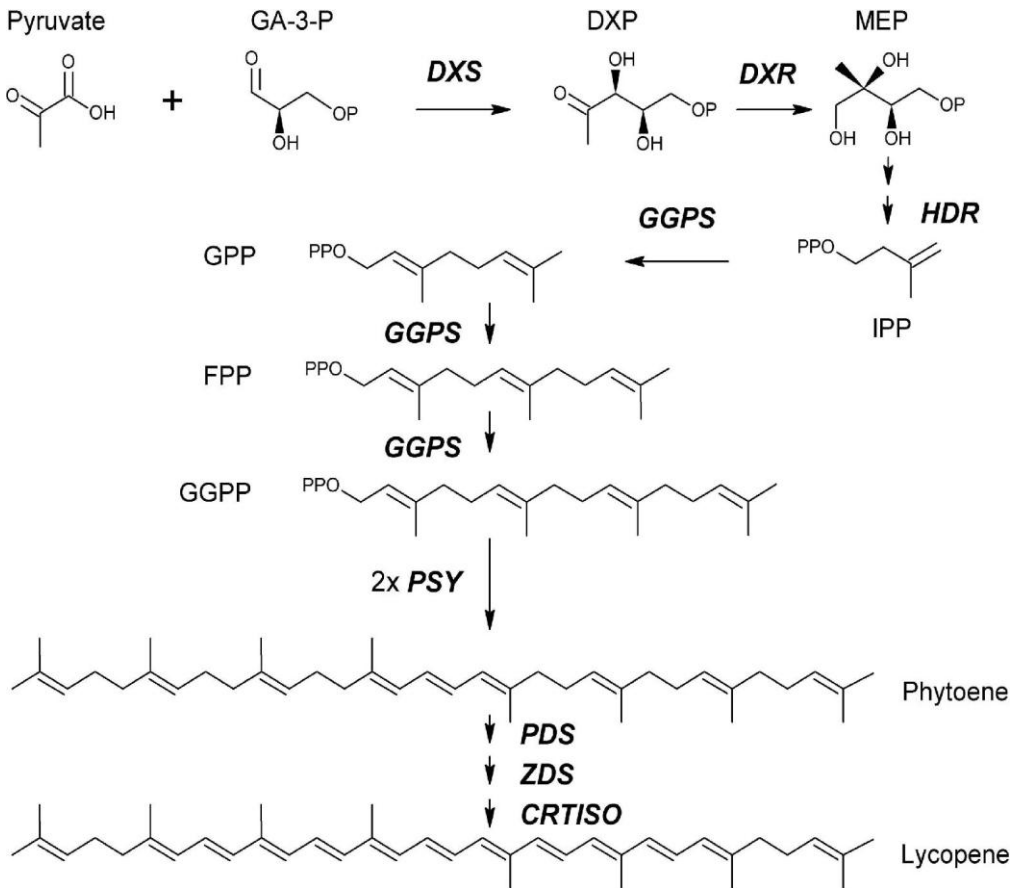


Figure 1.3. Outline of the plastidial/MEP pathway leading to all-*trans*-lycopene. Abbreviations of key enzyme names are given in bold italics: DXS, 1-deoxy-Dxylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; HDR, hydroxymethylbutenyl diphosphate reductase; GGPS, geranylgeranyl diphosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, W-carotene desaturase; CRTISO, carotenoid isomerase (from Taylor *et al*., 2005).

Phytoene does not normally accumulate in plant tissues (Hirschberg, 2001) but instead is converted to the red carotenoid lycopene *via* a series of four desaturation reactions catalysed by two functionally similar enzymes. The first, phytoene desaturase (PDS) converts phytoene to ζ-carotene by introducing two *trans* double bonds; this is subsequently converted to lycopene *via* ζ-carotene desaturase (ZDS) following the introduction of two *cis* double bonds (reviewed by Hirschberg, 2001; Howitt and Pogson, 2006). Following the formation of lycopene, the carotenoid biosynthetic pathway splits into an α- and a β- branch, each of which involves the formation of carotenoids with different functions (Taylor *et al.*, 2005).

1.2.3.2 The α- and β- branches of the pathway

The two alternative cyclisation routes of lycopene form a branch point in the carotenoid pathway, from which two compounds with different cyclic end groups, α-carotene and β-carotene, are formed (reviewed by Cuttriss and Pogson, 2004). In the α-branch, all-*trans*-lycopene is cyclised into α-carotene, with the addition of an epsilon (ε) ring at one end of the molecule, by the enzyme lycopene ε-cyclase (LCY E) and the addition of a β-ring at the other end by the enzyme lycopene β-cyclase (LCY B) (Cunningham *et al*., 1996). This α- branch of carotenoid synthesis leads to the formation of lutein and it has been reported that LYC E is the rate-limiting enzyme (Pogson *et al*., 1996).

In the β-branch, the formation of β-carotene involves addition of two β-rings to either end of all-*trans*-lycopene, catalysed by LYC B (Cunningham *et al*., 1994; Cunningham *et al*., 1996). Genes encoding the lycopene cyclases (LYC B and LYC E) are usually down-regulated in ripening tomatoes to allow the fruit to accumulate high levels of the red pigment lycopene (Pecker *et al*., 1996; Ronen *et al*., 1999). The LYC B gene is, however, up-regulated in mature flower chromoplasts, associated with synthesis of xanthophylls to create the bright yellow pigmentation to attract pollinators (Taylor *et al*., 2005). A chromoplast-specific LCY B gene has been identified in tomato (CYC B) and the tomato *Beta* (*B*) mutant causes continued expression of the gene (Ronen *et al*., 2000). This results in orange coloured ripe fruit, as the accumulation of β-carotene occurs at the expense of lycopene (Ronen *et al*., 2000). In the tomato mutants *old-gold* (*og)* and *old-gold crimson* (og^c), there is impaired function of the chromoplast-specific LYC B, leading to increased accumulation of lycopene in chromoplasts of mature petals due to the inability to synthesise β-carotene and the β-xanthophylls, with petals developing a red/gold colouration (Ronen *et al*., 2000; Galapaz *et al*., 2006). Carotenoid synthesis *via* the α- and β- branches of the pathway is tightly regulated and compensatory, being influenced by both environmental and genetic factors (Taylor *et al*., 2005). For example, the ratio of LCY B: LYC E mRNA increases when exposed to high light levels (Hirschberg *et al*, 2001; Taylor *et al*., 2005).

1.2.3.3 Xanthophyll formation/cleavage

The formation of xanthophylls occurs *via* the hydroxylation of carotenoids containing either ε–ring or β–rings. The α-carotene is hydroxylated on both rings by the enzymes ε–carotene hydroxylase (ECH) and β-carotene hydroxylase (BCH), to produce the end product of this branch in the pathway, the xanthophyll known as lutein (Howitt and Pogson, 2006). Lutein is the most abundant xanthophyll in the thylakoid membrane, forms part of the structural components of the light-harvesting complexes (Kühlbrandt *et al*., 1994; Horton *et al*., 1996), and is important in the protection of the photosynthetic apparatus in leaves (Rossel *et al*., 2002).

From β-carotene, the first xanthophyll in the pathway, all-*trans*-zeaxanthin is formed (Fig. 1.4). This occurs in two steps catalysed by the enzyme β-carotene hydroxylase (BCH) *via* the intermediate β-cryptoxanthin, in which the two symmetrical rings of β-carotene are successively hydroxylated (Howitt and Pogson, 2006). When a *BCH* gene was over-expressed in *Arabidopsis* with the objective of increasing xanthophyll accumulation, the concentration of xanthophylls doubled in leaf tissue, with only a small depletion of β-carotene content (Davison *et al.*, 2002). Antisense down-regulation of *BCH* in *Arabidopsis* caused a 64% reduction in β-carotene-derived xanthophylls (Rissler and Pogson, 2001). Whilst β-carotene is a precursor of the β-xanthophylls, it constitutes a major fraction of the total carotenoid content in photosynthetic tissues and the existence of such a large pool indicates that the expression of *BCH* genes is closely controlled (Taylor *et al*., 2005). Genetic control of the BCH enzyme is discussed in Chapter 7 (Section 7.1.1). The formation of zeaxanthin can in one sense be considered the starting point for ABA biosynthesis, as genetic lesions downstream of zeaxanthin result in plants with classic symptoms of ABA deficiency (e.g. wilty phenotype) rather than the characteristic photobleaching exhibited by plants deficient in carotenoids (Taylor, 1991; Taylor *et al*., 2000).

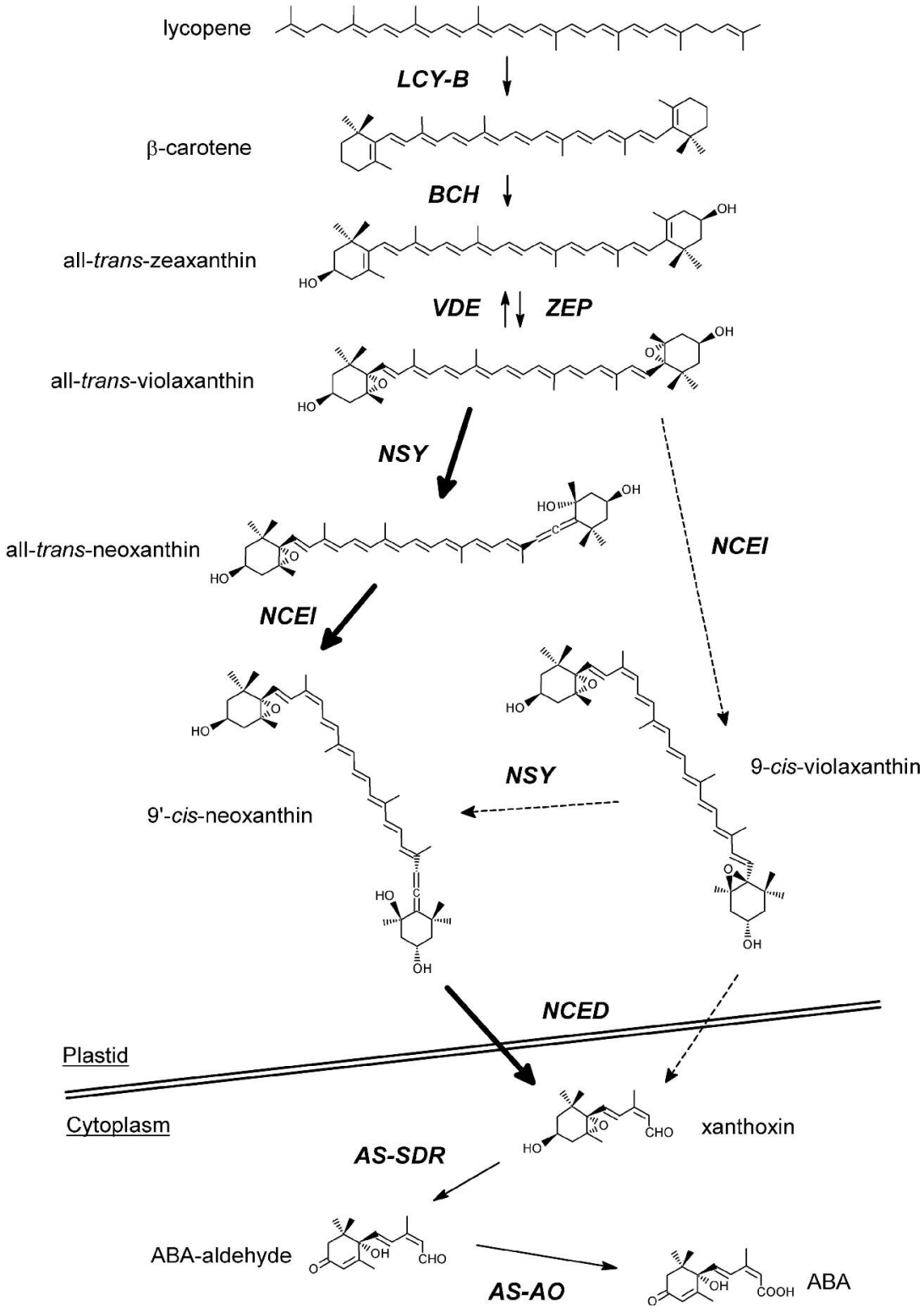


Figure 1.4. ABA biosynthesis from lycopene to ABA**.** Abbreviations of enzyme names are given in bold italics: LCY-B, lycopene-β-cyclase; BCH, β-carotene hydroxylase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin deepoxidase; NSY, neoxanthin synthase; NCEI, 9-*cis*-epoxycarotenoid-forming isomerase; NCED, 9-*cis*epoxycarotenoid dioxygenase; AS-SDR, ABA specific short-chain ehydrogenase/reductase; AS-AO, ABA specific aldehyde oxidase (from Taylor *et al*., 2005).

Zeaxanthin is symmetrically epoxidated on both rings to synthesise the major C_{40} xanthophyll known as all-*trans*-violaxanthin. This is catalysed by the enzyme zeaxanthin epoxidase (ZEP) and all-*trans*-violaxanthin is formed *via* the mono-epoxy intermediate antheraxanthin (North *et al.*, 2007). This reaction is reversible through the action of violaxanthin de-epoxidase (VDE) and is an important component of the xanthophyll cycle (Hirschberg, 2001), which is important for photoprotection during photosynthesis (Demmig-Adams and Adams, 1996).

The first *ZEP* gene was isolated from a transposon-induced mutant (*aba2)* of *Nicotinia plumbaginifolia* (diploid tobacco relative), in which zeaxanthin accumulation was substantially increased, indicating impaired ZEP activity (Marin *et al.*, 1996). This mutant also exhibited a wilty phenotype, caused by its reduced ability to close its stomata and reduced seed dormancy. These phenotypes were caused by a 48% decrease in ABA concentration in whole plants, and were reversed when exogenous ABA was applied (Marin *et al*., 1996). When a construct designed to give antisense expression of *ZEP* was used to transform tomato plants, zeaxanthin accumulation was increased, epoxycarotenoids (all-*trans*-violoxanthin and neoxanthin isomers) levels were reduced, and the tendency to wilt upon drought stress was increased (Thompson *et al*., 2000b).

ZEP expression is important in the control of ABA biosynthesis in seed (reviewed by Nambara and Marion-Poll, 2003). The level of ZEP mRNA peaks at the same point of seed development as ABA accumulation, suggesting a role for *ZEP* in regulating ABA concentration in the seed (Audran *et al*., 1998). *N. plumbaginifolia* seeds over-expressing *ZEP* exhibit slightly more delayed seed germination than WT seed, while antisense down-regulation in the expression of *ZEP* in tobacco caused rapid germination of freshly harvested seed, demonstrating that their natural dormancy was overcome in the absence of normal *ZEP* expression (Frey *et al*., 1999).

The xanthophyll cycle is involved in photoprotection and ZEP mRNA is more abundant in photosynthetic tissues than non-photosynthetic tissues (Audran *et al*., 1998). The fact that the ZEP mRNA content of tobacco and tomato leaves fluctuates coincident with light and dark cycles suggests a circadian/diurnal rhythm is involved

in controlling *ZEP* expression (Audran *et al*., 1998; Thompson *et al*., 2000b). Drought stress can induce ZEP expression in certain tissues but not in others. Thus, ZEP mRNA levels increased by five- and seven-fold respectively in the roots of drought stressed tomato and tobacco plants (Audran *et al*., 1998; Thompson *et al*., 2000b). By contrast, increased expression was not observed in the leaves of drought-stressed tomato plants (Burbidge *et al*., 1997), although the basal level of expression is much greater in leaves than in non-photosynthetic tissues (Audran *et al*., 1998). When *ZEP* was constitutively over-expressed in transgenic tobacco plants, there was no obvious phenotypic response in their leaves (Frey *et al*., 1999). This, and the fact that expression is not upregulated in response to drought, suggests that ZEP mRNA is not limiting for ABA biosynthesis in photosynthetic tissues. In non-photosynthetic tissues, however, where carotenoid levels are significantly lower than in leaves (Parry and Horgan, 1992), the step catalysed by ZEP appears to be rate-limiting for the synthesis of ABA, and the ZEP gene is up-regulated in response to water deficits (Taylor *et al*., 2005).

Conversion of all-*trans*-violaxanthin to form 9-*cis*-epoxycarotenoids precedes the formation of the first C_{15} intermediate (xanthoxin) by oxidative cleavage. The exact details of this section of the pathway are still ambiguous, largely due to the lack of ABA-deficient mutants impaired at the isomerisation step; however, possible alternative routes are outlined by Taylor *et al*. (2000). The lack of clarity mainly concerns the exact properties of the enzymes responsible for the synthesis and isomerisation of neoxanthin and the identity of the 9-*cis*-epoxycarotenoid compound that is the primary *in vivo* precursor of xanthoxin (Taylor *et al*., 2005). It has been suggested that *9′-cis*-neoxanthin is the most likely candidate to be the primary precursor of ABA, largely because it is the most abundant in photosynthetic tissues, although this has not been definitively confirmed (Schwartz *et al*., 2003). The two enzymes responsible for the conversion of all-*trans*-violaxanthin to produce all-*trans*-neoxanthin and then 9′-*cis*-neoxanthin are believed to be neoxanthin synthase (NSY) (North *et al*., 2007) and a putative 9-*cis*- epoxycaroteniod-forming isomerase (NCEI) (Strand *et al*., 2000). A putative *NSY* gene was independently cloned from tomato (Bouvier *et al*., 2000), but the DNA sequence for this *NSY* was found to be identical to the LCY B encoding tomato gene (*CYC B*). A possible dual role for LYC B has been suggested (Hirschberg *et al*., 2001), although there is, as yet, no *in*

vivo evidence for this (Taylor *et al.*, 2005). A membrane protein, ABA4, from *Arabidopsis* has recently been shown to be implicated in some way in neoxanthin synthesis (North *et al*., 2007). Mutant plants, with impaired *Ataba4*, accumulated all-*trans*-violaxanthin, in agreement with the expectation that plants defective in NSY would be blocked in the synthesis of the neoxanthin isomers from this precursor (North *et al*., 2007).

The final step in the ABA biosynthesis pathway to take place in the plastids is the oxidative cleavage of 9-*cis*-isomers of expoxycarotenoids (9′-*cis*-neoxanthin and/or 9-*cis*-violaxanthin) to create xanthoxin, a reaction catalysed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Burbidge *et al*., 1997; Tan *et al*., 1997). This reaction is arguably the first dedicated step of ABA biosynthesis (Parry *et al.*, 1988) and is a key regulatory step in the pathway (Qin and Zeevaart, 1999). Xanthoxin, the first C_{15} intermediate of ABA biosynthesis, is then relocated to the cytoplasm (North *et al*., 2007).

Study of a transposon tagged viviparous maize mutant, *vp14*, led to the discovery of this carotenoid cleavage enzyme. As *vp14* plants had the capacity to synthesise C_{40} xanthophylls and metabolise the C_{15} precursors of ABA, it was concluded that these mutants were probably unable to cleave the 9-*cis*-epoxycarotenoid, C_{40} precursors (Tan *et al*., 1997). A DNA sequence orthologous to that of the *Vp14* gene locus was identified in tomato and initially referred to as encoding a neoxanthin cleavage enzyme (NCE) (Burbidge *et al*., 1997). This enzyme was later renamed nine-*cis*-epoxycarotenoid dioxygenase (NCED) as it was capable of utilising both 9′-*cis*-neoxanthin and the 9-*cis* isomer of violaxanthin as *in vitro* substrates to produce xanthoxin (Liotenberg *et al*., 1999). The tomato NCED encoding gene *LeNCED1* was subsequently shown to be the wild type allele of the ABA-deficient, wilty tomato mutant, *notabilis*. This mutant has a null allele of *LeNCED1* containing a single A/T base pair deletion (Burbidge *et al*., 1999; Thompson *et al*., 2004), thus demonstrating the importance of *LeNCED1* in ABA biosynthesis. An orthologue of the *vp14* and *LeNCED1* genes was also cloned from *Phaseolus vulgaris* and named *PvNCED1* (Qin and Zeevaart 1999). It was demonstrated that in detached, water-stressed leaves, levels of *PvNCED1* mRNA increased within 30 minutes of stress being imposed, and this was followed by a subsequent rise in NCED protein levels

and ABA concentration (Qin and Zeevaart 1999). *NCED* genes have also been characterised in species such as avocado (Chernys and Zeevaart, 2000), cowpea (Iuchi *et al*., 2000), orange (Rodrigo *et al*., 2006), barley (Millar *et al*., 2006) and peanut (Wan and Li, 2006).

The expression of some *NCED* gene family members can be up-regulated in response to water stress in both roots and leaves (Qin and Zeevaart, 1999; Thompson *et al*., 2000a). An 8-fold increase in *LeNCED1* mRNA was observed for leaves collected from water-stressed plants and a 61-fold increase was reported in drying roots (Thompson *et al*., 2000a). The NCED protein levels also correlate with increased mRNA levels in the roots and leaves of water-stressed beans, providing evidence that increased expression of *NCED* regulates water-stress induced accumulation of ABA. Expression of *LeNCED1* was also demonstrated to be directly responsive to light, following a diurnal rhythm in non-stressed leaves (Thompson *et al*., 2000b); *LeNCED1* mRNA levels reached their peak soon after the start of the light period, remained high until the end of the light period, and then decreased rapidly and remaining low during periods of continuous darkness (Thompson *et al*., 2000b). Many other species have at least one *NCED* gene family member for which expression is rapidly upregulated in response to leaf dehydration, including cowpea (Iuchi *et al*., 2001), *Arabidopsis* (Iuchi *et al.*, 2001; Tan *et al*., 2003) and avocado (Chernys and Zeevaart, 2000).

In *Arabidopsis*, nine carotenoid cleavage genes have been identified following searches of the complete genome sequence; of these, some have been unequivocally confirmed as *NCED*s (Tan *et al*., 2003). One family member, *AtNCED3*, showed rapidly elevated expression in dehydrated leaves (Iuchi *et al*., 2001) and is probably orthologous to *LeNCED1*. Antisense suppression of *AtNCED3* resulted in drought sensitivity in *Arabidopsis* (Iuchi *et al*., 2001), indicating that this enzyme is responsible for the majority of ABA accumulation in stressed leaves. Although *AtNCED5* and *9* are mildly up-regulated following dehydration, the remaining *NCED* genes are probably largely involved in developmental control of ABA synthesis in different tissues, including seeds (Tan *et al*., 2003; Lefebvre *et al*., 2006).

1.2.3.4 C_{15} level of the pathway

After its formation, xanthoxin is transported from plastids to the cytoplasm, where it can be converted initially to abscisic aldehyde by the ABA-specific short chain dehydrogenase/reductase (AS-SDR) enzyme (Taylor *et al*., 2005). An *Arabidopsis* gene *ABA2*, encodes for the short chain desaturase (SDR1) enzyme which converts xanthoxin into ABA aldehyde (Cheng *et al*., 2002) and a null mutant of this gene (*aba2*) has been identified and mapped onto chromosome 1 (Leon-Kloosterziel *et al*., 1996). The mutant plants exhibited classic phenotypic symptoms of ABA-deficiency and a substantially reduced ability to convert xanthoxin to ABA-aldehyde (Schwartz *et al*., 1997). The favoured substrate for this enzyme is 2-*cis*, 4-*trans* xanthoxin, the natural product of 9-*cis*-epoxycarotenoid cleavage, rather than the 2-*tran*s, 4-*trans* isomer of xanthoxin. The enzyme ABA2 can perform the ring modifications and oxidation of the 4′-hydroxyl to a ketone group necessary for the conversion of xanthoxin to ABA aldehyde (González-Guzmán *et al*., 2002; Fig. 1.4). Whilst being predominantly expressed in roots and stems, *ABA2* is expressed in all tissues (Cheng *et al*., 2002) and its transcript levels do not increase significantly in response to water or salt stress (Schwartz *et al*., 1997a; González-Guzmán *et al*., 2002), implying a non-rate-limiting role for *ABA2* in water-stress-induced ABA biosynthesis (Chen *et al*., 2003).

In the final step of ABA biosynthesis, ABA aldehyde is oxidised by an ABA-specific aldehyde oxidase (AS-AO) to produce ABA (Seo *et al*., 2000; North *et al*., 2007), the catalytic activity of which requires a molybdenum cofactor (MoCo) (Taylor *et al*., 1988; Schwartz *et al*., 1997; Sekimoto *et al*., 1998). The first mutants shown to represent genetic lesions at this step were the wilty mutants of tomato, *sitiens* and *flacca* (Taylor *et al*., 1988). The *sitiens* mutant may be impaired in an (AS-AO), although the gene involved has not yet been identified (Seo *et al*., 2000). A mutant allele of *flacca* has been shown to have a 6 bp deletion in the MoCo sulfurase gene (Sagi *et al*., 2002). Other mutants deficient in the formation of MoCo synthesis rather than an AS-AO itself include the *aba3* mutant of *Arabidopsis* (Schwartz *et al*., 1997a) and *aba1* in *N. plumbaginifolia* (Leydecker *et al*., 1995). Four aldehyde oxidases have been identified in *Arabidopsis* (AAO1, AAO2, AAO3 and AAO4), although only AAO3 has activity specific for ABA aldehyde (Seo *et al*.,

2000) and was therefore classified as an ABA-specific aldehyde-oxidase (AS-AO, Taylor *et al*., 2005).

1.2.3.5 ABA Catabolism

ABA concentration in a specific tissue at any point in time depends on its rate of synthesis and catabolism (Verslues and Zhu, 2007), and continuous catabolic degradation of ABA occurs in most types of plant cell (reviewed by Cutler and Krochko, 1999). The major catabolism pathway involves initial hydroxylation of ABA, although esterification with glucose molecules also occurs. Hydroxylation of ABA at the 8′ methyl group involves a P-450 type monoxygenase, ABA 8'-hydroxylase (Cutler and Krochko, 1999), initially forming the unstable intermediate 8'-hydroxyl ABA (8'-OH-ABA). This compound then rearranges spontaneously to form phaseic acid (PA), which can be further reduced at the 4' position to form dihydrophaseic acid (DPA), a biologically inactive compound (Nambara and Marion-Poll, 2005). When ABA is exogenously applied, it is rapidly catabolised by this route (Huang *et al*., 2007); as 8'-hydroxylase is rate-limiting for ABA catabolism, it is up-regulated by ABA and in response to water stress (Windsor and Zeevaart, 1997; Krochko *et al*., 1998). This is a potentially important target for genetic engineering of ABA levels in plants as ABA accumulation can trigger its own degradation (Finkelstein and Rock, 2002). Consistent with this, the seed of *abi Arabidopsis* mutants accumulate up to threefold more ABA than WT seed (Koorneef *et al*., 1984), whereas supersensitive *sad1* mutants exhibit decreased ABA concentrations (Xiong *et al*., 2001).

ABA, PA and DPA can all be conjugated to glucose, the most common process being the conjugation of ABA and glucose to form the biologically inactive compound ABA-glucose ester (ABA-GE) (Cutler and Krochko, 1999). All ABA conjugates are biologically inactive, often being sequestered in the vacuole and intercellular spaces until they are released as free ABA *via* the activity of β-glucosidases (Sauter *et al*., 2002, Dietz *et al*., 2000; Sauter and Hartung, 2000).

1.2.4 Alternative functions of carotenoids and xanthophylls

Carotenoids are a diverse group of C_{40} plant pigments which are synthesised in various types of plastids (Hischberg, 2001) and are characterised by the presence

of a highly unsaturated polyene chain of alternating single and double bonds (Britton, 1995). Apocaroteniods have evolved hormonal functions in plants (Giuliano *et al*., 2003), providing precursors for the synthesis of ABA and other plant hormones (Cutriss and Pogson, 2004). However, the primary functions of carotenoids in plants include several physiological and biochemical roles, such as photoprotection, free-radical scavenging and light harvesting (Britton, 1995; Demmig-Adams *et al.*, 1996). Carotenoids are observed in high concentrations in fruit and flower chromoplasts (Cunningham and Gantt, 1998) and are the pigments responsible for giving the red, yellow and orange colouration to these organs, functioning as attractants for pollination and seed dispersal purposes (Goodwin, 1980). Carotenoids are integral constituents of the thylakoid membranes of chloroplasts in plants, algae and cyanobacteria (Cunningham and Gantt, 1998). They are involved with many proteins to form the light harvesting and reaction centre complexes of the photosynthetic apparatus (Howitt and Pogson, 2006), absorbing wavelengths of solar radiation that are not absorbed by chlorophyll and transferring the energy to chlorophyll for photosynthesis (Taiz and Zeiger, 2006), thus improving the effectiveness of light capture (Cutriss and Pogson, 2004).

In addition to their role as accessory pigments in the photosynthetic apparatus, carotenoids also serve as photoprotective agents. The photosynthetic membrane is easily damaged by excess energy that is absorbed by pigments, but not utilised in the process of photosynthesis. The excited state of chlorophyll can react with oxygen molecules to form an excited form of oxygen, termed singlet oxygen ($^1O_2$), which can damage plant cells, especially lipid components (Taiz and Zeiger, 2006). Carotenoids contain relatively low levels of energy in their excited state compared to chlorophyll (Bassi *et al*., 1993), meaning they can rapidly quench the excited state of chlorophyll, allowing excess energy to be lost as heat.

The second protective and regulatory mechanism is the quenching of chlorophyll fluorescence, termed non-photochemical quenching (NPQ). Intense illumination causes excitation in the antenna system and NPQ adjusts the flow of excitations to the PSII reaction centre, protecting the photosynthetic machinery against damage due to over-excitation, quenching excess excitation through conversion to heat (Demmig-Adams and Adams, 1992). Three xanthophylls, violaxanthin,

antherxanthin and zeaxanthin are involved in this process. In high light, the increase in the pH gradient across the thykaloid membranes induces violaxanthin to be converted to antherxanthin and then zeaxanthin by the enzyme violaxanthin de-epoxidase (VDE) (Demmig-Adams and Adams, 2002) in a process which can be reversed when light levels decrease by ZEP. Protons and zeaxanthin are bound to the light harvesting antennae proteins, facilitating quenching of excitation by dissipating heat (Demmig-Adams and Adams, 1992; Horton *et al*., 1996). The *Arabidopsis npq1* mutant, which is unable to de-epoxidise violaxanthin, shows increased photosensitivity (Niyogi *et al.,* 1998). When this deficiency is combined with the *lut2* mutant, in which the formation of lutein is blocked (Pogson *et a*l., 1996), the induction of NPQ was severely inhibited (Niyogi *et al*., 2001). The importance of xanthophylls in photoprotection mechanisms has been further demonstrated using a range of *Arabidopsis* lines combining three different mutants (*npq1*, *npq2* and *lut2)* (KalituIho *et al*., 2007).

1.2.5 Manipulation of ABA accumulation in plants

It has been demonstrated on several occasions that elevation of ABA concentrations in response to mild drought stress can increase WUE (Davies *et al*., 1978; Jones, 1993; Turner, 1997; Lamberts *et al.*, 1998) by restricting stomatal opening (*cf.* Chapter 5, Section 5.1.2.2 for discussion). When this response was artificially mimicked through long-term exogenous application of ABA, WUE was also improved (Bradford *et al.,* 1983). The PRD method of irrigation exploits the intrinsic water-stress response mechanisms of plants to reduce water use and, in some cases, improve WUE (Section 1.1.1.2). It is therefore possible that, due to the role of ABA in regulating stomatal behaviour, long-term increases in endogenous ABA levels may also improve WUE under well-watered conditions.

Genetic manipulation of key regulatory enzymes in the ABA biosynthesis pathway is one method of achieving elevated ABA accumulation under non-stressed conditions. The ever-increasing knowledge of the ABA biosynthesis pathway, coupled with general advances in transgene technology, is allowing the production of transgenic plants with upregulated activity of one or more key ABA biosynthetic enzymes. It has, however, been suggested that the potentially beneficial effects of such upregulation could be hindered by the activation of a negative feedback system

which would increase the rate of ABA degradation, thereby preventing over-production of ABA (Cutler *et al*., 1997).

It has been demonstrated that the enzyme 9-*cis*-epoxycarotenoid-dioxygenase (NCED) catalyses a key rate-limiting step in ABA biosynthesis and, as such, it has been a target for the manipulation of ABA biosynthesis. It has been shown that upregulation of a single *NCED* gene in tomato (Thompson *et al*., 2000a), tobacco (Thompson *et al*., 2000a; Qin and Zeevaart, 2002) and *Arabidopsis* (Iuchi *et al*., 2001) results in plants exhibiting 'high ABA' concentrations. Thus, in transgenic tobacco plants, the inducible over-expression of *LeNCED1* led to a significant increase in whole plant ABA concentration (Thompson *et al*., 2000). In plants grown in tissue culture, this inducible *NCED* upregulation increased ABA concentration by ten fold in leaves (Thompson *et al.*, 2000a). Over-expression of *AtNCED3* in *Arabidopsis* resulted in significant increases in ABA concentration, with associated reduction in transpiration and visible improvement in drought tolerance (Iuchi *et al*., 2001). Similarly, Qin and Zeevaart (2002) reported that the inducible over-expression of *PuNCED1* in tobacco increased ABA accumulation. In this case, the authors also reported an increased accumulation of phaseic acid (an ABA catabolite), at least partially confirming the suggestion that ABA over-production would be accompanied by an increased ABA degradation.

Thompson *et al*. (2000a) characterised three tomato primary transformants, which over-expressed *LeNCED1* using a strong constitutive promoter, the Gelvin 'super-promoter'. Homozygous true breeding lines from two of these primary transformants exhibited increased steady-state *LeNCED1* mRNA levels, resulting in an elevated bulk seed and leaf ABA concentrations. These plants exhibited several phenotypes consistent with those expected for an increased level of ABA, including reduced stomatal conductance under unstressed conditions, increased seed dormancy and an over-guttating phenotype that was attributed to a combination of increased root hydraulic conductivity and stomatal closure (Thompson *et al*., 2000a). In view of the observation that the elevated ABA levels in these transgenic lines alter stomatal behaviour, they may potentially provide the basis for improving the WUE of commercial tomato crops. This hypothesis was tested by the research described in this thesis.

2 AIMS AND OBJECTIVES

ABA influences transpirational water loss, and hence plant water status, by restricting stomatal conductance. It has been shown that WUE can be improved under mild water stress, possibly because there is a non-linear relationship between the decrease in water loss and any accompanying reduction in carbon assimilation when mean stomatal aperture is slightly reduced. Transgenic tomato lines, previously transformed with constructs designed to constitutively over-express the *LeNCED1* gene, exhibit increased bulk leaf ABA concentrations (Thompson *et al*., 2000a). These 'high ABA' tomato lines constantly generate a water stress signal, even when grown under non-stressful conditions, and have already been shown to exhibit reduced stomatal aperture under environmental conditions in which WT control plants exhibit high stomatal conductances (Thompson *et al*., 2000a). It is therefore hypothesised that manipulation of the modified stomatal behaviour shown by the 'high ABA' lines may be used to curb the excessive transpiration typical of WT plants, so increasing their WUE. The primary aim of the work reported here was to assess the potential effectiveness of various transgenic tomato lines that are 'pre-adapted' to water stress *via* their increased ABA biosynthesis to improve the WUE of commercial tomato crops.

The 'high ABA' tomato lines currently available also provide a new opportunity to expand current knowledge of the role of ABA in controlling plant growth. It has been suggested that, whilst limiting transpiration, the restriction of stomatal opening may, in some circumstances, adversely affect assimilation rate. There is also a possibility that increased concentrations of bulk leaf ABA may directly affect shoot growth and alter plant development, potentially impairing the productivity of 'high ABA' lines. The project examined the effects, both positive and negative, of increased ABA accumulation on growth and development.

In transgenic tomato plants over-expressing *LeNCED1* alone, it is possible that the sustainability of ABA over-production is limited by availability of the β-xanthophyll precursors of ABA. Creating plants with simultaneous over-expression of *LeBCH2* and *LeNCED1* may provide greater substrate availability to the NCED enzyme,

thereby sustaining an increased flux through the ABA biosynthetic pathway. The research evaluated the effect of combining lines containing constructs designed to over-express these two ABA biosynthetic genes on whole plant WUE. Their use as potential 'high ABA' rootstocks was also evaluated. In this alternative approach, transgenic rootstocks were grafted to WT scions with the objective of moderating stomatal behaviour. Finally, a preliminary evaluation of the potential of an incomplete transgenic tomato line over-expressing three ABA biosynthetic genes as a 'high ABA' rootstock was undertaken.

The primary aims were to:

(1) Identify suitable irrigation regimes/experimental methods under which differences in water use and growth/productivity between WT and 'high ABA' lines could be identified.

(2) Use the most appropriate experimental method to evaluate the WUE of a range of 'high ABA' lines which over-produce ABA to varying degrees to determine the optimal level of ABA over-production required to maximise water economy without affecting growth and productivity.

(3) Evaluate the effects of increased ABA accumulation on germination, seedling establishment and long-term growth, development and productivity, using a range of 'high ABA' lines which over-produce ABA to varying degrees.

(4) Determine the effectiveness of combining the over-expression of two or more ABA biosynthetic genes to improve whole plant WUE or for use as 'high ABA' rootstocks.

3 GENERAL MATERIALS AND METHODS

3.1 Seed stocks

At the beginning of the research project, all seed was obtained from Dr. Ian Taylor (The University of Nottingham) and Dr. Andrew Thompson (Warwick HRI) and subsequently stored at room temperature in the laboratory. Unless otherwise stated, a homozygous tomato mosaic virus (TMV) resistant line of *Solanum lycopersicum* L. (species formally known as *Lycopersicon esculentum* Mill.) cv. Ailsa Craig, GCR267 *Tm-2*^{2} (Lanfermeijer *et al*., 2003), was included in experiments as the wildtype control (WT).

3.1.1 *sp::LeNCED1* seed (pure breeding lines sp12 and sp5)

The *sp::LeNCED1* transgene was constructed as described by Thompson *et al*. (2000a), based on cDNA clones that had been isolated at The University of Nottingham (*cf*. Section 3.1.2 for more details), and this construct was used in the *Agrobacterium tumefaciens* mediated transformation (Bird *et al*., 1988) of wild type tomato (*Solanum lycopersicum* L. cv. 'Ailsa Craig') at Warwick HRI (as described in Thompson *et al*., 2000a). Three primary transformants, sp::*LeNCED1*-9 (originally termed D9 but later changed to sp12, *cf*. Thompson *et al*., 2007a), sp*::LeNCED1*-5 (sp5) and sp*::LeNCED1*-6 (sp6) all exhibited symptoms of ABA over-production and were selected for more detailed study (Thompson *et al*., 2000a). True breeding lines for sp12 and sp5 were subsequently identified (Thompson *et al*., 2007a).

3.1.2 *35S::LeCrtRb2* seed (pure breeding line BCH12)

A full length *LeBCH-2* cDNA clone, identical to the coding sequence of the tomato gene *CrtR-b2* (accession number Y14810; Hirschberg, 1998), was isolated by screening a λ ZAPII wilt-related tomato leaf cDNA library prepared at the University of Nottingham (Burbidge *et al*., 1997). This was then converted to a phagemid (pBluescript) and used to make the *35S::LeCrtRb2* construct required for transformation. The construct was then utilised in the *Agrobacterium tumefaciens* mediated transformation (Bird *et al*., 1988) of (*Solanum lycopersicum* L. cv. Ailsa

Craig, *Tm-2*^{2}), using the same protocol described previously (Thompson *et al*., 2000a). One primary transformant, termed BCH12, with a single T-DNA insertion locus was subsequently selected and made homozygous for the transgene by selfing, with its progeny confirmed as homozygous *via* a PCR-based segregation analysis (Sonneveld *et al*. in prep.).

3.1.3 *sp::LeNCED1* and *35S::LeCrtRb2* 'double transgene' seed (pure breeding lines G28 and G29)

To create transgenic lines which simultaneously over-express *LeBCH2* and *LeNCED1*, a homozygous *35S::LeCrtRb2* over-expressing line (BCH12) was crossed in turn with the two homozygous *sp::LeNCED1* lines (sp12 and sp5). In order to select 'double transgene' lines, F_1 plants, identified as containing both the *sp::LeNCED1* and *35S::LeCrtRb2* constructs were selfed. The subsequent F_2 generation was screened by Q-PCR using a method based on that of German *et al*. (2003). This allowed the selection (Sonneveld *et al*. in prep.) of two homozygous, true breeding lines, G28 (BCH12/sp12) and G29 (BCH12/sp5).

3.1.4 *rbcS3C*::*LeNCED1* seed (pure breeding rbcS-4, -10, -17, 18)

The tomato promoter region from a RuBisCO small subunit gene, *rbcS3C* (GenBank accession number X05986; Sugita *et al.*, 1987) was amplified from a plasmid (pSCV1.6RBCS3CP; Gittins *et al.*, 2000) and the PCR product was cloned and used to produce the *rbcS3C*::*LeNCED1* construct. This was then transformed into tomato (*Solanum lycopersicum* L cv. Ailsa Craig, *Tm-2*^{2}) at Warwick-HRI (as described in Tung *et al*., 2008). The presence of the *rbcS3C*::*LeNCED1* transgene was confirmed by PCR and true breeding homozygous lines were identified in progeny from the primary transformants numbered 4, 10, 17 and 18. These four lines were chosen to represent a relatively wide range of severity of phenotype.

3.1.5 Seed Harvesting

The ripe fruits were sliced transversely across the middle and seed was removed from each locule. The seed was then briefly rinsed with water in a sieve before being soaked in a 1 g l^{-1} pectinase (Boots pharmaceuticals)/ 12mM HCl (Fisher) solution

for 24 hours (Silva *et al*., 1982). The seeds were subsequently rinsed in water and air-dried on Whatman filter paper (Whatman, Clifton, NJ) at ambient temperature and humidity for 24 hours.

3.2 Standard plant husbandry

All experiments were conducted in a partially environmentally controlled glasshouse. The minimum day and night temperatures were 24^{o}C and 18^{o}C respectively (±0.5^{o}C), with ventilation if temperatures exceeded 25^{o}C. Artificial light was used to supplement natural light as necessary, to achieve a 16/8 hour day/night regime. Unless otherwise specified, plants were watered by hand and fed (1:1:3 NPK) *via* the irrigation water.

3.3 Seed germination protocols

3.3.1 Norflurazon treatment

A 1mg ml^{-1} stock solution of norflurazon was prepared by dissolving 10mg of pure norflurazon (Sigma, UK) into 10ml sterile distilled water (SDW). This solution was then stored in the dark at -4°C (for a maximum of 6 months). The appropriate norflurazon dilution was prepared as required (*cf.* Table 3.1) using SDW. A 9cm diameter Whatman No. 1 filter paper (Whatman, Clifton, NJ) was folded to produce a raised central ridge and placed in a 9cm Sterilin petri dish and the norflurazon solution was applied, leaving a reservoir of solution either side of the ridge. Ten seeds were placed on either side of the ridge with the filter paper acting as a wick to imbibe the seeds in the norflurazon solution, whilst preventing them from being submerged. The petri dishes were placed in a controlled environment growth room (12h/12h day/night regime, 25^{o}C). On completion of the appropriate duration of norflurazon treatment, seeds were removed from the petri dish and washed thoroughly in water using a sieve to remove any residual norflurazon.

3.3.2 Sowing into compost

Seeds were sown into Levington F2s compost in individual 9 cm pots at a depth of 1 cm. The compost was carefully compacted to ensure close contact with the seed and

prevent any possibility of desiccation. The seeds were kept under standard glasshouse conditions (*cf*. Section 3.2). To maintain high humidity in the air space above the compost and facilitate the softening of the testa, each pot was covered with the lid of a 9 cm Petri dish (Fig. 9.1). These were removed following emergence of the hypocotyl hook to avoid hindering seedling development, allowing the cotyledons to emerge into the ambient atmospheric environment of the glasshouse. Germination was judged to have occurred when both cotyledons had fully emerged from the testa

Table 3.1. Schedule of sowing and norflurazon treatment required for the synchronisation of growth of 'high ABA' lines with WT at point of germination and alternatively at the four fully expanded leaf stage. N.B. This recommended sowing schedule was devised during the course of the present research programme based on pooled data from a large number of different experiments.

Line	Norflurazon Treatment Concentration	Norflurazon Treatment Duration	Advanced sowing (d) before WT in order to synchronise growth at:	
			Germination	Four fully expanded leaf stage
BCH12	N/A	N/A	0	2
sp12	N/A	N/A	5	8
sp5	0.1mg l^{-1}	24 hours	7	16
G28	1mg l^{-1}	72 hours	8	16
G29	1mg l^{-1}	96 hours	8	20

3.4 Plant physiological analyses

3.4.1 Leaf gas-exchange

Gas exchange measurements were made using a CIRAS-1 Infrared Gas Analyser (PP Systems, Hitchin, Herts, UK) coupled with a 2.5cm^2 Parkinson leaf cuvette. The leaf chamber was illuminated with ambient light levels, CO_2 concentration was set to 350 μmol mol^{-1} and relative humidity was set to 60% of ambient. Gas-exchange calculations were based on the changes in the CO_2 concentration and moisture content relative to the reference air and water vapour provided by the cuvette. Measurements were made by placing the cuvette onto the terminal leaflet of the youngest fully expanded leaf, avoiding the midrib, allowing

the leaf to equilibrate within the cuvette for 1minute. Readings for the leaf stomatal conductance, transpiration rate and assimilation rate were then recorded.

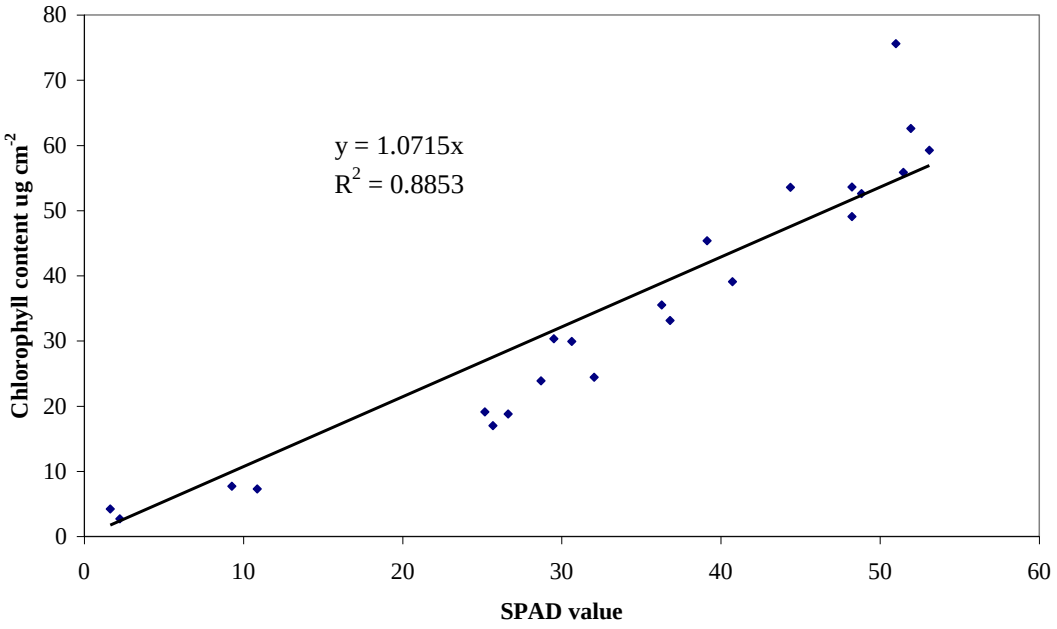


Figure 3.1. Calibration for determining leaf chlorophyll content from SPAD values, showing a linear relationship.

3.4.2 Leaf chlorophyll content

Leaf chlorophyll content was measured non-destructively using a portable Minolta SPAD-502 meter, which measures the radiation emitted by leaves at 650nm and 940nm, where chlorophyll absorption is at its highest and lowest respectively. Measurements were made for the terminal leaflet of the youngest fully expanded leaf, in which four readings were taken within the leaflet, avoiding the midrib and these were averaged to obtain a mean SPAD value. SPAD readings have a more or less linear relationship with the actual extractable leaf chlorophyll content of tomato leaf tissue. In order to validate this, a calibration (Fig. 3.1.) between SPAD values and leaf chlorophyll concentration was achieved by sampling leaves of a range of ages (and therefore chlorophyll contents) from four tomato lines (WT, sp12, sp5 and rbcS-10).

A mean SPAD reading for each chosen leaflet was obtained and then four 3 mm^2 leaf samples were independently collected in 1.5 ml amber eppendorf tubes and immediately cooled in liquid nitrogen. The leaflet was weighed, ground down to a

fine powder, and extracted overnight (12-24 h) in 1 ml of 80% aqueous acetone. The extracted sample was diluted to a final volume of 10 ml with distilled water in a 20 ml polypropylene tube. Chlorophyll concentration of each sample was determined spectrophotometrically by measuring absorbance of chlorophyll *a* at 664 nm and chlorophyll *b* at 647 nm using a UV spectrophotometer. The total chlorophyll content of fresh tissue of the sample was determined using the empirical equations published in Inskeep and Bloom (1985):

$$\text{Total Chlorophyll (mg l}^{-1}) = 17.9*\text{Abs}_{647nm} + 8.08*\text{Abs}_{664.5nm}$$

The mean total leaf chlorophyll content was calculated for each sample and plotted against the corresponding SPAD values to provide a calibration curve (Fig. 3.1). A near linear relationship between chlorophyll content and SPAD reading was observed ($R^2 = 0.8853$) and this was used to convert SPAD readings to total leaf chlorophyll concentration (μg cm^{-2}) during the plant analyses described in this research programme.

3.4.3 Plant growth measurements during destructive harvest

Stem height, internode number and the length of all leaves was first determined. Plant height was taken as the distance between the cotyledons and the top of the last internode greater than 2mm. The number of internodes along the stem over this same interval was noted, allowing mean internode length to be calculated, before excising the shoot below the cotyledons. Leaves were individually detached from the stem and the length was recorded. The stem, petiole, lamina and fruit (where present) components of the shoot were then separated and the fresh weight of each component was recorded (Sartorius Model 2574, Calibration Precision Balance Services). Leaf area was assessed by passing individual leaflets from each plant through a leaf area meter (LiCOR 3100, Lexicon Instruments, Lincoln, Nebraska). Lamina, petiole and stem materials were then put in separate paper bags and placed into a Gannet Model L6499 oven (LTE Scientific), where they were dried for 48 hours at 80 ^{o}C and weighed (Sartorius Model 2574, Calibration Precision Balance Services) to determine dry weight.

4 LONG TERM GROWTH AND DEVELOPMENT UNDER DIFFERING IRRIGATION REGIMES

4.1 INTRODUCTION

4.1.1 Commercial Tomato Production

4.1.1.1 Global Production

Domestication of the tomato crop is thought to have occurred in Central America in the 15th century (Harvey *et al.,* 2002). Today, tomatoes are an important economic crop in many countries, and global production (fresh and processed) has increased by *c.* 300 % in the last four decades. China is the biggest producer of tomatoes, followed by the USA, Turkey and India (Costa and Heuvuelink, 2005). Cultural practices vary between countries due to differing climates and types of tomato crop produced. In Northern Europe, tomatoes are mostly grown in controlled environments, i.e. glasshouses or "poly" (polythene) tunnels, whereas large scale field production is more common in USA, Spain, Mexico and Australia.

European tomato production can currently be divided into two major systems. The Northern European system is capital-intensive, using modern technology involving greenhouse structures, climate control and crop protection. This system is highly productive and focuses on the fresh tomato market. In Southern European countries with Mediterranean climates, the crop is produced mainly in open fields for processing and under poly-tunnels for fresh production (Harvey *et al.,* 2002). In Mediterranean regions, currently significant environmental and social pressures are impacting on water supply, as over-exploitation of aquifers by horticultural crop production has adversely affected both water quality and quantity (Martinez-Fernandez and Selma, 2004). One example of this is the Almeria region of Spain, which has the largest concentration of poly-tunnels in the world, with around 40,000 ha of protected crops being grown predominantly under flat-roof greenhouses (Cantliffe and Vansickle, 2000). However, water use for tomato production in the Almeria region is unsustainable, currently being five times greater than the annual rainfall (Chapagain and Orr, 2009). With increased demands on water resources, the irrigation required for high input crop production systems will become more

expensive. As climate change predictions suggest that overall rainfall may be reduced and become more erratic, both types of production system would benefit from tomato crops that can produce similar yields with a smaller input of water. Genetically modified (GM) tomatoes will become increasingly important for production in America, China and many other countries, but in areas such as the UK, where production of GM crops is not allowed, information arising from genetic modification might potentially be translated into the breeding of improved varieties using more conventional means (Costa and Heuvuelink, 2005).

4.1.1.2 Irrigation

Water availability has a crucial role in determining the yield of tomatoes (Rudich and Luchinsky, 1986). The yields of commercial crops are therefore dependent on frequent, controlled irrigation, with different irrigation regimes being used in glasshouse (Welles, 2005) and field grown situations (Sizinsky, 2005) to more effectively distribute water throughout the crop.

Tomato crops require large quantities of high quality water for transpiration, which serves both to cool leaves and trigger the transport of nutrients from the roots to leaves and fruit. Mature tomato crops use 2-3 litres of water per plant per day when radiation levels are high (OMFRA, 2003), using *c*. 90% of this water for transpiration and only 10% for growth. One irrigation method used in the production of many crops is drip or trickle irrigation, a term which refers to the frequent application of small quantities of water at low flow rates and pressures. The use of drip irrigation allows precise application of water directly to plants and, with frequent application of irrigation, plants suffer almost no water stress, as their water status remains almost constant. Under these conditions, it can be argued that a high proportion of the water supplied is being wasted, as transpiration is completely unrestricted. The use of novel irrigation practices is discussed in Section 1.1.1.

4.1.2 Increasing ABA production may affect economic yield

There is a close relationship between the transpiration of tomato crops and seasonal radiation patterns, meaning that under sunny conditions commercial varieties may open their stomata above the optimal level, thus wasting water without providing any photosynthetic benefit (*cf.* Section 10.4 for discussion of optimal levels of stomatal opening). When glasshouse heating systems are operating during the winter period in temperate environments, up to 50% of the total transpiration may occur at night, compared with only 5-8% for field-grown crops in summer (Portree, 1996). Therefore plants selected to be more restrained in their nocturnal stomatal opening would have substantially reduced water use overall. The yield of tomato crops is, however, determined by biomass production, biomass partitioning and the dry matter content of the fruit. If these parameters were adversely affected by breeding for crops which are pre-adapted to water stress and are more efficient in their water use, the advantage of reduced water input would be outweighed by adverse effects on growth and, ultimately, fruit production.

4.1.2.1 Biomass Production

The relationship between endogenous ABA levels and growth during water stress is still not fully understood. ABA accumulation often appears to be correlated with reductions in leaf and shoot growth (Zhang and Davies, 1990; Davies, 1995), but may not be its cause (Hussain *et al.*, 1999). Many studies which conclude that ABA is a growth inhibitor used methods involving application of exogenous ABA to unstressed plants, and the extent to which such treatments are predictive of the role of increases in endogenous ABA caused by water stress is highly uncertain, to say the least. Work by Gowing *et al.* (1991) using techniques involving split root systems showed that plants with half of their root system growing in dry soil had lower leaf growth rates than plants with all of their root system in wet soil. When the roots in contact with dry soil were severed, leaf growth rate increased to that of control plants, despite the fact that there was no increase in the quantity of water available to the roots. Whether ABA and/or some other chemical/physiological signals are involved in controlling this growth response to drying soil, has been a matter of controversy for many years.

Studies of the interactions between ethylene and ABA have sparked an interest in reassessing the role of ABA in regulating shoot growth in response to water stress (e.g. Sharp and LeNoble, 2002). Ethylene production is enhanced in the shoots of the ABA deficient tomato mutant *flacca* (Tal *et al.*, 1979), and many ABA-deficient mutants exhibit morphological symptoms characteristic of excess ethylene, including symptoms resembling leaf epinasty and increases in adventitious rooting (e.g. Tal, 1966). ABA-deficient mutants are usually shorter and have smaller leaves than the corresponding wild type (Quarrie, 1987), and several authors have demonstrated that this 'dwarfism' phenotype can be reversed by the application of ABA (e.g. Tal and Nevo, 1973). Following on from these observations, Sharp *et al.* (2000) and others have examined the possibility that increased ethylene levels are a cause of decreased shoot growth in ABA-deficient mutants. They showed that normal endogenous ABA levels are required to maintain shoot development and leaf expansion in well watered tomato plants, and that the impaired growth of ABA-deficient plants is at least partly attributable to excess ethylene. It has often been assumed that the restoration of growth following the application of ABA to mutant plants could simply be explained by the effect of ABA in restoring leaf water potential and turgor to levels conducive to growth. The observation that ABA-deficient tomato plants exhibit dwarfism when grown in normal atmospheric conditions and cannot be restored to normal growth rates under high humidity indicates that the reduction in growth is not simply an effect of excessive water loss (Sharpe *et al*., 2000).

It has also been suggested that, in some circumstances, ABA may increase the rate of leaf senescence, potentially decreasing the transpiring surface area (Zacarias and Reid, 1990). At a given stomatal conductance (g_s) and vapour pressure deficit (VPD), the rate of transpiration essentially depends on leaf area, meaning plants may be able to reduce transpiration during periods of water stress by reducing leaf area (Hsiao, 1973). When grown at high relative humidity, ABA over-producing transgenic tomato plants often display an overguttating phenotype (Thompson *et al*., 2000a), in which interveinal flooding and, in some cases, chlorosis of leaves can occur. The severity of these symptoms increases with increased levels of overproduction (Tung *et al*., 2008), although these effects are less dramatic in large, mature plants than in young plants. Conceivably, this effect of ABA over-production

may sometimes restrict the green leaf area available for photosynthesis and hence productivity (Heuvelink and Dorais, 2005).

Leaf growth is significantly more inhibited than root growth by adverse soil conditions, thus increasing root to shoot ratio and providing access to a larger soil volume compared to a situation where root and shoot growth were inhibited equally (Munns and Sharp, 1993). Increased ABA concentration in the roots may modify membrane properties (Hartung and Jeschke, 1999) partly through ABA-induced changes in the activity and abundance of aquaporins (Hose *et al*., 2000; Seimens and Zwiazek, 2004), so increasing root hydraulic conductivity and hence the potential water uptake per unit surface area and time (Glinka and Reinhold, 1971). Manipulating ABA concentration in the roots may therefore have the potential to increase the flow of water to the shoot. It has already been demonstrated that ABA over-producing tomato plants have increased root hydraulic conductivity (Thompson *et al.*, 2007a).

4.2 Aims

The experiments described in this Chapter involved the use of differing trickle irrigation regimes to investigate the long-term growth, physiological and developmental responses of transgenic plants engineered to accumulate consistently high concentrations of ABA, under both optimal and sub-optimal levels of irrigation.

ABA influences transpirational water loss, and therefore plant water status, by controlling stomatal conductance. As 'high ABA' plants continuously generate a water stress signal even when grown under non-stressful conditions, this may mean that they are not wasteful when water is still plentiful, thereby conserving soil moisture for future use. This could allow rapid growth rates to be sustained for longer periods relative to WT plants under sub-optimal irrigation. It is hypothesised that by manipulating stomatal behaviour in 'high ABA' plants, excessive transpiration may be prevented, thereby increasing WUE. The potential use of plants that are 'pre-adapted' to water stress to allow a reduction in total water use whilst sustaining growth and productivity, was assessed. However, it is possible that, whilst limiting transpiration, this restriction of stomatal opening may, in some

circumstances, adversely affect assimilation rate. The possibility that increased levels of bulk leaf ABA may directly reduce growth and therefore potentially impair the productivity of ABA over-producers was therefore also evaluated.

Maintaining levels of total biomass production is not the only consideration when assessing the productivity of these 'high ABA' plants, as high biomass does not necessarily result in high value yield. The fruit produced by tomato plants is the part of economic interest and so the effect of long term elevated levels of ABA on reproductive ability and fruit quality is also of great importance. As the fruit yield of tomato plants is determined by the number and weight of individual fruit high yields are also dependent on proper fruit set and reproductive development (Ho and Hewitt, 1986). Preliminary studies of the fruit production of 'high ABA' plants were carried out and the occurrence of any physiological disorders apparently associated with increased ABA levels was noted.

4.3 MATERIALS AND METHODS

4.3.1 Long Term Growth under Water-Rationed Conditions (Experiment 1)

This experiment compared the responses of three 'high ABA' genotypes and WT controls to progressive drought, induced by rationing the daily supply of water.

4.3.1.1 Plant Material

The assessment of the water-saving capacity of 'high ABA' plants involved using the Wild Type (WT) tomato variety Ailsa Craig and three transgenic tomato lines sp12, sp5, rbcS-10, which differed in the severity of ABA over-production (*cf.* Chapter 2 for full description of transgenes). Transgenic sp5 plants have regularly been found to overproduce ABA to a greater extent than sp12 (Thompson *et al*., 2000; Appendix 7), while rbcS-10, an 'ultra-high' ABA line, exhibits more extreme ABA over-accumulation symptoms (Tung *et al.,* 2008).

All plants were grown in well-watered conditions in the glasshouse until the main part of the experiment began. Although the sowing protocol for sp5 was subsequently refined and dramatically improved, germination of these transgenic

seeds was very poor in this experiment, with the result that only three plants were available for study. 12 plants of the transgenic genotypes, sp12 and rbcS-10 and 21 WT Ailsa Craig plants were selected for use in this growth experiment, based on uniformity in height and leaf area. Three representative plants from each genotype (excluding sp5) were analysed when the experimental period commenced (0 days), to determine the biomass at the start of the experiment and ensure that any subsequent trends in growth or physiology were not simply due to pre-existing differences in growth during the germination and seedling establishment phase. The remaining 9 plants for each treatment (three for sp5) were then potted into round 9 litre pots containing the commercial compost Levington M3 (Scott's Professional, Suffolk, U.K.).

4.3.1.2 Experimental Design

The first growth trial (Experiment 1) contained four water-rationing treatments, WT Ailsa Craig 50%, sp12 50%, sp5 50%, rbcS-10 50% and well-watered WT Ailsa Craig (WT 100%), arranged in a split-plot randomised block design (Fig. 4.1). Each block included three plants per treatment, arranged as a sub-plot. One plant from each sub-plot (treatment) was harvested at each of the three harvests. A split plot system was used to allow uniform spacing of plants, as the middle plant of each sub-plot of three was used for the final harvest. The three sp5 plants that were available were included within the split plot design, with pots containing only compost on either side to make up the sub-plot of three. The transgenic line sp5 could therefore only be included in the final destructive harvest at 96 days after the experiment commenced.

4.3.1.3 Irrigation Rationing Treatment

The pots were watered to field capacity until the experimental period commenced. After arranging the pots along the three irrigation lines on plastic sheeting to reduce risk of TMV infection, the experiment began and water was supplied by drip irrigation. Water was delivered automatically using solenoid valves programmed to supply water at regular intervals for specified durations. WT 100% plants received irrigation *via* two drippers, where as WT 50%, sp12 50% and rbcS-10 50% received water *via* one and were therefore given half the volume of water applied to WT

100% plants. Feed (1:1:3 NPK) was supplied via the irrigation water throughout the experimental period.

A progressive drought was bound to develop during this experiment as a fixed quantity of water was supplied despite the inevitable increase in demand as the plants grew larger. Water was supplied at 4 h intervals during the 24 h cycle starting at 0400 h and ending at 2400 h. The duration of delivery was set at 3 min for each irrigation until Harvest 2 (68 days). Thereafter, the water ration was increased to a 5 min delivery to prevent terminal drought stress from developing in plants which were to be grown until Harvest 3 (96 days). The volume of any excess water draining from the compost in which the plants were grown was recorded on a weekly basis. Vermiculite was placed on top of each pot to minimise evaporation from the soil surface.

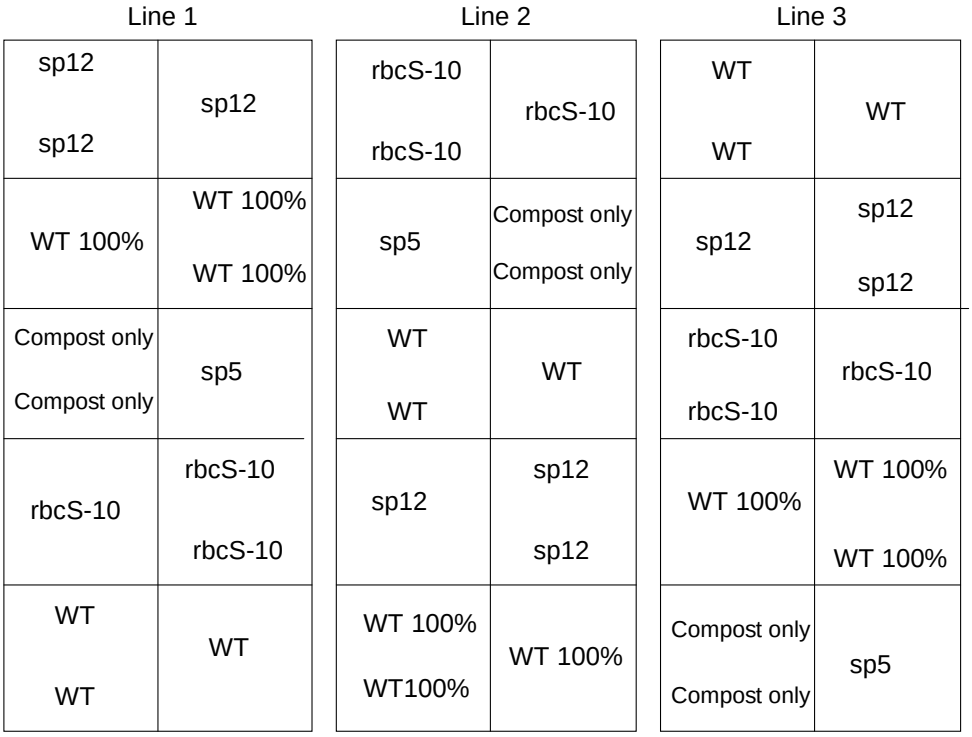


Figure 4.1. Experimental design of the water-rationing experiment.

4.3.1.4 Non-destructive Physiological Measurements

Measurements were made on the central plant in each sub-plot, as these plants were reserved for use in the final harvest (96 days); this approach meant that the plant sampled in sub-plot was consistent throughout the experiment. Gas exchange measurements were made using a CIRAS-1 Infrared Gas Analyser (PP Systems, Hitchin, Herts, UK) coupled with a 2.5cm^2 Parkinson leaf cuvette. Leaf chlorophyll content was measured non-destructively using a Minolta SPAD-502 meter. Measurements were made for the terminal leaflet of the youngest fully expanded leaf

4.3.1.5 Growth Analysis

Plant growth was determined by destructive analysis of one plant from each genotype x treatment combination at 45, 68 and 96 days after transplanting into the seven litre pots. Stem height, internode number and the length of all leaves were determined before excising the shoot below the cotyledons. Stem, petiole, lamina and fruit fresh weights were recorded. Fruit fresh weight was calculated for all fruit present at harvest, many of which were still immature. Leaf area was assessed by passing the leaves from each plant through a leaf area meter (LiCOR 3100, Lexicon Instruments, Lincoln, Nebraska). Lamina, petiole and stem materials were then placed in separate paper bags, dried to constant weight at 98 ^{o}C and weighed to determine their dry weight.

4.3.1.6 Data Analysis

Analysis of variance (ANOVA) for a split-plot block design was carried out for all data obtained using Genstat 8th edition (Lawes Agricultural Trust, Rothamstead, Herts, UK).

4.3.2 Genotype-Specific Management of Irrigation (Experiment 2)

4.3.2.1 Experimental design

Following the differences in growth and physiology identified between WT and sp12 in the initial water-rationing experiment, a second experiment was carried out to examine any differences in the growth of these two genotypes under optimal, well-watered growth conditions. Irrigation treatments were chosen to allow the pots containing sp12 plants to remain at field capacity, whereas the paired sample pots containing WT control plants received a water supply based on the daily amount used by the corresponding sp12 partner. This was achieved by having an experimental design incorporating four irrigation lines. Two lines (1 and 3) had an irrigation delivery rate set at a level that allowed both WT and sp12 plants to be grown under optimal conditions (WTC), with soil moisture levels maintained at field capacity in all pots; any excess water draining from each pot was measured on a weekly basis. Lines 2 and 4 provided an irrigation rate designed to maintain only the sp12 plants at field capacity (sp12C), with corresponding WT controls in these lines receiving the same amount of water as the transgenic plants irrespective of their own daily requirements. Plants were arranged in pairs along the irrigation lines (Fig. 4.2).

Figure 4.2. WT and sp12 plants arranged along four irrigation lines. Irrigation lines 1 and 3 supplied the amount of water required to maintain pots containing WT plants at field capacity (WTC). Irrigation lines 2 and 4 supplied the amount of water required to maintain pots containing sp12 plants at field capacity (sp12C).

4.3.2.2 Method of estimating overall water requirements for each genotype

As mentioned to earlier, a system was developed to enable any drainage water to be collected and accurately recorded on a daily basis, allowing total water supply to each plant to be calculated. This involved standing each pot in a container with a reservoir underneath capable of holding up to 2 litres of water (Fig. 4.3). The drainage water was used to ensure soil moisture levels remained at field capacity where required and to adjust irrigation rates accordingly. WT or sp12 plants were collectively considered to be at field capacity when no fewer than 50% of each genotype had some water draining from the pot each day. Soil evaporation was minimised by using Uvi ground cover disks placed on top of the compost (Fig. 4.3). Before the experiment commenced, the irrigation lines were set running for five days and the volume of water from each individual output was measured. These observations confirmed that the irrigation system uniformly distributed water along the lines and identified the volume of water delivered by each line per unit time.

Figure 4.3. Plants were grown in 9 litre pots covered with Uvi matting to minimise evaporation. Pots were placed in a container which acted as a reservoir to allow accurate measurement of the volume of drainage water.

4.3.2.3 Soil Moisture Content

Soil moisture content was measured using a Theta probe (Delta-T Devices, Burwell, Cambridge, UK). This was placed in the compost of each pot at three pre-determined locations to provide a mean value for each pot. This procedure was repeated three times during measurement weeks (weeks 2, 4, 6, 8, 10, 12 and 14) to provide weekly mean values for compost moisture content for all genotypes and watering treatments.

4.3.2.4 Plant Analyses

Gas exchange measurements were made using the CIRAS-1 IRGA and 2.5cm^2 Parkinson leaf cuvette during weeks 1, 8 and 16. Two destructive harvests were carried out, each using half of the plants, when all growth parameters recorded in the water-rationing experiment were determined. The influence of ABA accumulation on flower initiation was also analysed, as any agronomic advantage resulting from improvements in WUE might not be commercially viable if reproductive development and fruit quality were impaired. Time until first anthesis was recorded for each plant and fruit were harvested at a pre-determined stage of ripening (early breaker) to allow more accurate measurements of fruit production than those obtained in Experiment 1.

4.3.2.5 Data Analysis

Data were analysed using paired sample Student's t-tests within a Microsoft Excel worksheet.

4.4 **RESULTS**

4.4.1 Effects of elevated endogenous ABA concentration on long term growth under water- rationed conditions (Experiment 1)

4.4.1.1 Growth Analysis

The first harvest, 45 days after imposing the experimental treatments, showed no significant differences in vegetative growth (dry weight) between WT 100%, WT 50% and sp12 50% plants, indicating that WT and sp12 plants receiving half the irrigation volume of WT 100% plants had not yet had their growth rates reduced by the onset of water stress. This pattern also appeared to apply 68 days after imposing the watering treatments. However, at final harvest, after 96 days of growth, vegetative dry weight (DW) was greater in the sp12 plants than in WT 50% plants ($P < 0.05$; Fig.4.4). Mean vegetative dry weight was 49 g plant^{-1} in WT 50%, compared to 81.1 g in sp12 (l.s.d.: 24.37); thus, despite having received the same volume of water, the 'high ABA', sp12 plants produced 66% more vegetative biomass than the WT plants. It is also interesting to note that, even though WT 100% plants received twice as much water as the sp12 50% treatment, their vegetative DW was not significantly greater.

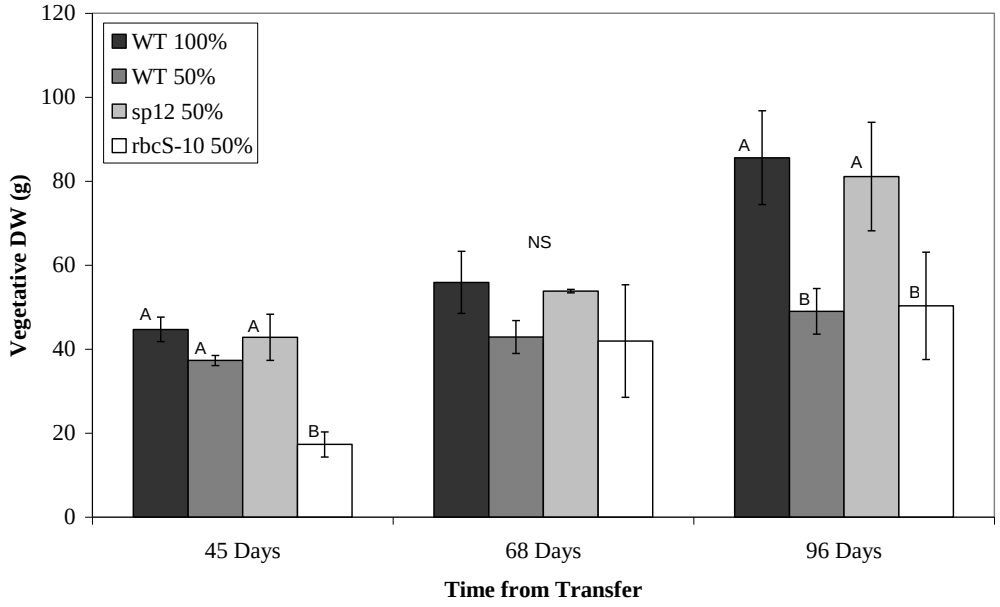
The 'ultra high' ABA rbcS-10 plants initially had a far smaller biomass than all other treatments at day 45 ($P<0.05$; Fig.4.4), an observation consistent with the hypothesis that more extreme levels of ABA over-production than those found in sp12 or sp5, might limit biomass production under well-watered conditions. However, after 96 days of growth under sub-optimal irrigation, the vegetative biomass of rbcS-10 plants was comparable to that of WT plants receiving the same quantity of water. Visual observations indicated that, although the WT 100% plants were beginning to show signs of water stress (e.g. wilting) by the end of the experimental period, the 'ultra high' ABA rbcS plants were not visibly stressed, indicating that they were able to conserve soil moisture better than the other genotypes examined.

As the experiment ended after 96 days, most of the fruit produced was immature and so provides only an approximate estimate of crop yield. However, it should be noted

that no significant difference in fruit production between sp12 50% and WT 50% plants was apparent at any of the harvests. WT 100% plants consistently produced the greatest number of fruit, possibly indicating that fruit production was affected by the water stress induced when WT plants received only half of the irrigation supplied to control plants. This difference between fruit production of WT 50% and WT100% plants was significant by harvest 3 ($P < 0.05$), in agreement with the pattern for vegetative biomass. rbcS-10 plants produced no fruit by Harvest 1, but had produced a limited number of fruit by Harvest 2 (68 days) and Harvest 3 (96 days) (Fig. 4.4). By contrast with the milder ABA over-producer (sp12), the 'ultra high' over-production in rbcS plants had negative implications for growth and development.

The absence of any difference in height between WT 100%, sp12 50% and rbcS-10 50% plants shows that the WT plants achieved no increase in height despite receiving twice the quantity of water. Therefore, the 'high ABA' plants had grown almost twice as tall per unit of water supplied. However, the transgenic genotypes were consistently taller than the corresponding WT 50% plants, with the difference being highly significant by Harvest 3 ($P < 0.005$; Fig. 4.5). The height achieved by WT 50% plants at Harvest 3 was 48% less than the mean value for the transgenic plants, as the WT plants exhibited restricted growth close to the apex due to lack of available water towards the end of the 96 day experimental period. The 'high ABA' plants conserved soil moisture more effectively and so were less affected. In contrast to the differences between sp12 and rbcS-10 plants in DW and fruit number, there was no significant difference in height at any of the harvests. At Harvest 1 (45 days) and 3 (96 days), internode length was greater in rbcS-10 plants than in WT plants ($P < 0.05$).

A



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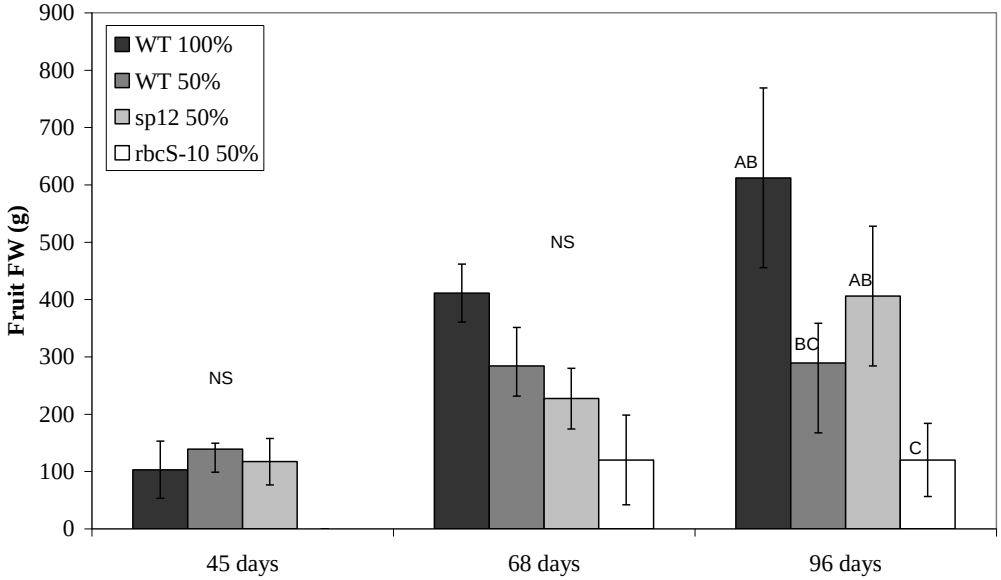
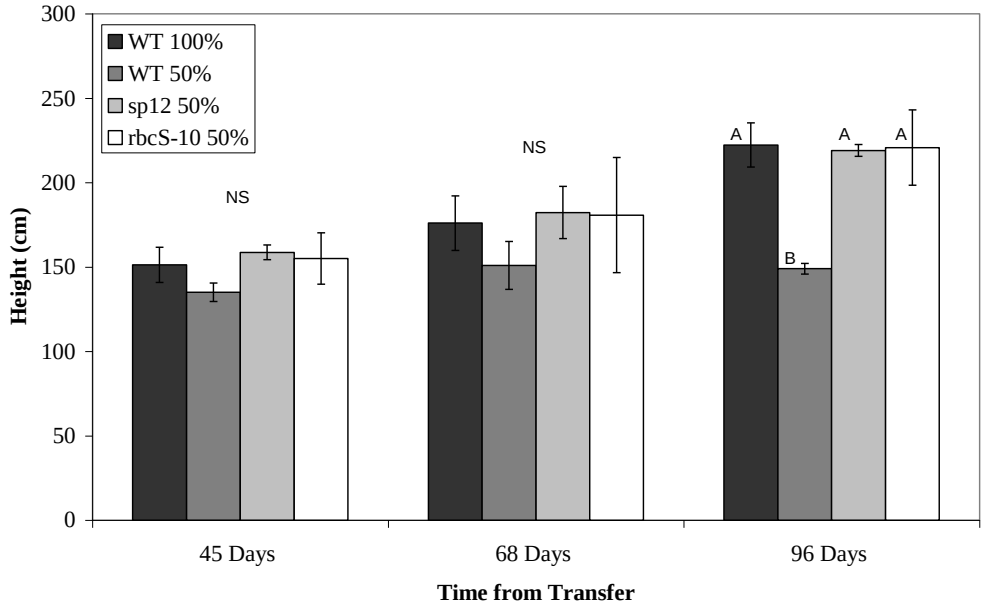


C

	Harvest 1 (45 days)		Harvest 2 (68 days)		Harvest 3 (96 days)	
	Veg DW	Fruit FW	Veg DW	Fruit FW	Veg DW	Fruit FW
F pr.	*0.005*	*0.056*	*0.602*	*0.093*	*0.024*	*0.011*
d.f.	6	6	6	6	6	6
s.e.d.	4.98	41.1	12.53	92.5	10.3	94.1
l.s.d.	12.18	100.6	30.65	226.5	24.37	222.5

Figure 4.4. A: Above-ground dry weight at 45, 68 and 96 days from transfer. **B:** Fruit fresh weight at 45, 68 and 96 days from transfer. **C:** Accompanying statistical summary. Bars with differing letters indicates a significant difference, NS denotes no significant differences across whole data set. Error bars represent standard error of the mean.

A



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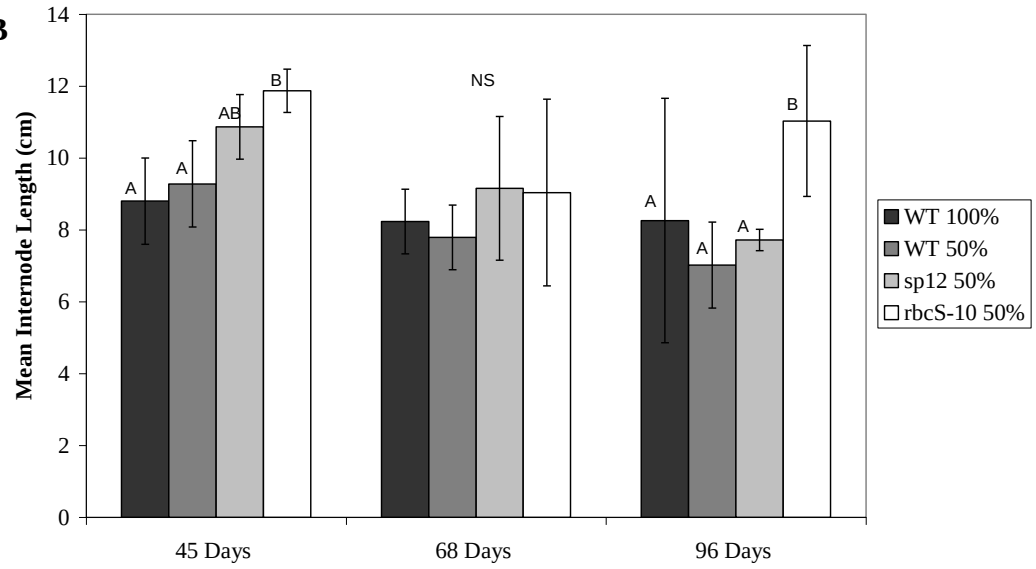


C

	Harvest 1 (45 days)		Harvest 2 (68 days)		Harvest 3 (96 days)	
	Height	Int length	Height	Int length	Height	Int length
F pr.	*0.154*	*0.043*	*0.667*	*0.222*	*0.005*	*0.003*
d.f.	6	6	6	6	6	6
s.e.d.	9.28	0.884	27.82	0.662	14.4	0.64
l.s.d.	22.7	2.163	68.07	1.621	34.06	1.512

Figure 4.5. A: Plant height and **B**: mean internode length at 45, 68 and 96 days after the experiment commenced. **C:** Accompanying statistical summaries. Bars with differing letters indicate significant differences; NS denotes no significant difference. Error bars represent standard error of the mean.

At Harvest 1 leaf area was comparable in WT 50%, sp12 50% and WT 100% plants and was greater than in rbcS-10 plants. By Harvest 2, however, the ranking of leaf area had altered, being lower in WT 50% plants than in both sp12 and rbcS-10 (Table 4.1), although the differences were not significant. This indicates that limited irrigation had a greater effect on leaf production and expansion on WT plants than in the transgenic lines which were able to conserve water during the initial growth period. Indeed, the leaf area of sp12 50% plants increased between Harvests 2 and 3, whilst that of WT 50% decreased (Table 4.1). This suggests a difference in the severity of water stress between the two genotypes, with sp12 50% plants continuing to grow during this final period of the experiment when WT 50% plants experienced severe water stress. At Harvest 3, sp12 had the greatest leaf area of all treatments; despite receiving only half the volume of water supplied to WT 100% plants. Transgenic plants consistently had longer leaves throughout the experiment and by Harvest 3, the leaves of sp5, sp12 and rbcS-10 were significantly longer than in WT 50% plants ($P<0.05$; Table 4.1).

The differing severity of progressive stress imposed on WT 100% and WT 50% plants did not affect partitioning of above-ground vegetative biomass (Fig. 4.6). The fact that rbcS-10 exhibited significantly longer internodes at Harvest 3 ($P<0.05$) was reflected by the distribution of biomass, as rbcS-10 plants partitioned a greater proportion of their assimilate to the stem than all other genotypes.

Table 4.1. Mean leaf length and leaf area at 45, 68 and 96 days after the experiment commenced, with accompanying statistical summary.

	Harvest 1 (45 days)		**Harvest 2 (68 days)**		**Harvest 3 (96 days)**	
	Mean leaf length (cm)	Leaf Area (cm^2)	Mean leaf length (cm)	Leaf Area (cm^2)	Mean leaf length (cm)	Leaf Area (cm^2)
WT 100%	39.6	5371	36.8	3930	38.7	4370
WT 50%	37.7	5414	34.5	2802	33.2	1900
sp12 50%	40.6	4941	40.5	3177	40.4	5362
sp5 50%	N/A	N/A	N/A	N/A	42.5	3825
rbcS-10 50%	41.2	2667	39.8	3147	38.7	2320
Mean	39.8	4598	37.9	3264	38.7	3556
Fpr.	*0.075*	*0.052*	*0.074*	*0.731*	*0.006*	*0.087*
d.f.	6	6	6	6	7	7
s.e.d.	1.104	854.6	1.976	1010.3	1.571	1141.9
l.s.d	2.702	2091.1	4.836	2472	3.716	2700.1

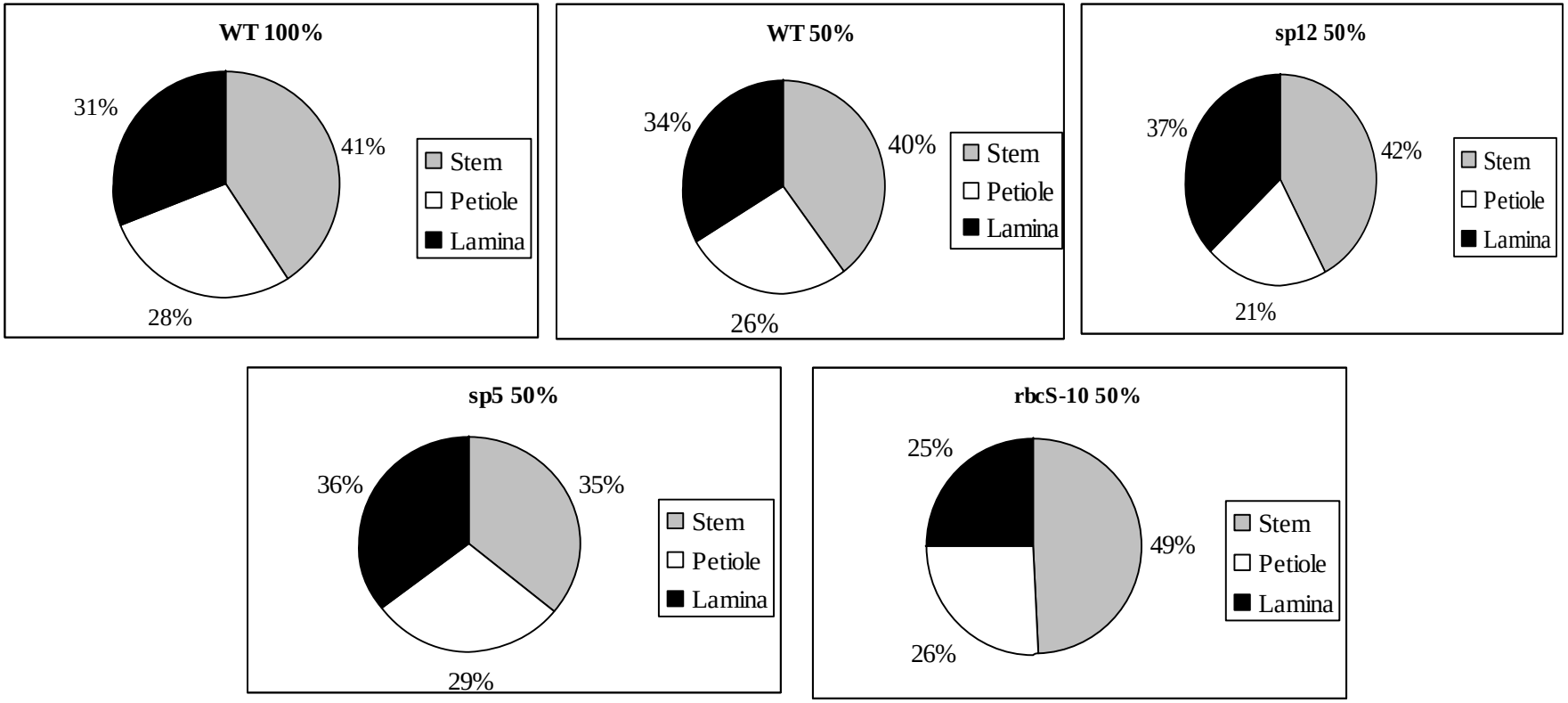


Figure 4.6. Partitioning of above ground vegetative biomass at harvest 3 (after 96 days of growth).

4.4.1.2 Stomatal Conductance

The greatest differences in stomatal conductance were observed during the initial period, before plants started to experience water stress (Fig. 4.7). Measurements made on Day 14 demonstrate that, under optimal conditions, WT 50% and WT 100% plants had higher stomatal conductances than the ABA over-producing genotypes ($P<0.001$). By this time, 14 days of water rationing treatment had significantly reduced the maximal stomatal conductance of WT 50% plants relative to the fully watered WT 100% treatment ($P<0.001$). By Day 80, WT 50% plants had depleted their soil water reserves due to their more rapid transpiration during the early phase of the experiment and were starting to show visible symptoms of water stress. Their stomatal conductances reflect this as they decreased to similar levels to sp12 and rbcS-10 plants. These results show that the greatest gains in water conservation through reduced transpiration occurred under optimal conditions, during the early stages of the experiment, i.e. before the WT plants experienced water stress and stomatal conductance was reduced in WT plants. As WT plants acted more like the ABA over-producer genotypes during the later stages of the experiment, differences between genotypes become undetectable.

4.4.1.3 Chlorophyll Content

Measurements of chlorophyll content made using a SPAD meter on seven dates during the experimental period (Fig.4.7) showed no significant difference between WT 50%, sp12 50% and WT 100% plants. It has been observed that high ABA transgenic tomato plants often display an overguttating phenotype (Thompson *et al*., 2000), during which interveinal flooding of leaves may occur and chlorosis may develop due to a decline in chlorophyll content. There were, however, no visual signs of over-guttation or interveinal flooding in sp12 plants, probably because these effects usually occur under conditions of low evaporative demand.

The rbcS-10 50% plants exhibited a ‘yellow’ phenotype, which was most extreme in the first four leaves produced. Consistent with this observation, these plants had a lower leaf chlorophyll content than sp12 and WT plants on all measurement dates ($P<0.001$; Fig. 4.7). The lower chlorophyll content of the ‘ultra high’ rbcS-10 plants

may reduce their photosynthetic capacity and contribute to their lower growth rates under optimal conditions (Fig 4.4).

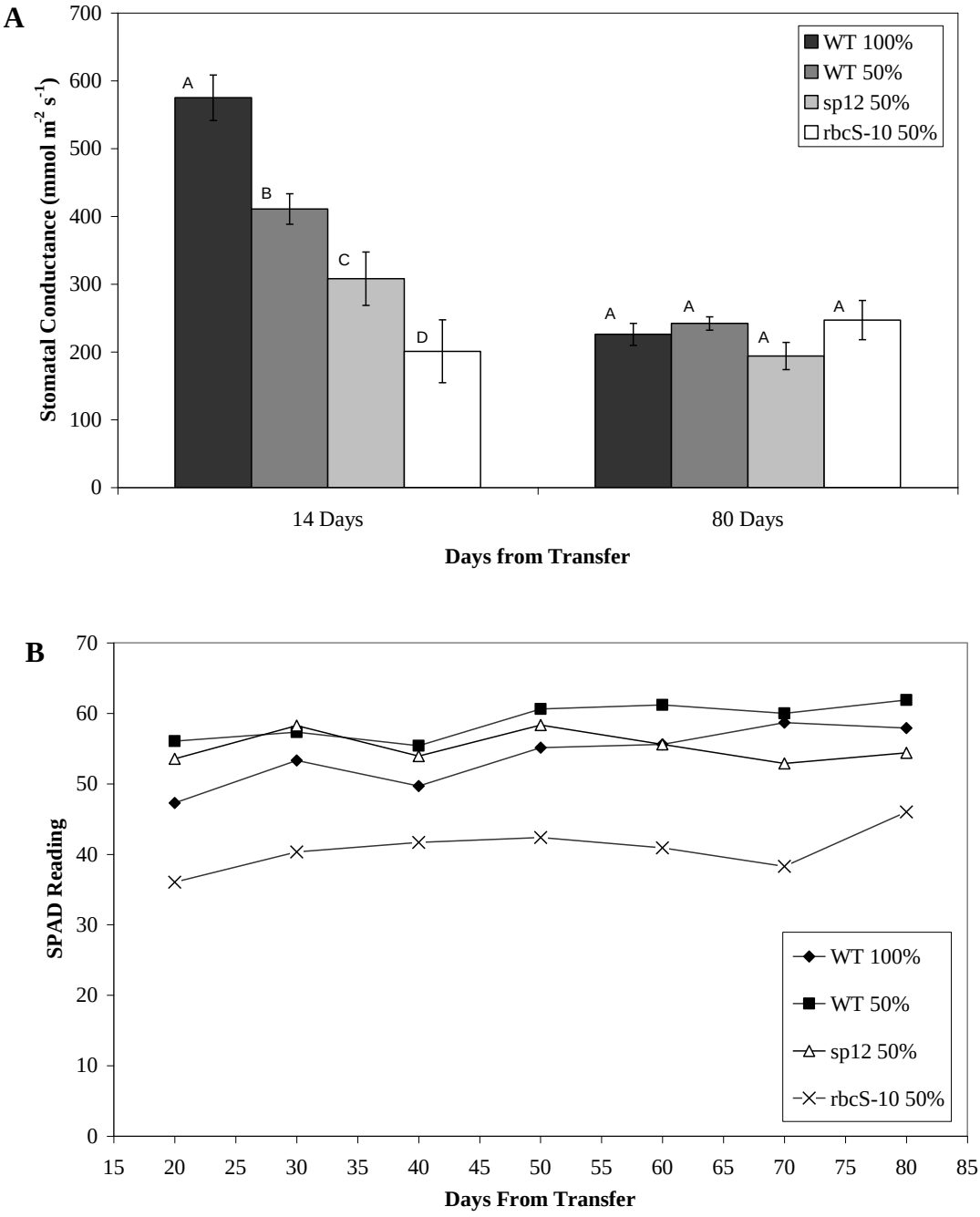


Figure 4.7. A: Stomatal conductance in all treatments on Days 14 and 80. (Day 14: P<0.001, s.e.d. 48.1, d.f. 30); Day 80: P>0.05, s.e.d. 45, d.f. 6). **B**: SPAD values showing time courses of leaf chlorophyll content, n=30. Bars with differing letters indicate a significant differences (P<0.05) between genotypes. Error bars represent standard error of the mean

4.4.2 Genotype-Specific Management of Irrigation

Drainage from pots was recorded daily to determine the volume of irrigation water not used by each genotype and to provide a means of controlling the quantity of irrigation required for each treatment. Measurements of mean drainage for each genotype calculated on a weekly basis allowed the water saving capacity of sp12 plants to be compared with WT plants.

4.4.2.1 WT Controlled Irrigation

WT plants consistently used a larger fraction of the water applied, as a greater volume of water drained from the sp12 pots throughout the experimental period (Fig. 4.8). On average, drainage was 48% greater from sp12 plants than WT plants, and this difference was significant ($P < 0.001$) for all except Week 4 (Fig. 4.8). WT plants therefore almost invariably used a greater percentage of the irrigation supplied (Fig. 4.8).

4.4.2.2 sp12 controlled irrigation

When the volume of irrigation supplied was determined by the needs of sp12 plants, WT plants used up all of the irrigation water delivered each day such that drainage declined to zero, and became water stressed, during the last four weeks of the experiment (Fig. 4.8). The sp12 plants were maintained at or close to field capacity throughout the experimental period, with expected levels of drainage occurring on a daily basis. This demonstrated that WT plants consistently transpired greater quantities of water than sp12 plants, thus exhausting soil water reserves (Fig.4.8). When the irrigation volume was determined by the demand of sp12 plants, a smaller volume of irrigation was required daily. This was especially clear during the later part of the experiment (weeks 10-15), when demand for water was greatest. Thus, a mean of 1545 ml of irrigation water was delivered to ‘sp12-controlled’ plants (sp12C) on the final day of the experiment, compared to 1695 ml when demand by WT plants controlled irrigation levels.

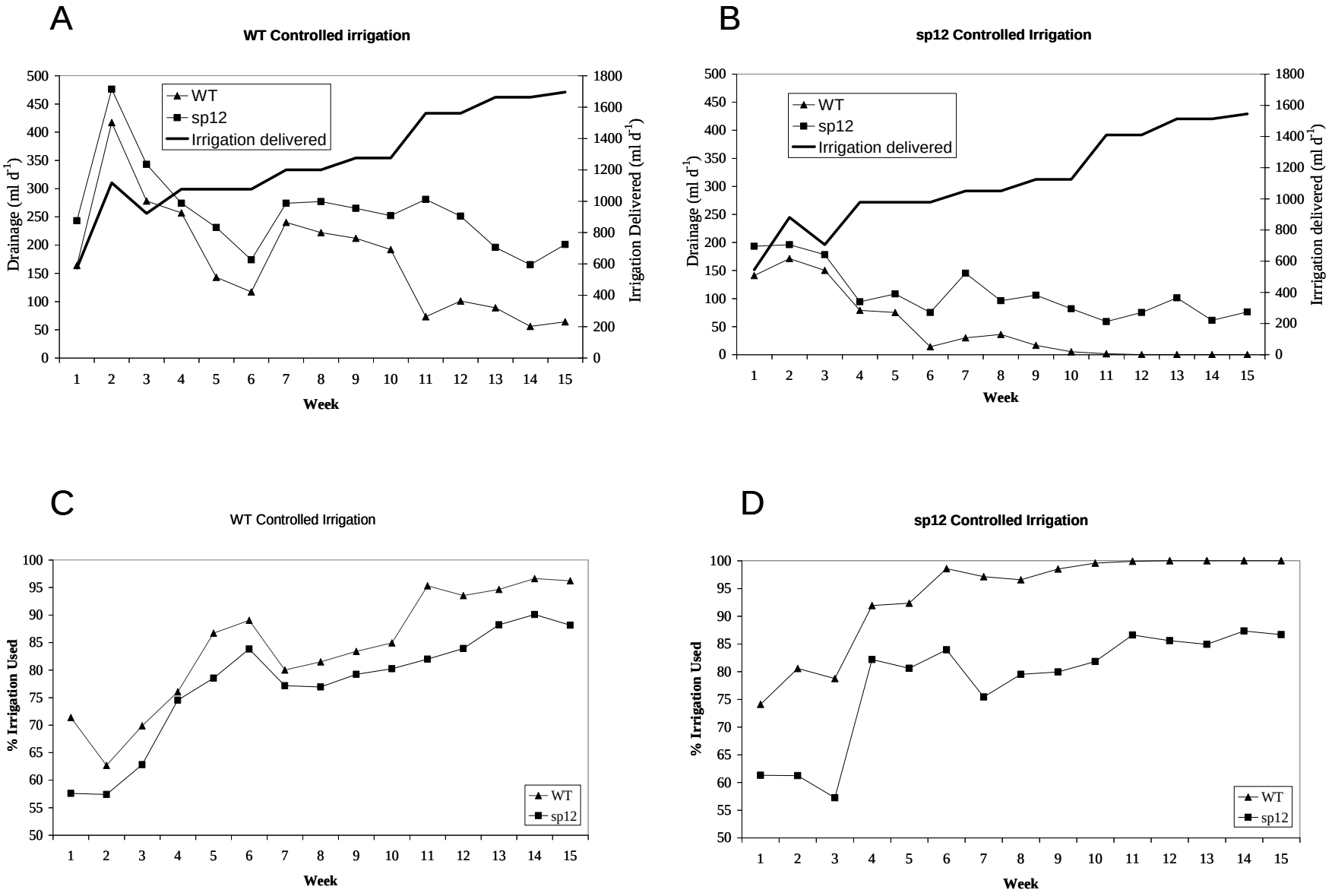
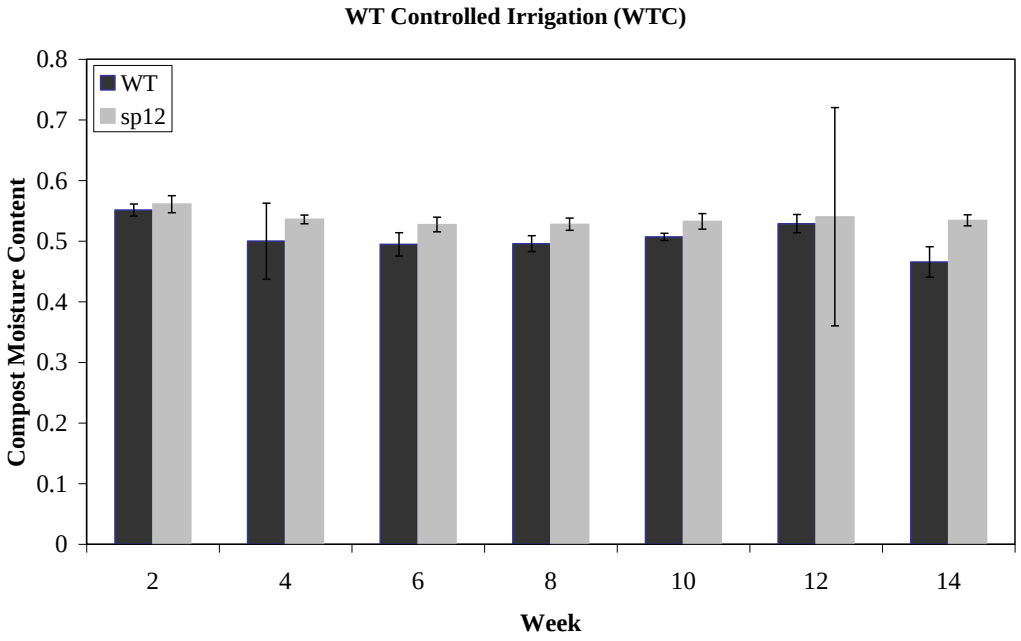


Figure 4.8. Mean daily drainage from WT and sp12 pots and irrigation volume delivered (A and B) and percentage of irrigation water consumed (C and D) when irrigation was controlled by water use of WT plants (A and C) or sp12 plants (B and D).

A



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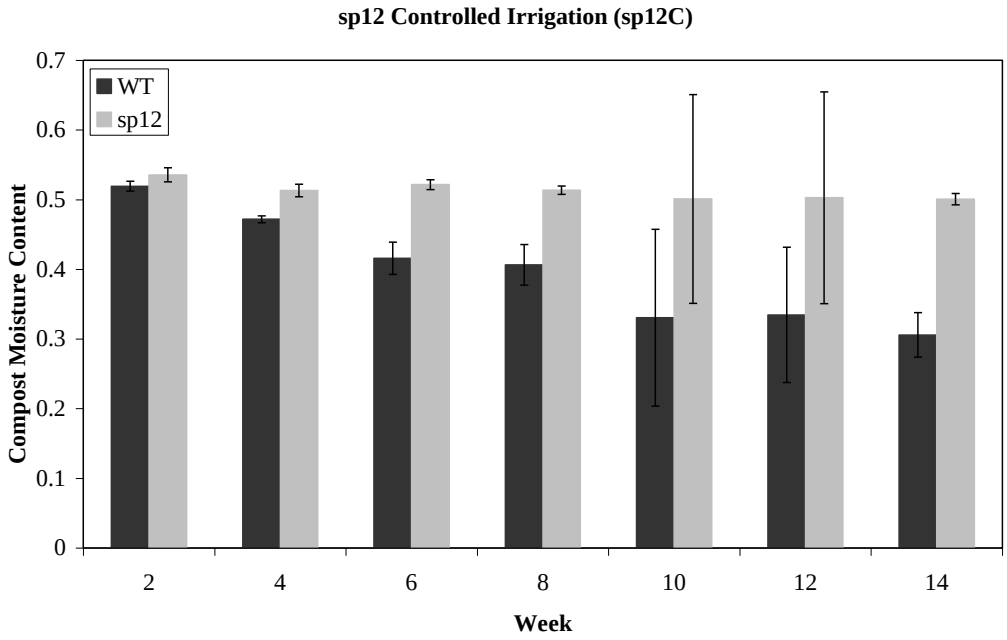


Figure 4.9. Compost moisture content for the WT and sp12 treatments when irrigation was controlled by **A** water use of WT plants and **B** the water use of sp12 plants. Error bars represent standard error of the mean.

4.4.2.3 Compost Moisture Content

Measurements of compost moisture content when sufficient irrigation was supplied to maintain sp12 plants at field capacity (sp12C-WT) demonstrated that WT plants depleted available water supplies, especially from Week 4 onwards ($P<0.001$; Fig. 4.9). Compost moisture contents in the other three treatments (WTC-WT, WTC-sp12, sp12C-sp12) remained relatively constant throughout the experimental period, demonstrating that field capacity was effectively maintained.

4.4.2.4 Stomatal Conductance

Measurements of stomatal conductance within Week 1 demonstrated that, under optimal conditions, the stomatal conductances of the WT control plants were on average 68% greater than in the 'high ABA' sp12 plants ($P<0.001$; Fig. 4.10). Stomatal conductance remained higher in WT than in sp12 plants in both irrigation treatments in Week 8 ($P<0.001$), but the WT plants receiving sp12-controlled irrigation had begun to show symptoms of water stress as there was no significant difference in conductance between the WTC sp12 and sp12C WT plants ($P>0.05$). By Week 16, there was still a difference between WT and sp12 plants grown under optimal conditions (WTC), with sp12 plants showing a two-fold reduction in stomatal conductance relative to WT plants. When irrigation demand was determined by sp12 plants, however, stomatal conductance was greatly reduced in WT plants due to the formation of water stress. However, this effect was much less severe than in the previous water-rationing experiment (Fig. 4.7), demonstrating that the demand-led irrigation system caused less severe water stress, as water supply was increased to some extent as the plants grew larger and therefore required increasing quantities of water. These plants showed a four-fold reduction of stomatal conductance relative to WT plants experiencing well-watered conditions (WTC-WT).

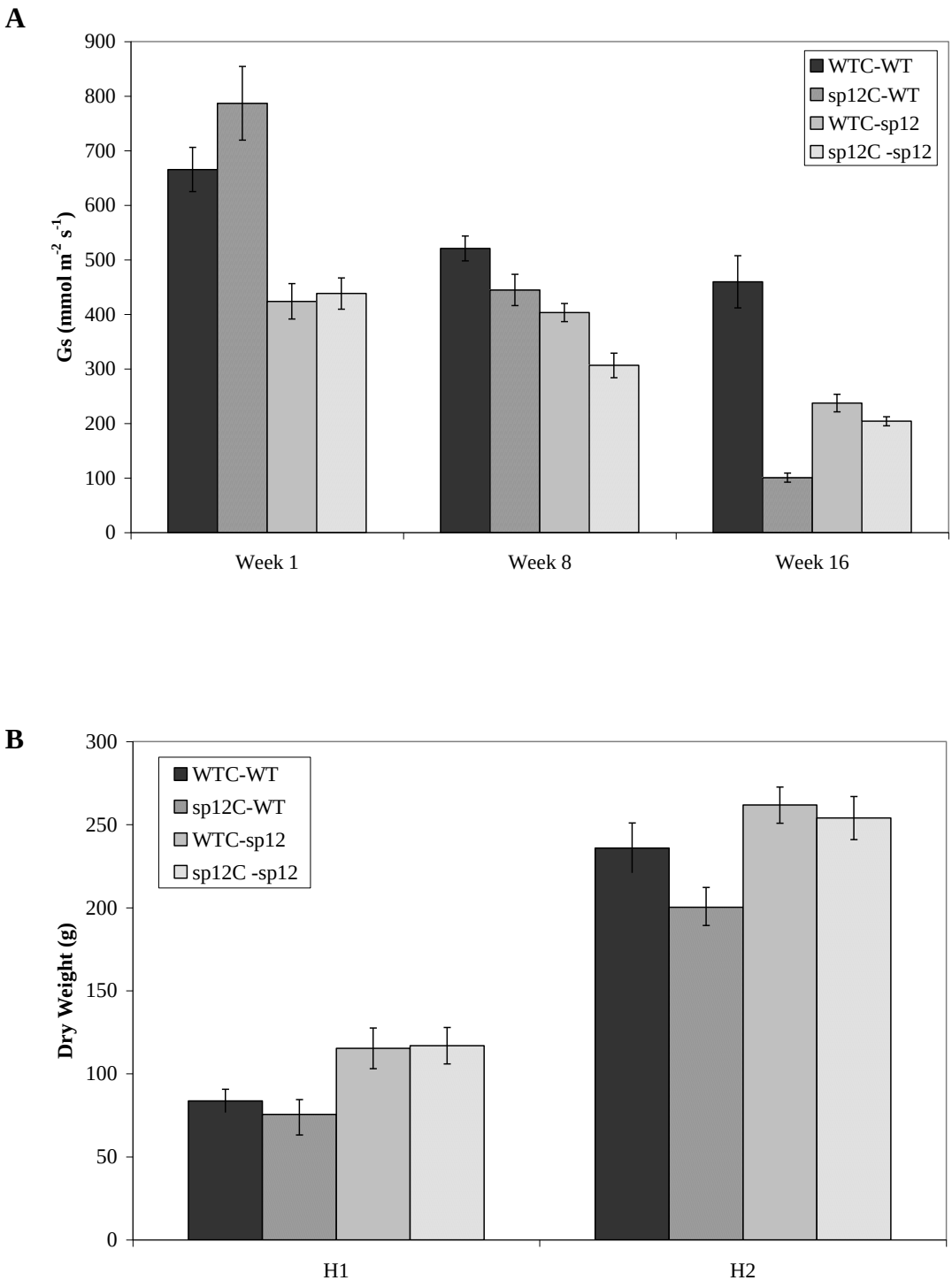


Figure 4.10. A: Mean stomatal conductance in Weeks 1, 8 and 16 (WTC and sp12C respectively denote irrigation controlled by WT plants or sp12 plants) **B:** Vegetative dry weight at Week 8 (H1) and Week 16 (H2) for plants grown under WT controlled irrigation (WTC) or sp12 controlled irrigation (sp12C). Error bars represent standard error of the mean.

4.4.2.5 Biomass Production

Transgenic sp12 plants exhibited significantly greater biomass production than WT plants ($P<0.001$) at Harvest 1 (H1), after 8 weeks of growth, even when both genotypes were grown at field capacity (WTC). There was also no significant difference in biomass production after 16 weeks of growth at Harvest 2 (H2), when plants of both genotypes were well-watered (WTC). However, when irrigation was controlled by sp12 plants (sp12C), sp12 substantially outperformed WT, with a mean biomass of 254 g compared 200 g in WT plants ($P<0.001$). This demonstrates that irrigation levels sufficient to sustain sp12 plants at field capacity provided sub-optimal irrigation for WT plants, especially during the last eight weeks of the experiment (Fig. 4.8). This sub-optimal irrigation level induced water stress and reduced biomass production relative to both sp12 plants ($P< 0.001$) and WT plants ($P>0.05$) grown at field capacity.

4.4.2.6 Fruit Production

Mild TMV symptoms became apparent in Weeks 12 and 13 of the 16 week experiment. As TMV may affect fruit set, data are presented only for truss 1 of each plant, as all fruit had been harvested from this truss by Week 12 (Table 4.2). Under well-watered conditions (WTC), no difference was observed between WT and sp12 plants, in the total fresh weight of fruit produced by truss 1. This contrasts with the preliminary fruit production results from the water rationing experiment which appeared to indicate that sp12 plants produced fewer fruit than WT controls. In this experiment, WT and sp12 plants also produced similar numbers of fruit per truss and mean weights per fruit. There was no variation in the percentage of fruit experiencing blossom end rot (BER) as only 1.2% of WT and 1.6% of sp12 fruit displayed this physiological disorder, which occurs when the blossom end of a green fruit starts to develop a water-soaked area around the blossom scar.

When sp12 plants dictated irrigation (sp12C), sp12 plants produced a greater number of fresh fruit than WT plants ($P>0.05$), again demonstrating that, even though the volume of irrigation supplied was sufficient to sustain sp12 plants, WT plants experienced water-stress and exhibited reduced biomass production. It is likely that this effect would have been more strongly illustrated by subsequent trusses produced

during the last few weeks of the experiment, when WT plants were most severely affected by depleted soil moisture content.

Table 4.2. Fruit number, mean individual weight and total fruit weight for WT and sp12 plants, with accompanying statistical summary.

		WT	**sp12**	***F pr.***	**t-Stat**	**t-Critical**
WT-Controlled	Fruit Number	6.65	8.45	*0.075*	-1.883	2.093
	Mean Weight	37.1	36.6	*0.070*	1.915	2.093
	Total Weight	265.2	262.6	*0.946*	0.068	2.093
sp12-Controlled	Fruit Number	5.80	7.81	*<0.001*	-2.922	2.093
	Mean Weight	38.2	31.2	*0.006*	2.751	2.093
	Total Weight	202.4	270.1	*0.034*	-2.288	2.093

4.5 DISCUSSION

4.5.1 'high ABA' genotypes show more effective management of available water supplies

Availability of water is of key importance when growing tomato crops (Rudich and Luchinsky, 1986), and with predicted climate change and increased competition for increasingly scarce water resources, it is important that future crops become more efficient in their use of water (Parry *et al.*, 2005). Previous experiments involving applications of exogenous ABA suggest that increasing bulk leaf ABA concentration reduces transpiration rate (Zhang and Davies, 1991; Munns, 1992). Both irrigation strategies used in Experiments 1 and 2 demonstrated that 'high ABA' genotypes exhibit more restrained use of water. In the water rationing experiment (Experiment 1), the transgenic sp12 line, outperformed WT plants at sub-optimal irrigation levels and, most interestingly, maintained similar growth rates to WT plants receiving double the quantity of irrigation water.

Stomatal conductance directly influences transpiration rate and hence the rate and extent of soil water depletion. Stomatal conductances under optimal conditions were greater in WT than in sp12 during the early stages of the experiment, but as there was no associated gain in biomass production this was effectively a waste of available water. The water conserved by 'high ABA' plants, during this period enabled them to maintain greater substrate moisture reserves which were used to sustain growth during later stages of the experiment, when WT plants were experiencing severe water stress. This gain in growth was clearly demonstrated by the greater biomass of sp12 compared to WT plants which had received the same volume of irrigation.

In Experiment 2, water use and the consequent demand for replenishment of soil moisture reserves were used to determine the volume of irrigation delivered. The 'high ABA' sp12 plants again showed the ability to conserve water, even when availability was high. When WT plants were used to determine the quantity of irrigation applied, sp12 plants had lower stomatal conductances and used less water than the WT controls. The much greater stomatal conductance of WT plants in Week 1 demonstrated their profligacy in the use of water when its supply was plentiful. WT plants therefore rapidly depleted soil moisture reserves during the early weeks of the experiment when stomatal conductance was at its maximum, resulting in the formation of progressive water stress when the lower irrigation input led by the demand of sp12 was imposed. The decline in stomatal conductance in these WT plants during the later stages of the experiment suggests that, when WT plants become water stressed, they produced additional ABA, with the result that their stomatal behaviour became similar to the 'high ABA' line. This observation is consistent with findings that ABA concentrations in the leaves, roots and xylem sap of WT plants were similar to those in sp12 when both genotypes were subjected to a water deficit (Thompson *et al*., 2007a).

The lower stomatal conductance and transpiration rate in 'high ABA' plants is consistent with the findings of Thompson *et al*. (2000a), in which the stomatal conductances of sp12 plants were found to be only 59% of the WT control. Improved tolerance or avoidance of drought stress has also been reported in several other species that over-express *NCED* genes (Iuchi *et al*., 2001; Aswath *et al*., 2005;

Deswarte *et al*. in prep.). The experiments reported here clearly show that WT plants, which are not pre-adapted to water stress, do not restrict their stomatal behaviour, and use water rapidly when water supplies are optimal. This strategy may be beneficial in natural environments, where there is no advantage in conserving soil moisture and allowing competitive neighbours to utilise it; this is not, however, the case in crop production systems, where neighbouring plants are not competitors and water is an expensive commodity.

4.5.2 Effect of 'ultra high' ABA accumulation on water use and growth

Although transgenic 'ultra high' ABA over-producer genotypes such as rbcS-10 exhibit a huge potential for conserving water, this extreme water saving ability is accompanied by several undesirable characteristics. When water was severely restricted during the latter stages of the water rationing experiment, these 'ultra-high' ABA over-producers remained turgid when plants of other genotypes showed visible wilting, and had equivalent vegetative growth rates to WT controls. However, during the early stages of the water rationing experiment, when all plants were experiencing well-watered conditions, the milder sp12 genotype maintained growth rates similar to WT control plants, whereas rbcS-10 plants exhibited far slower growth rates and did not produce fruit until much later than the other genotypes. The moderate decrease in stomatal conductance in sp12 plants had no negative impact on biomass production, whereas rbcS-10 plants exhibited a much greater reduction in mean stomatal conductance. This resulted in a major reduction in the quantity of water required to maintain growth, but was also accompanied by a significant reduction in growth rate. These 'ultra-high' over-producers also had significantly lower chlorophyll concentrations than the milder sp12 and WT control genotypes. Other studies have shown these plants to exhibit perturbed cotyledon release from the testa during germination, substantially reduced carotenoid content, interveinal flooding, chlorosis and photobleaching (Tung *et al*., 2008). The severe phenotype observed in the rbcS-10 plants is further characterised and discussed in Chapter 7.

4.5.3 Effect of mild ABA over-production on growth and physiology

Whilst more effective management of water use during the production of tomato crops is desirable, it is important that there is no biomass penalty for the more restrained use of water, as long term restriction of stomatal opening is likely to reduce photosynthesis and carbon assimilation (Jones, 2004). The growth analysis carried out in both experiments demonstrated that mild ABA over-producers maintain growth rates similar to WT controls. Transgenic 'high ABA' genotypes have been shown to be slower to establish compared with WT plants (Chapter 9), but once the four-leaf stage has been reached (the point at which both experiments commenced), 'high ABA' plants showed equal, if not greater, rates of growth. The higher ABA concentration in the transgenic plants did not affect of above-ground biomass, with 'high ABA' plants being visually similar to WT controls. This is consistent with a previous study in which the dry weight and height of sp5 plants did not differ significantly from WT controls after 13 weeks of growth (Thompson *et al*., 2007a). Aswath *et al*. (2005) also reported that, under non-stress conditions, the growth of transgenic bent grass (*Agrostis palustris* L.) plants over-expressing the *VuNCED1* gene from cow pea, was visually similar to that of equivalent WT (non-transgenic) control plants.

Thompson *et al*. (2007a) reported that petiole length was greater and epinasty was reduced in sp5 than in WT plants, and these characteristics were also observed in sp5 plants in the water-rationing experiment as their leaves were significantly longer than in WT control plants. Possible explanations for this effect are increased turgor or an antagonistic interaction between ABA and ethylene. ABA-deficient mutants typically exhibit phenotypes characteristic of excess ethylene, such as increased epinasty and adventitious rooting (Tal and Imber, 1970; Tal *et al*., 1979). Whilst it has been suggested that ABA may act as an inhibitor of shoot growth (Creelman *et al*., 1990), it has more recently been proposed that exogenous ABA promotes leaf expansion due to its antagonistic interaction with ethylene (Sharpe, 2002).

Although the development of tomato genotypes which use less water and produce equal or greater growth than current varieties has potentially major agronomic advantages, 'high ABA' genotypes would not be commercially viable if their

reproductive ability was impaired or their fruit quality was reduced, as high yields depend on proper fruit set and development (Ho and Hewitt, 1986). Preliminary data from the first water-rationing experiment appeared to indicate that sp12 plants might have impaired fruit production, although these data only took account of the largely unripe and underdeveloped fruit present at the time of harvest. This effect might also have been influenced by water stress experienced by plants in the water-rationing experiment, possibly by increasing incidence of flower abortion and reducing fruit size and quality (Saltveit, 2004).

It is important to note that when the fruit were harvested and weighed at the end of the demand-led irrigation experiment, no significant difference between genotypes in the number, weight and size of the fruit produced were detected when the plants were well-watered (WTC). This indicates that in optimal, well-watered conditions there is no yield penalty associated with the 'high ABA' genotype. When sp12 plants dictated irrigation levels and the volume of water delivered to the WT plants was less than optimal, sp12 plants produced a greater total weight. This appears to indicate that in situations where the amount of irrigation is limited, due to the 'high ABA' plant's ability to conserve water, these plants could potentially produce a greater yield. A separate study of the reproductive ability and fruit quality of plants grown under optimal conditions (*cf*. Chapter 7) indicated there was no difference in the time taken to initiate inflorescences or the number of inflorescences produced and 'high ABA' sp12 plants appeared to have an ability to set fruit comparable to that of WT Ailsa Craig control plants.

The market value of fruit is largely determined by its quality (Saltveit, 2004). Brix analysis can be used to determine fruit quality and this provides a measure of the percent total soluble solid (TSS) within a given mass of juice and the application of partial root drying treatments has been shown to increase the Brix value of tomato fruit by 21% (Davies, 2000). A large effect of NCED over-expression on the carbohydrate content of leaves and roots has previously been shown (Thompson, 2004), with both tissues showing increases in sugars and starch. Brix analysis of fruit harvested from 'high-ABA' genotypes suggested that fruit was of equal quality to WT controls (Appendix 4). There are many physiological disorders that significantly impact on the quality of fruit, including blossom end rot, which occurs

when calcium becomes deficient at the distal end of the fruit and is often associated with water stress (Guichard *et al.*, 2001). Blossom end rot was observed during the water rationing experiment and subsequently monitored during the genotype-specific irrigation experiment. No significant difference in the frequency of blossom end rot was found between 'high-ABA' and WT control plants although, as with all other fruit quality evaluations carried out in the present study, these results can be regarded as encouraging but only preliminary. For this reason a larger scale, commercial production style experiment involving measurement of a greater number of quality parameters is necessary to establish unequivocally that the plants with permanently elevated ABA concentrations has no detrimental effect on fruit quality.

4.5.4 Evaluating the extent of improvement in WUE in 'high ABA' plants

Whilst high ABA plants that are pre-adapted to water stress exhibited more restrained stomatal behaviour and reduced transpiration, this did not reduce biomass production in the milder 'high ABA' sp12 line, possibly because partial stomatal closure restricts water loss more quickly, and to a larger extent than CO_2 influx. WUE would therefore be improved due to the non-linear relationship between decreases in water loss and associated reductions in carbon assimilation (Jones, 1976; Lamberts *et al.*, 1998). WUE at the whole plant level can be described as the ratio of biomass production to the quantity of water transpired (Jones, 2004). Bradford *et al.* (1983) used applications of exogenous ABA to young tomato leaves to demonstrate that, despite operating with reduced stomatal conductances, the leaves had the same photosynthetic capacity as control plants.

Both irrigation regimes employed in experiments 1 and 2 demonstrated the ability of 'high ABA' plants to conserve water. Under sub-optimal irrigation (Experiment 1), ABA over-producers conserved soil moisture, permitting growth over prolonged periods; however, the greatest advantages were observed under optimal conditions where WT plants are profligate in their use of water. In the second experiment, specific management of irrigation was used to compensate for increasing plant size, with the result that they experienced more optimal conditions as irrigation delivery rates were adjusted after monitoring amount of water draining from the compost.

This style of irrigation management is often utilised in soilless media (Peet, 2005). Whilst these trickle irrigation experiments have revealed the potential water saving ability of ABA over-producers under both progressive drought and optimal irrigation systems, more detailed studies in which water use by individual plants was accurately recorded would enable more precise examination of the extent of any advantage in WUE and allow the optimal level of ABA over-production to be determined. Therefore an experiment involving daily gravimetric determination of water use and destructive analysis of biomass production would allow accurate calculation of WUE for individual smaller plants over a shorter time period. The use of this approach is described in Chapter 5.

5 WATER USE EFFICIENCY OF SINGLE TRANSGENIC GENOTYPES

5.1 INTRODUCTION

5.1.1 Water Use Efficiency (WUE)

5.1.1.1 Demand for improved WUE in global agriculture

Water stress is the main environmental factor limiting plant growth and yield worldwide (Boyer, 1982). However, around 80% of the world's fresh water resource is already consumed by irrigated agriculture (Condon *et al.,* 2004), with 40% of global food produce being grown on irrigated land (Somerville and Briscoe, 2001). Although the water use efficiency (WUE) of crops has not historically been a major issue for growers, the restrictions and costs associated with the abstraction of water are currently growing. There is also likely to be increased competition for water resources from industrial, leisure and domestic users, so forcing an increase in its cost, as availability decreases. This situation is compounded by the predicted change in climate, as increased temperatures and decreased precipitation are expected to occur in some parts of the world as a result of global warming (Mannion, 1995). There is therefore a pressing need to improve WUE for both rain-fed and irrigated crop production (Anderson *et al*., 1999; Hamdy *et al.,* 2003; Parry *et al.,* 2005). A comprehensive review of the demand for improved WUE in global agriculture is presented in Chapter 1).

5.1.1.2 Definition and Measurement of WUE

The definition of WUE depends on the context in which it is being discussed. WUE is usually described either in terms of an instantaneous measurement of efficiency in relation to carbon gain, or as an integral of such efficiency over more extended periods (Bacon, 2004). A basic definition of WUE at the whole plant level is the ratio of the quantity of biomass produced to the quantity of water transpired (Jones, 2004). However, instantaneous WUE is defined as the ratio of instantaneous net CO_2 assimilation rate (A) to transpiration rate (E_l) i.e. A/E_l (Farquhar and Richards, 1984). The ratio of the quantity of dry matter accumulated to the quantity of water lost through transpiration can also be termed the transpiration coefficient or transpiration

ratio (Jones, 2004), and was historically referred to as transpiration efficiency (Maximov, 1929).

Various methods may be used to determine instantaneous or long-term WUE. Gas exchange studies can provide estimates of instantaneous WUE (mmol CO_2 mol^{-1} H_2O), whilst destructive growth analysis or isotope discrimination give time-integrated estimates (g DM kg^{-1} H_2O). The development of infra-red gas analysers (IRGAs) has allowed accurate measurements of CO_2, O_2 and H_2O fluxes between leaves and the surrounding environment by enclosing a known leaf area within a transparent chamber, through which air flows. This technique allows the calculation of net photosynthesis, stomatal conductance and transpiration rate under near-ambient growth conditions, as well as determination of responses to light and atmospheric temperature, humidity and CO_2 concentration (von Caemmerer and Farquhar, 1981; Field *et al*., 1989; Parsons *et al*., 1997). This technique allows rapid screening of gas-exchange trends in populations of plants, but only provides a snap-shot of stomatal conductance and gas fluxes at specific points in time.

Measurements of WUE derived from isotopic analysis are often used as an indicator of long term trends in the internal regulation of carbon assimilation and water loss. WUE has been shown to be negatively correlated with values for carbon isotope discrimination (Δ) predicted from the theory of leaf gas-exchange (Farquhar *et al*., 1982; Farquhar and Richards, 1984). This relationship applies under both well-watered and sub-optimal supply conditions. Measurements of total water loss and biomass gain over defined time periods may also be used to estimate long-term, above-ground transpiration efficiency. The most common method of calculating total transpiration of greenhouse crops, such as tomato production in Northern Europe, is to apply the Penman-Monteith Equation (Monteith and Unsworth, 1990; Jolliet and Bailey 1992; Boulard and Jemaa 1993). This model, however, requires data for stomatal resistance and the aerodynamic resistances within the glasshouse. A more direct method of determining transpiration efficiency per plant is to use a gravimetric and destructive harvest approach, whereby each plant is weighed daily to determine total water loss via transpiration (e.g. Xu *et al*., 2006).

5.1.2 Potential for improving WUE by manipulating stomatal behaviour

Plants require access to the atmosphere to acquire carbon dioxide (CO_2) for photosynthesis, even though this is accompanied by water loss by transpiration. Water loss is greatest when the leaf to air vapour pressure deficit and irradiance are high and the stomata are wide open. Stomatal aperture may be reduced to avoid excessive water loss but, if sustained over prolonged periods, this may restrict the absorption of CO_2 and can therefore limit photosynthesis and carbon assimilation (Jones, 2004). As mentioned earlier, WUE may be defined as the ratio between the quantity of CO_2 assimilated into plant biomass and the quantity of water lost via transpiration (Bacon, 2004). Both processes are influenced by changes in stomatal aperture and the concentration gradients of water vapour and CO_2 between the interior and exterior of the leaf. WUE is therefore continuously influenced by stomatal behaviour and environmental conditions during each diurnal cycle.

5.1.2.1 ABA and the control of stomatal opening

Stomatal conductance is determined by a combination of the number of stomata per unit leaf area and how widely open they are and is a key factor in the regulation gas exchange. Indeed, stomatal conductance is the primary means by which plants regulate water flow through the soil-plant-atmosphere continuum (Saliendra *et al*., 1995). One of the most important targets for ABA action within plants is the stomatal complex, where it contributes to the regulation of gas exchange by influencing stomatal opening and closure (Zeevaart and Creelman, 1988). Plants have been shown to reduce stomatal aperture when their roots encounter drying soil, even when leaf water status remains constant. This suggests that one or more non-hydraulic root-sourced signals enable plants to sense and respond to drying soil (Zhang and Davies, 1989).

ABA is a fundamental component of the complex mechanisms which allow plants to co-ordinate water use with supply. It was initially thought that a decline in leaf water status was the key signal allowing plants to respond to water stress, although this simple view has been complicated by the evidence that leaf water potential and turgor may remain unchanged even though water-conserving responses have been

induced (Davies and Zhang, 1991; Schachtman and Goodger, 2008). Researchers have used several different methods to demonstrate this, including maintaining leaf water status as soil dries by pressure balancing techniques (Gollan *et al.*, 1986), or by splitting roots between wet and dry soil (Stoll *et al.*, 2000; Mingo *et al.*, 2003).

The concentration of ABA in the xylem stream has been proposed to provide at least one component of a positive measure of soil water availability, allowing plants to regulate their physiology, growth and development accordingly (Zhang and Davis, 1991). When soil drying occurs, roots experience water stress and respond by initiating changes in their physiology, metabolism and gene expression (Griffiths and Bray, 1996). Xylem sap ABA concentrations typically increase with decreasing soil water potential (Davies and Zhang, 1991) and this may be associated with water conservation responses in the shoot, including stomatal closure. Significant ABA synthesis may also occur in the shoots themselves, either directly as a result of changes in leaf water status induced by lack of water availability in the soil or dry atmospheric conditions (Wright, 1977; Wilkinson, 2004) or perhaps indirectly in response to signals from the roots. It is usually difficult to determine whether the xylem sap ABA is root-sourced in the sense of being synthesised in the roots, or represents recycled ABA originally synthesised in the shoot.

ABA concentration was shown to increase in water-stressed leaves by Hiron and Wright (1973) and substantial evidence has subsequently accumulated to support the view that ABA is involved in stomatal regulation (McAinsh *et al.*, 1990; MacRobbie, 2000). In early studies, Jones and Mansfield (1970) reported a link between stomatal closure and ABA concentration following exogenous applications of ABA to the leaves of *Xanthium pennsylvanicum*. The importance of endogenously synthesised ABA as a regulator of transpiration *via* control of stomatal aperture has been demonstrated by characterisation of mutants with a wilty phenotype. A major characteristic of three wilty mutants of *Solanum lycopersicum* (previously known as *Lycopersicon esculentum*), *sitiens*, *flacca* and *notabilis*, is their failure to close their stomata sufficiently during conditions of water stress, leading to extreme wilting symptoms (Tal and Nevo, 1973). Several authors have demonstrated that the phenotype of these mutants is attributable to their low endogenous ABA concentrations caused by specific impairments in the biosynthetic pathway (see

Taylor *et al.*, 2000 for a review). These can be largely reversed by the application of exogenous ABA (Linforth *et al.*, 1987; Taylor *et al.*, 1988; Nagel *et al.*, 1994; Marin and Marion-Poll 1997; Burbidge *et al.*, 1999).

Stomatal pores are enclosed by a pair of guard cells which regulate stomatal conductance through osmotic shrinking and swelling of these cells. This mechanism is facilitated by the movement of K^+, H^+ and Cl^- between guard cells and neighbouring cells, and the metabolism, movement and synthesis of organic ions, especially malate (MacRobbie, 1988; Talbott and Zeiger, 1998). High solute concentrations in the cytosol and vacuoles increase guard cell turgor and hence volume, leading to stomatal opening. Alternatively the loss of solutes from guard cells reduces cell turgor and volume, causing guard cell shrinkage and stomatal closure (Pei and Kuchitsu, 2005). ABA influences ion transport in the apoplast surrounding guard cells, reducing guard cell turgor and causing stomatal closure (Assmann, 1993). The apoplast surrounding guard cells is regarded as the most important site of ABA action on stomata, as it allows potential access to receptors on exterior membranes and is the only compartment from which ABA can be taken up inside the guard cells themselves. In response to water stress, ABA concentration in the guard cell apoplast may increase by up to 30-fold (Zhang and Outlaw, 2001; Outlaw, 2003). Regulation of stomatal aperture is considered in more detail in Chapter 1.

5.1.2.2 Relationship between carbon gain and water loss

Plants require access to the atmosphere *via* open stomatal pores to absorb CO_2 for photosynthesis, the process whereby many bacteria, algae and vascular plants convert solar energy to chemical energy contained in various organic molecules (Lawlor, 2000). The end-product of photosynthesis may be regarded as the production of assimilates required for growth. In C3 species, CO_2 in the chloroplast stroma is removed by ribulose bisphosphate (RuBP) carboxylase and a CO_2 concentration gradient develops across the chloroplast envelope, cytosol, cell membranes and walls to the intercellular spaces and, *via* the stomata, to the ambient air (Lawlor, 2000). This gradient provides the driving force for CO_2 diffusion, although the rate at which this occurs depends on conductance of CO_2 in the gas and liquid phases in the leaf and atmosphere, as well as the external CO_2 concentration.

The loss of water vapour by transpiration is sometimes simplistically regarded as a necessary 'evil' associated with organisms exchanging CO_2 and O_2 with the atmosphere. The rate of transpiration from leaves depends on the difference in water vapour concentration between the intercellular spaces and the external environment and the diffusive resistance of this pathway (Taiz and Zeiger, 2006). Transpiration rate and biomass production usually tend to be fairly closely related as both processes are under stomatal control.

A major objective of efforts to improve WUE is to enhance the capacity of crops to produce biomass per unit of water transpired (Wallace, 2000). As mentioned earlier, WUE can be defined as the ratio of CO_2 assimilation (*A*) to the water loss *via* transpiration (*E*) (Bacon 2004). Regulation of stomatal aperture is central to the WUE of plants, as *A* and *E* are both influenced by the extent of stomatal opening, although not necessarily to the same degree. Although CO_2 and water vapour share the same stomatal diffusion pathway, the concentration gradient driving water loss is far larger than that for CO_2 uptake. Thus, an increase in stomatal conductance usually enhances stomatal diffusion and may increase photosynthetic rate, but usually also increases transpiration rate. As photosynthetic rate in C3 species is co-limited by the carboxylation of CO_2 and regeneration of RuBP, the actual photosynthetic rate depends on CO_2 availability (Chaves *et al*., 2004). Assuming the concentration gradients of water vapour (*Wi*-*Wa*) and CO_2 (*Ci*-*Ca*) between the interior of the leaf (*Wi* and *Ci* respectively) and the external atmosphere (*Wa* and *Ca* respectively) are independent, the intrinsic water use efficiency is a complex negative function of *Ci*/*Ca*.

When stomatal aperture is restricted, the efflux of water vapour is reduced to a greater extent than CO_2 influx due to the differing diffusive resistances encountered in the transport pathways for these gases. This means that WUE can be improved due to the non-linear relationship between the decrease in water loss and any accompanying reduction in carbon assimilation. Under any particular set of environmental conditions, the driving force for CO_2 uptake will be enhanced by a decrease in *Ci*, whilst the driving force for water loss remains relatively unchanged, so increasing WUE (Bacon, 2004). For example, in response to mild water stress, a small decrease in stomatal conductance will cause a linear decrease in transpiration

(if VPD remains constant), but as demand for CO_2 in the chloroplasts remains unchanged, the *Ci-Ca* gradient will increase. Intrinsic WUE commonly increases when plants experience mild water deficits (Chaves *et al*., 2004) and stomata begin to close (Jones, 1993). It is possible to mimic the effect of mild water stress by long-term exposure to elevated internal ABA concentrations; for example, Bradford *et al.* (1983) applied ABA to young tomato leaves and found that, despite having reduced stomatal conductances, photosynthetic capacity was comparable to control plants, suggesting that stomatal behaviour may be manipulated by increasing endogenous ABA production under unstressed conditions to enhance WUE.

5.2 Aims

Detailed knowledge of water use characteristics is an important requirement in developing approaches to improve WUE in crop species. Whilst previous studies have shown the potential water-saving abilities of ABA over-producers in both well-watered and water limited environments (Chapter 4), accurate estimation of transpiration efficiency (TE_p) for each 'high ABA' line is necessary.

The experiment reported here was designed to determine whether a gravimetric method for determining WUE would reliably reveal differences between WT and 'high-ABA' plants. This would provide a preliminary evaluation of the potential for using tomato lines that are pre-adapted to water stress *via* increased ABA biosynthesis to obtain improvements in whole plant WUE. This method was evaluated for potential future use to compare other 'high ABA' genotypes which have recently become available, and to determine an optimal level of ABA over-production to achieve sustained crop growth using reduced water inputs.

5.3 MATERIALS AND METHODS

As previous experiments here and elsewhere (Chapter 4; Thompson *et al*., 2000a) have demonstrated the potential water saving ability of ABA over-producers under both progressive drought and optimal irrigation, a gravimetric analysis experiment was designed to determine the extent of any advantage in whole plant transpirational efficiency (TE_p) for the 'high-ABA' transgenic tomato lines (sp12 and sp5) compared to WT plants.

5.3.1 Plant Material

Seeds were sown according to the appropriate protocol for each genotype to synchronise development by the end of the seedling establishment phase (Chapter 3). As the sp12 and sp5 genotypes displayed prolonged dormancy, it was necessary to sow these on different dates to achieve uniform seedling size at the beginning of the experiment (Chapter 9). Batches of sp5 seed were sown 19 days before sp12 seed and 24 days before WT seed. Plants were watered daily to field capacity until the start of the experiment.

5.3.2 Experimental Design

Six uniform plants of each genotype were selected at the four leaf stage for inclusion in the experiment. Three plants of each genotype were destructively harvested to determine their dry weight at the beginning of the experimental period. The three remaining replicates of each genotype (WT, sp12 and sp5) were re-potted into 7 litre pots and arranged in a Latin square design in the glasshouse. The compost (Levington M3) was then watered until saturated. Once excess water had drained from the pots, each pot was weighed to ascertain its weight at field capacity. Uvi ground cover disks (Growing Technologies, Derby, UK) were placed on top of the compost to prevent evaporation from the soil surface. The pots were weighed daily to determine the quantity of water used by each plant and were given an equivalent volume of water to return the compost to field capacity for the duration of the 25 day experimental period. The daily weight loss of individual pots was taken as the weight of water lost via transpiration. Daily transpiration rates were summed to give total

water loss per plant over the 25 day experimental period. All plants were then harvested and above-ground dry weight, plant height and the length of each leaf were recorded. Mean biomass for each genotype at the beginning of the experiment was subtracted from the corresponding value at final harvest to determine biomass accumulation during the experimental period. Transpiration efficiency (TE_p) was calculated for all plants.

5.3.3 Instantaneous Gas Exchange

On days 21, 22, 23 and 24, gas exchange measurements were made using a CIRAS-1 Infrared Gas Analyser (PP Systems, Hitchin, Herts, UK) and a Parkinson leaf cuvette with an enclosed leaf area of 2.5 cm^2 for all plants using the terminal leaflet of the youngest fully expanded leaf.

5.3.4 Data Analysis

Daily water use was analysed using paired sample Student's t-tests, within a Microsoft Excel worksheet. Analysis of variance (ANOVA) for a Latin square block design was carried out for all other data, using Genstat 8th edition (Lawes Agricultural Trust, Rothamstead, Herts, UK).

5.4 RESULTS

5.4.1 Instantaneous Gas-Exchange

Mean stomatal conductance measured using a CIRAS-1 on day 21 was 31 and 37% lower in sp12 and sp5 plants than in WT plants ($P < 0.05$; Table 5.1). There were no significant differences in assimilation rate, indicating that the 'high ABA' genotypes transpired less rapidly than WT plants in the absence of any accompanying decrease in photosynthetic rate. Interestingly, these measurements suggest that, even on days of optimal light conditions, WT plants gained nothing in terms of CO_2 assimilation by opening their stomata more widely and transpiring more rapidly.

WT plants invariably had the highest stomatal conductances of all genotypes on all sampling dates; this difference was most pronounced on days 21 and 23. Mean stomatal conductance of 'high ABA' genotypes was only 40% of that for WT control plants on day 23, although this difference was not significant, even though WT plants had a significantly greater transpiration rate at this time ($P<0.05$; data not shown).

No significant differences in assimilation rate (*A*) were found between genotypes for any of the sampling dates. The *A* value was greatest across all genotypes on day 21, when significant differences in stomatal conductance were observed between genotypes ($P<0.05$). The mean *A* value of all genotypes was 4.54 µmol m^{-2} s^{-1} on day 21, in comparison with 1.81 µmol min^{-1} s^{-2} across the other 3 time points (days 22-24). Of the four measurement dates, day 21 was also the date when mean water use, determined gravimetrically, was greatest (Fig.5.1). On this day mean stomatal conductance and assimilation rate for all genotypes was greater than on any other measurement date ($P<0.05$). This observation shows that differences between WT and 'high-ABA' plants were greatest on days when conditions were optimal, when WT plants opened their stomata sufficiently to be apparently at their most wasteful in their use of water. It is important to note that the stomata of 'high ABA' plants were highly dynamic and responded more strongly to varying environmental conditions, as demonstrated by the greater stomatal apertures on day 21 compared to other days ($P<0.05$); but were more restrained than WT plants in the extent of their stomatal opening.

5.4.2 Gravimetric Measurements of Transpiration

Over the 25 day experimental period, WT plants transpired more water than the sp12 and sp5 lines, with overall mean daily values of 340, 225 and 175 ml d^{-1} respectively ($P<0.05$). This difference was not due to variation in plant size, as there was no significant difference between the three genotypes in terms of their dry weight at the start of the experiment, or the quantity of biomass accumulated during the experimental period (Fig. 5.2). WT plants consistently exhibited the highest transpiration, followed successively by sp12 and sp5. This ranking was as predicted from the levels of ABA over-production, as sp5 plants have typically shown higher

transgene expression and ABA concentrations than sp12 (Thompson *et al.*, 2007b). Over the 25 day period, sp12 plants used less water than WT on 12 occasions, whilst sp5 plants used less water than WT on 17 of the 25 days ($P<0.05$). Despite consistently ranking lower than sp12 for daily transpiration, sp5 used significantly less water than sp12 on only one occasion ($P<0.05$), when the data for individual days were analysed (Fig.5.1). When the daily water use of sp12 and sp5 plants was compared using a paired sample t-test, sp12 plants had a greater daily water use than sp5 ($P<0.001$).

Interestingly, on days 22, 23 and 24, when there was no significant difference in stomatal conductance determined using the CIRAS-1, the gravimetric measurements of daily water use showed that WT plants used more water than sp12 or sp5 ($P<0.05$), thereby demonstrating that instantaneous measurements of stomatal conductance and transpiration rate may fail to reveal significant differences in water use during the full 24 hour cycle.

5.4.3 Biomass production

As noted previously, there was no significant differences in mean biomass between genotypes at the start of the experiment (3.31, 3.50 and 3.29 g respectively for WT, sp12 and sp5), or in the quantity of biomass accumulated during the subsequent 25 day period. The similar growth rate of all genotypes was also reflected by the fact that there was no significant difference in plant height between genotypes (Fig.5.2).

Table 5.1. **A:** Stomatal conductance (g_s) and **B:** assimilation rate (A) on Days 21-24 of the experimental period with accompanying ANOVA summary (n=3).

A

	Stomatal Conductance (mmol min^{-2} s^{-1})			
	Day 21	**Day 22**	**Day 23**	**Day 24**
WT	429	202	386	198
sp12	297	133	128	103
sp5	272	128	186	107
Fpr.	*0.018*	*0.129*	*0.088*	*0.064*
s.e.d.	33.4	30.7	87.6	31.1
l.s.d.	92.6	85.3	243.2	86.4

B

	Assimilation Rate (µmol min^{-2} s^{-1})			
	Day 21	**Day 22**	**Day 23**	**Day 24**
WT	4.83	1.57	2.3	1.83
sp12	4.3	1.70	1.8	1.67
sp5	4.5	1.57	1.9	2.03
Fpr.	*0.511*	*0.882*	*0.183*	*0.794*
s.e.d.	0.426	0.303	0.235	0.526
l.s.d.	1.184	0.841	0.652	1.459

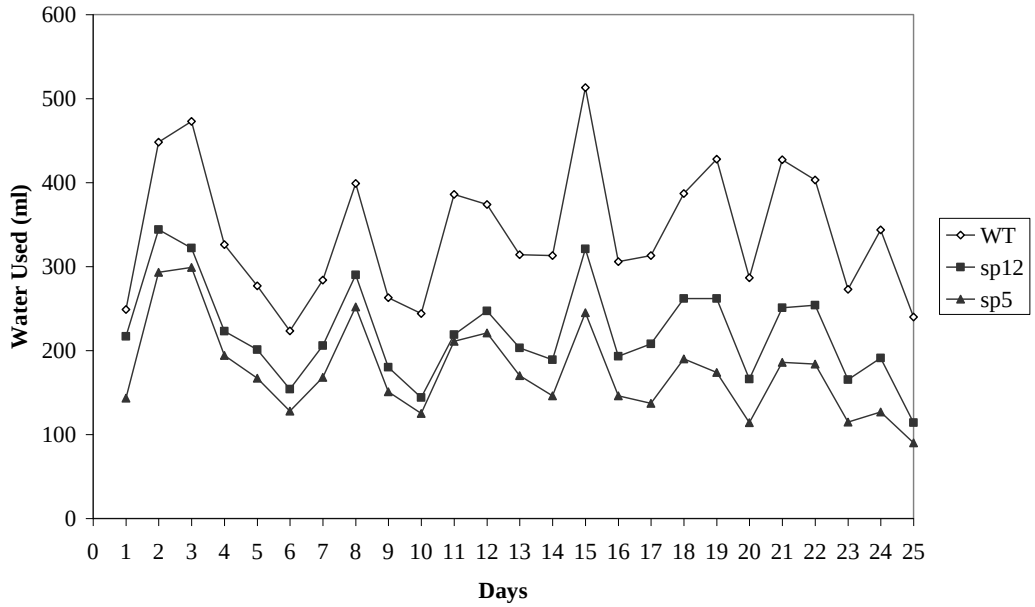
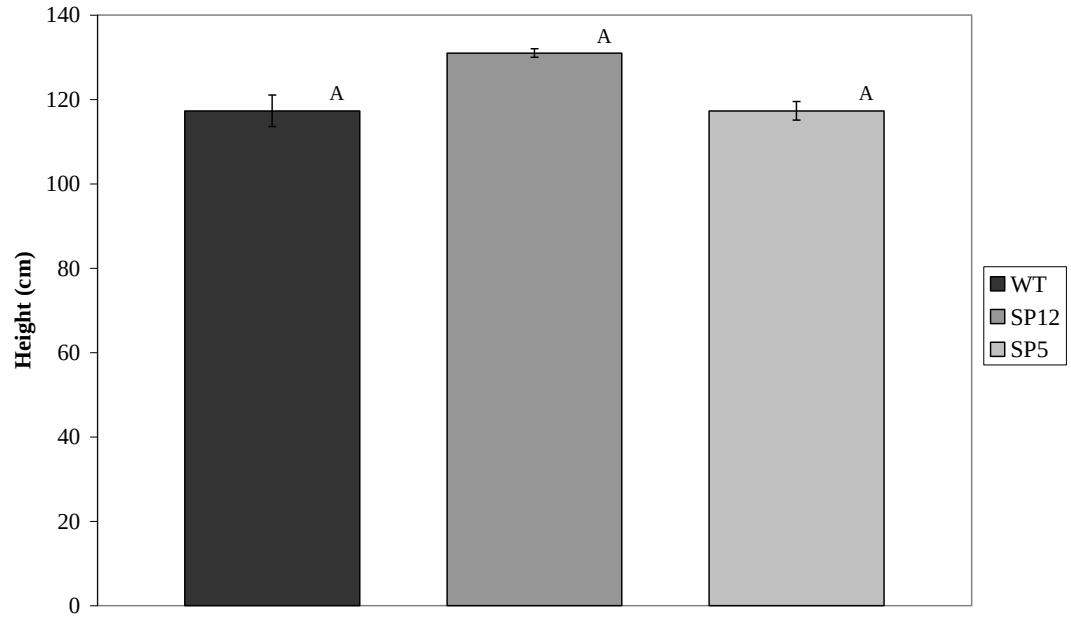
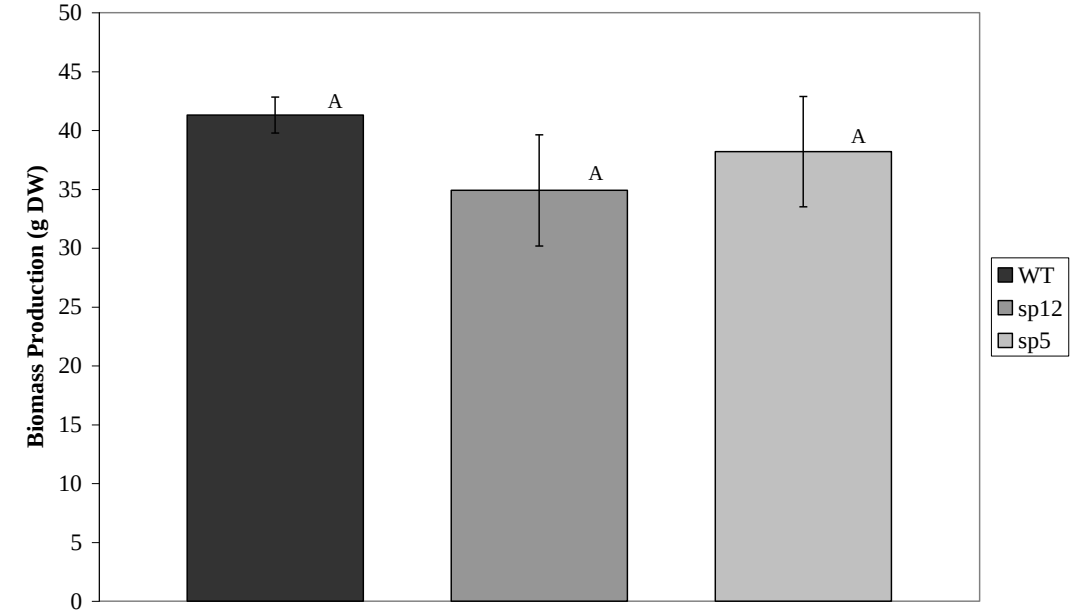


Figure 5.1. Daily transpiration measured gravimetrically for WT, sp12 and sp5 plants during the 25 day experimental period.

A



B

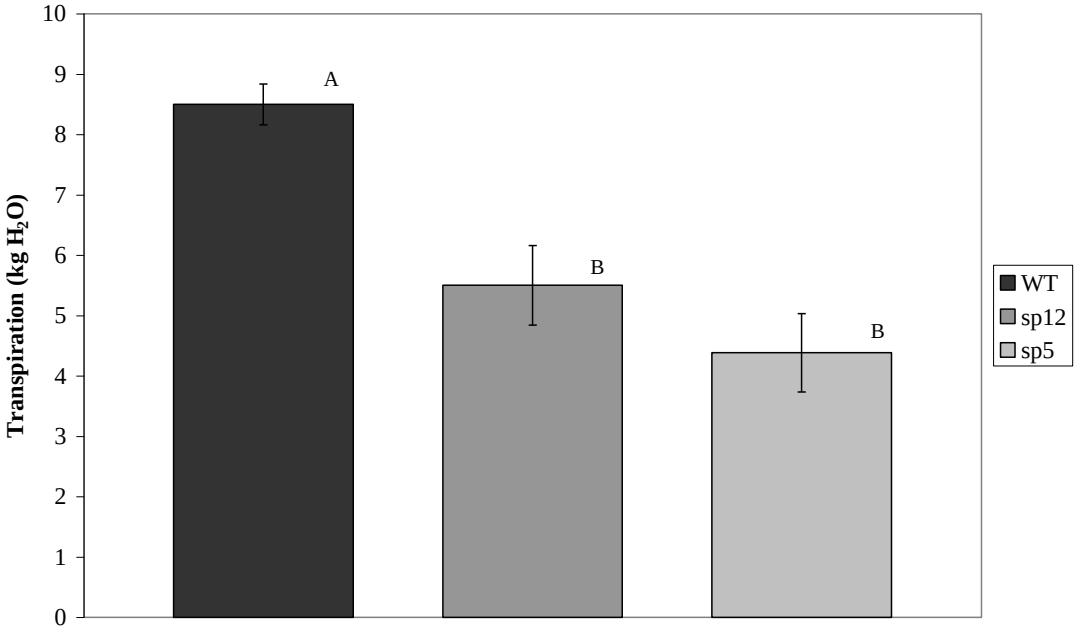


C

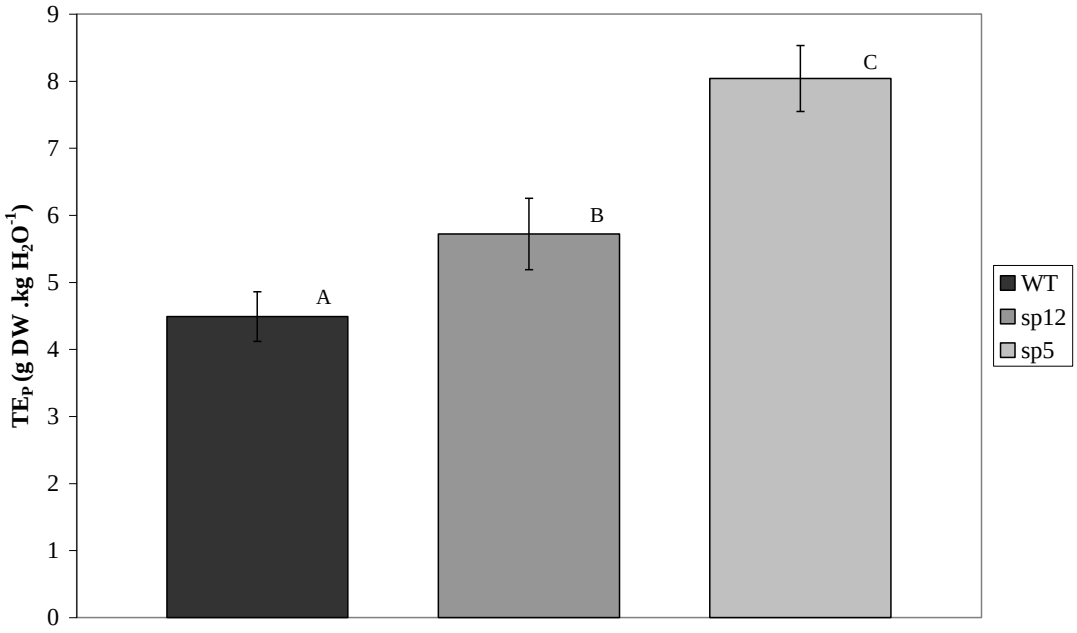
	Height (cm)	Biomass Accumulation (g DW)
F pr.	*0.055*	*0.667*
s.e.d	4.38	6.75
l.s.d	12.16	18.75

Figure 5.2. A: Mean plant height after 25 days of growth; **B:** Biomass accumulation during the 25 day experimental period. **C:** statistical summary from AVOVA (n=3). Bars with differing letters indicate significant differences. Error bars represent standard error of the mean.

A



B



C

	Transpiration (kg H_2O)	***TE_p*** **(g DW kg H_2O^{-1})**
F pr.	*0.019*	*0.002*
s.e.d	0.85	0.37
l.s.d	2.35	1.02

Figure 5.3. A: Total water use during the 25 day experimental period;. **B:** mean TE_p during the 25 day experimental period; **C:** statistical summary from AVOVA (n=3). Bars with differing letters indicate significant differences. Error bars represent standard error of the mean. Error bars represent standard error of the mean.

5.4.4 Transpiration Efficiency (TE_p)

Because WT plants transpired more water during the 25 day experimental period ($P<0.05$), but there was no significant difference in total biomass production between genotypes, TE_p was respectively 27% and 79% higher in sp12 and sp5 plants than in WT plants ($P<0.05$; Fig.5.3). Thus, sp5 plants used 44% less water to produce the same amount of biomass compared to WT controls. These gravimetric data are consistent with previous observations based on instantaneous gas exchange data and carbon isotope analysis approaches which support the findings that TE_p was typically greater in sp12 and sp5 than in WT plants (Thompson *et al.*, 2007a), in which Figures 5.2 and 5.3, have already been published.

5.5 DISCUSSION

5.5.1 Tomato lines constitutively over-expressing *LeNCED1* (sp12 and sp5) showed greatly improved transpiration efficiency.

It has been suggested that increasing endogenous ABA concentrations by manipulating the ABA biosynthetic pathway may severely reduce growth rate (Condon *et al*., 2004), not only by reducing stomatal aperture and hence assimilation rate, but also because ABA has been reported to have negative impacts on plant growth in some experiments (Creelman *et al*., 1990). Some more recent studies have attempted to de-couple the more direct effects of ABA on growth from its effects on water balance, challenging the notion that ABA always acts as a growth inhibitor (Sharp and LeNoble, 2002). The growth analyses at the start and end of the 25 day experiment showed no difference in the rate of biomass accumulation between WT and the 'high ABA' sp12 and sp5 plants. These findings are consistent with previous evidence (Chapter 4), in which these genotypes were consistently capable of either equalling or exceeding the growth rates of WT control plants under both well-watered and sub-optimal irrigation conditions. Gas exchange measurements also confirmed that there was no difference in photosynthetic rate between WT and the sp lines on four consecutive days, in agreement with previous studies of the growth of plants over-expressing ABA biosynthetic genes. For example, Aswath *et al*. (2005) reported that, under unstressed conditions, the growth of transgenic bent grass

(*Agrostis palustris* L.) plants over-expressing the cowpea *VuNCED1* gene was visually similar to equivalent WT (non-transgenic) controls.

Whilst the 'high ABA' (sp) lines maintained 'normal' growth rates compared to WT controls, their transpiration rate was greatly reduced. Interestingly, the stomata of all three genotypes responded dynamically to environmental fluctuations, transpiring more rapidly under conditions favouring optimal photosynthesis and growth. However, the two sp lines were almost always more restrained in their daily water use and appeared to limit maximum stomatal aperture to a greater extent than WT plants. Plants use most water when VPD is greatest around midday and, under well-watered conditions, sp plants exhibit a stronger response to VPD than WT plants (Thompson *et al*., 2007a). It is possible that sp lines restrict water wastage when VPD is high but act like WT plants when VPD is low, so maintaining CO_2 uptake when water loss would be greatest. Also, growth rates may be maintained but water is not wasted when photosynthesis is restricted by factors other than stomatal aperture (e.g. by saturating c_i) as any increase in stomatal aperture would not increase carbon assimilation. The effect of over-expressing *NCED* genes on stomatal conductance and transpiration has also been investigated in Arabidopsis. Iuchi *et al.* (2001) found that the over-expression of *AtNCED3* also produced 'high ABA' plants with reduced rates of water loss, although they did not report any detailed measurements of WUE.

Loveys *et al.* (2004) suggested that reducing transpiration rate by manipulating the ABA biosynthetic pathway might reduce evaporative cooling and significantly increase leaf temperature. In some environments, this might increase transpiration rate sufficiently to negate the potential benefit of reduced stomatal conductance and increase the risk of heat stress. However, under the glasshouse conditions used here, the subtle reductions in stomatal apertures achieved in the mild ABA over-producing lines meant that plants were still transpiring rapidly on sunny days (e.g. day 21), but in a slightly more restricted way than WT plants. There was no indication of any obvious adverse effects of heat stress caused by reductions in transpiration, although leaf temperature was not determined directly, for example by thermal imaging.

The gravimetric experiment demonstrated that plants over-expressing *LeNCED1* have greatly improved transpiration efficiency, despite the fact that their growth rate was comparable to that of WT controls. This is consistent with Wong *et al.* (1979), who showed that, when stomatal aperture was reduced, *A* was lowered to a lesser extent than transpiration when stomatal conductance was relatively high. Other early reports also suggest that plants subjected to increased internal ABA concentrations exhibit decreased stomatal aperture without significant reductions in photosynthetic capacity (Kriedemann *et al*., 1975; Bradford *et al*., 1983). This non-linear relationship between reduced stomatal conductance and the resulting reduction in *A* was also reported by Thompson *et al*. (2007a). When *A* was plotted against stomatal conductance for WT, sp12 and sp5 plants, a hyperbolic relationship was observed (r^2 = 0.82), confirming that a given reduction in stomatal conductance has a smaller effect on assimilation than on transpiration rate when stomatal conductance is high.

Various experimental approaches have been used to show that, in plants which are pre-adapted to water-stress, the long term reductions in stomatal conductance, and hence transpiration rate, are proportionally greater that any reduction in carbon assimilation. The results obtained here, using a combination of gravimetric and harvest analysis methodologies, are supported by instantaneous gas exchange measurements and isotope analysis approaches (Thompson *et al*., 2007a). When WT, sp12 and sp5 plants were analysed, a positive relationship between $\delta^{13}C$ and A/g_s was found, as predicted by Farquhar *et al.* (1982). Transgenic (sp12 and sp5) plants had higher $\delta^{13}C$ values, indicating that the improvement in intrinsic transpiration efficiency for these plants was consistent over the timescale of leaf development. Gas exchange measurements showed 1.7 and 2.5-fold increases in A/g_s for sp12 and sp5 plants respectively compared to WT controls (Thompson *et al*., 2007a). In transgenic *Arabadopsis* plants, also over-expressing *LeNCED1* using the rbcS tomato promoter, both gravimetric (WUE_p) and instantaneous gas-exchange (WUE_{iGE}) methods have been used to show that the transgenic lines exhibited a significant increase in WUE (Deswarte *et al.* in prep.). The above findings suggest that it is possible to manipulate stomatal behaviour by increasing ABA production to improve WUE under non-stressed conditions.

5.5.2 Optimal levels of ABA over-production to produce WUE improvement without significantly reducing dry matter accumulation

It is well established that NCED enzymes are important in the control of stress responses (Burbidge *et al.*, 1999; Qin and Zeevaart, 1999; Thompson *et al.*, 2000). Improved resistance to drought stress has been noted in several species which have been genetically manipulated to over-express *NCED* genes (Iuchi *et al*., 2001; Aswath *et al*., 2005; Thompson *et al*., 2007a; Deswarte *et al*. in prep.). Under well-watered conditions, the 'high-ABA' (sp) lines of tomato exhibited stomatal conductances similar to mildly stressed WT plants (Thompson *et al*., 2007a). However, once a certain level of water stress was applied, both genotypes exhibit similar stomatal behaviour. Therefore the greatest differences and most useful gains in terms of the agronomic application of these plants occur when water is readily available and WT plants are profligate in its use.

However, this is not the case for all ABA over-producing tomato lines. For example, the use of the promoter sequence of the tomato rbcS3C gene, which encodes the small-subunit of RuBisCo to drive *LeNCED1* transgene expression in tomato, allowed the recovery of many transgenic plants with very high levels of ABA (Tung *et al*., 2008). The rbcS3C promoter is strongly active at high levels in the light and is switched off in the dark (Wanner and Gruissem, 1991). The relatively low light (or high sucrose media) environment in the tissue culture room may have prevented full expression of the rbcS3C::*LeNCED1* construct until the transformants were transferred to the higher light conditions provided by the glasshouse, allowing the recovery of a large number of transgenic plants from tissue culture (Tung *et al*., 2008; Appendix 7). These *rbcS3C*::*LeNCED1* primary transformants were self-pollinated to produce homozygous lines which exhibited highly restricted stomatal conductances and severely reduced growth rates (Chapter 6). Although plants from these transgenic lines (rbcS3C lines) have the ability to drastically reduce water consumption, the severity of phenotype is too great within an agricultural context as this reduction in water use is coupled with restricted biomass production (See Chapter 6 for further analysis of these rbcS lines).

Thus, these rbcS plants may exhibit ABA over-production above the optimal level for agronomic benefit, in contrast to the sp lines which have lower levels of constitutive over-expression of *LeNCED1* and do not appear to exceed the optimal level. This optimum could be defined as achieving the maximal possible reduction in transpiration before growth rates are adversely affected. During the original transformation protocol used to obtain these sp lines, inadvertent selection against high levels of transgene expression during transformation may have meant that only lines with moderate over-expression were recovered. It has been suggested that lines with higher transgene expression and therefore higher ABA over-accumulation might not have been recovered from tissue culture due to the adverse effects on growth and chlorophyll content of regenerating transgenic shoots (Tung *et al*., 2008). An alternative promoter may therefore be required to reliably generate transgenic lines that over-express *LeNCED1* at the level required to produce ABA over-production greater than sp lines, but not as severe as in rbcS plants. An alternative approach would be to combine the over-expression of *LeNCED1* in the existing sp lines with simultaneous over-expression of another ABA biosynthetic gene designed to increase the flux of ABA precursors, so increasing potential ABA accumulation, as is discussed further in Chapter 7.

6 EFFECTS OF 'ULTRA-HIGH' ABA OVER-PRODUCTION

6.1 INTRODUCTION

6.1.1 The *rbcS3C* promoter

True breeding transgenic tomato lines (sp12 and sp5), over-expressing NCED using constructs involving a constitutive promoter (Gelvin superpromoter), show relatively mild increases in ABA accumulation under non-stress conditions compared to those typically found in water-stressed WT plants. These mild ABA over-producers exhibit reduced stomatal conductance with no loss in biomass production, resulting in significant increases in WUE (Thompson *et al*., 2007a). The only adverse effect on plant growth of constitutively over-producing ABA lines occurred during the propagation phase, when sp12 and sp5 plants exhibited significantly delayed germination (Thompson *et al.,* 2000a) and a longer early establishment period (*cf.* Chapter 9). It is therefore desirable that over-expression of the *LeNCED1* gene is restricted to tissues in which increased ABA over-production is beneficial. An alternative, light-regulated (tomato *rbcS3C*) promoter (Carrasco *et al*., 1993; Gittins *et al*., 2000), was therefore chosen to drive *LeNCED1* expression with the major objective of eliminating the undesirable increased seed dormancy phenotype of the sp lines. Use of this light-inducible, strong promoter in the construct design allowed the creation of new transgenic genotypes which over-produced ABA to a greater extent than in the previous transgenic lines (sp12 and sp5), providing an opportunity to evaluate the effects of ultra high ABA levels on plant growth and development (Tung *et al*., 2008).

6.1.1.1 Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO)

Photosynthesis is the process whereby green plants, algae and some bacteria absorb and use light energy to synthesise organic compounds (initially carbohydrates). Ribulose-1,5-bisphosphate carboxylase/oxygenase, commonly known as RuBisCO, is an enzyme used in the Calvin cycle during the first major step of carbon fixation to catalyse carboxylation (Quayle *et al*., 1954) or oxygenation (Ogren and Bowes,

1971) of ribulose-1,5-bisphosphate (RuBP). The RuBisCO protein constitutes 50% of the soluble protein in leaves and is the most abundant protein on earth (Ellis, 1979).

RuBisCO comprises eight large (L) and eight small (S) polypeptide chains, or subunits (Jensen and Bahr, 1977). In plants and green algae, the 50-55 kDa large subunit (LSU) is encoded by the chloroplast *rbcL* gene (Sugita and Gruissem, 1987), whilst the 12-18 kDa small subunit (SSU) is coded by a family of nuclear *rbcS* genes (Sugita *et al*., 1987). LSUs are formed constitutively in most plants but remain inactive until SSU is present to form the complete enzyme; production of RubisCO therefore depends on the control of rbcS gene expression (Rodermel *et al*., 1988).

6.1.1.2 rbcS genes in tomato

The *rbcS* gene family in tomato comprises five genes (rbcS1, -2, -3A -3B and -3C) which are differentially expressed during the growth cycle (Sugita *et al*., 1987). All five *rbcS* genes are transcribed in photosynthetically active, chloroplast-containing organs, including leaves of mature plants and cotyledons of seedlings grown in light (Wanner and Gruissem, 1991). In other tissues rbcS genes are differentially expressed, with rbcS1, -2 and -3A all being transcribed in dark-grown seedlings and water-stressed mature leaves, whilst rbcS-3B and -3C transcripts are not detected (Wanner and Gruissem, 1991). rbcS3C mRNA is not present in immature or ripe fruit of tomato (Sugita and Gruissem, 1987).

6.1.1.3 Ultra-high ABA rbcS tomato lines

Transformation of tomato with constructs using rbcS3C to drive *LeNCED1* over-expression provided the opportunity to create transgenic lines with increased specificity of NCED transgene expression. Prior use of a chimaeric "super-promoter" (sp) potentially providing strong constitutive expression (Ni *et al*., 1995), yielded few over-expressing primary transformants (Thompson *et al*., 2000a). During transformation inadvertent selection against strong transgene expression appears to have occurred, as only sp lines with limited over-expression were recovered, possibly due to the adverse effects of high transgene expression on growth, meaning that lines

possessing higher ABA levels were not recovered from tissue culture (Tung *et al*., 2008). The use of alternative constructs, in which the rbcS3C promoter was substituted for the super-promoter substantially improved recovery of ABA over-producing transformants from tissue culture. As mentioned earlier, the relatively low light environment in the tissue culture facility coupled with a high sugar content of the tissue culture media may have prevented full expression of the rbcS3C::*LeNCED1* construct until the transformants were transferred to the higher light conditions provided by the glasshouse, allowing the recovery of a greater number of transgenic plants from tissue culture. The primary *rbcS3C*::*LeNCED1* transformants showed more severe symptoms than the sp::*LeNCED1* lines (Tung *et al*., 2008). The rbcS lines exhibited higher levels of leaf *LeNCED1* mRNA than WT, sp5 or sp12 plants and leaf ABA concentrations were 2.3-fold higher than in non-stressed WT plants (Tung *et al*., 2008; Appendix 7). These putative ultra-high ABA over-producers therefore allow evaluation of the effects of more substantially increased ABA production on plant growth and physiology than had previously been possible.

6.1.2 Ultra-high endogenous ABA levels may adversely affect growth and reproductive development

6.1.2.1 Biomass production

Elevated ABA concentrations in leaves may reduce stomatal conductance and transpiration rate, thereby improving "drought tolerance" when water is withheld (Iuchi *et al*., 2001; Qin and Zeevaart 2002). Similar responses were observed when constitutive promoters were used to over-express *NCED* in tomato (Chapter 5) and bent grass (Aswath *et al*., 2005). To date, the use of constitutive promoters to drive ABA over-production has provided limited increases in endogenous ABA concentrations compared to those typically found in water-stressed WT plants, resulting in little or no adverse effect on biomass production in mature plants compared to WT control plants. However, the long-term growth of plants with ultra-high ABA concentrations may be more severely disrupted by direct and indirect effects of the construct, including long term severe reductions in leaf gas exchange.

6.1.2.2 Reproductive development

Formation of flowers is essential for fruit or grain production and delays in flowering may severely delay fruit production (Atherton and Harris, 1986). After the first truss has formed, most tomato cultivars produce repeating developmental units comprising three leaves and internodes terminated by an inflorescence, which build up the sympodial stem (Heuvelink, 2005). The rate at which new trusses appear is closely linked to leaf appearance rate, which is closely related to temperature (DeKoning, 1994). Continued flower development after initiation is strongly influenced by temperature (Picken, 1984), although limited assimilate supplies during flower development often induce flower abortion. If photosynthetic rate is limited by reduced stomatal aperture, and hence CO_2 influx in ultra-high ABA plants, flower and fruit production may be adversely affected through an indirect mechanism whereby substantially increased ABA concentrations may impact on flowering.

In terms of more direct effects of ultra-high endogenous ABA levels, it should be noted that exogenous supplies may delay flowering in *Arabidopsis* (Razem *et al.*, 2006). High ABA levels in transgenic tomato plants may therefore affect flowering and fruit production. However, it is difficult to compare the effects of applications of exogenous ABA with the processes that may occur naturally in plants producing high endogenous ABA concentrations. The range of 'ultra-high' ABA transgenic plants available within tomato therefore provide a valuable tool for evaluating possible effects on developmental characteristics, such as time to initiation of the first inflorescence and the number of leaves preceding the first inflorescence (NLPI). In general, NLPI for tomato is 6-8 leaves (Heuvelink, 2005), but this may be affected by environmental conditions, particularly shading. The sensitive period determining the position on the stem where the first inflorescence develops lasts for *c*, 10 days, starting from the initiation of cotyledon expansion (Dieleman and Heuvelink, 1992).

6.2 AIMS

In the water rationing experiment (Chapter 4) involving a small preliminary sample of rbcS-10 plants, demonstrated that this transgenic line exhibited the most extreme phenotype of all homozygous genotypes available at that time. However, it has subsequently been shown that several rbcS lines exhibit ultra-high ABA accumulation and that steady-state levels of *LeNCED1* mRNA in well-watered leaves are comparable to water-stressed WT plants (Tung *et al*., 2008). In the water rationing experiment, several unusual aspects of the rbcS phenotype were noted, including visible leaf yellowing, consistently low values for stomatal conductance and net assimilation, slow growth rate and abnormal flowering patterns. The growth experiments described here were aimed at utilising the four independent rbcS transformants which subsequently became available as homozygous lines to further investigate these aspects of phenotype and to analyse the effects of ultra-high ABA accumulation on growth, physiology and development. The effectiveness of using a tissue-specific promoter to separate the negative consequence of increased seed dormancy from other high-ABA shoot phenotypes was also evaluated.

6.3 MATERIALS AND METHODS

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rbcS-10 rbcS-17 WT rbcS-18 rbcS-4	rbcS-4 rbcS-10 rbcS-18 rbcS-17 WT
rbcS-18 rbcS-17 WT rbcS-4 rbcS-10	WT rbcS-4 rbcS-10 rbcS-18 rbcS-17
rbcS-18 WT rbcS-4 rbcS-17 rbcS-10	rbcS-4 rbcS-18 rbcS-17 rbcS-10 WT

Figure 6.1. Experimental design for the ultra high ABA growth experiment.

6.3.1 Plant Material and Experimental Design

Seeds of four homozygous rbcS lines (rbcS-4, rbcS-10, rbcS-17, rbcS-18) and WT (*Tm-2*^{a} homozygous) Ailsa Craig were germinated using the method described in Chapter 3. Six uniform plants of each genotype were then arranged in a randomised block design (Fig. 6.1) and grown under well-watered conditions for 18 weeks, before being destructively harvested

6.3.2 Analysis of reproductive development

Plants were monitored daily for signs of the initiation of inflorescences and first anthesis of each flower truss. Time to initiation of the first inflorescence, number of leaves preceding the first inflorescence (NLPI) and number of leaves between each truss on the sympodial stem was recorded for all plants. Time to initiation of the first inflorescence was found to be more reliable than time to first anthesis, as many rbcS plants aborted all flower buds on the earliest trusses produced.

6.3.3 Non-destructive physiological measurements

Gas exchange measurements were made using a CIRAS-1 Infrared Gas Analyser (PP Systems, Hitchin, Herts, UK) coupled with a 2.5 cm^2 Parkinson leaf cuvette. Leaf chlorophyll content was determined non-destructively using a Minolta SPAD-502 meter. All measurements were made for the terminal leaflet of the youngest fully expanded leaf after 5 and 17 weeks of growth.

6.3.4 Seed germination

Twenty seeds of each genotype (WT, rbcS-4, rbcS-10, rbcS-17, rbcS-18) were included in a germination trial. Pre-imbibed seeds were sown directly into individual 7 cm diameter pots containing moist Levington F2s compost at a depth of 1 cm; the compost was carefully compacted to ensure close contact with the seed and avoid desiccation. Pots were covered with 9 cm Petri dish lids to maintain a humid environment until the hypocotyl hook emerged from the compost, when the lids were removed. Pots were monitored daily and the date of germination of each seed was recorded.

6.4 RESULTS

6.4.1 Seed germination of rbcS lines

There was a slight delay of 2-6 d, in the time taken to reach 50% germination in the rbcS lines compared to WT seeds (Fig. 6.2); with rbcS-10 seeds being the least dormant and rbcS-4 being the slowest to germinate. rbcS-10 and rbcS-17 seeds both achieved final germination percentages, similar to WT seeds, but rbcS-18 and rbcS-4 seeds only achieved 65 and 70% germination respectively by the end of the 20 d experimental period. The 'lollipop' phenotype reported by Tung *et al*. (2008) in rbcS lines was not observed when using the germination protocol described in Chapter 3, which allowed no opportunity for the testa to dry out before it was shed.

6.4.2 Vegetative growth of ultra-high ABA plants

In contrast to the high ABA genotypes derived from over-expression of ABA biosynthetic genes driven by the constitutive Gelvin super-promoter (sp5 and sp12), the homozygous rbcS lines obtained following transformation with the *rbcS3C*::*LeNCED1* construct differed visibly from WT plants. The most striking observation was the drastically reduced growth rates of the ultra-high ABA transgenic lines, especially during the early stages. This was monitored by recording the heights of plants grown for 35 days after emergence (DAE) under well-watered conditions. At the end of this period, the mean height of WT plants was 48.1 cm compared to 15.5, 12.4, 7.9, and 5.1 cm respectively for the corresponding homozygous rbcS-10, -17, -18 and -4 plants ($P < 0.001$). The visual observations and height analysis suggest that the greatest reduction in growth occurred in rbcS-18 and rbcS-4 plants, with the rbcS-10 and rbcS -17 lines being slightly less severe in this phenotypic characteristic.

The lower biomass production for all homozygous rbcS lines was also consistent for the longer growth periods. When grown under well-watered glasshouse conditions for 18 weeks (Fig.6.3), lines rbcS-10, rbcS-17, rbcS-18 and rbcS-4 respectively showed 48, 59, 88 and 89 % reductions in vegetative biomass (DW) production ($P<0.001$). The severity of phenotypic effects across all four rbcS lines was

consistent with previous observations, as rbcS-10 and rbcS-17 plants were less severely affected than rbcS-18 and rbcS-4.

The marked reduction in biomass production was less strongly reflected by differences in height, especially in rbcS-10, as lines rbcS-10, rbcS-17, rbcS-18 and rbcS-4 showed 4, 21, 53 and 58 % reductions respectively relative to WT controls. There was no significant difference in plant height between WT and rbcS-10 after 18 weeks (Fig. 6.3), whereas rbcS-17, rbcS-18 and rbcS-4 were all significantly shorter than WT controls ($P<0.001$). This contrasted with the values recorded after 35 days of growth, when rbcS-10 plants exhibited a 68 % reduction in height compared to WT. This recovery in height between harvests reflects the unique phenotype of relatively long internodes observed in rbcS-10 plants but not in the other transgenic lines. This phenotype was found only in rbcS-10 plants and was also observed in previous long-term trials using this transgenic line (Chapter 4). The greater height achieved by rbcS-10 plants means that this line resembled WT plants most closely than the other homozygous rbcS lines. However, when considering other parameters such as SPAD values (Fig. 6.4), biomass production (Fig. 6.3) and general visual observation, this was not the case, as rbcS-17 plants generally exhibited the least severe phenotype overall. Lines rbcS-18 and rbcS-4 were less than half as tall as WT plants, although this difference was smaller than the reduction in plant biomass produced by these ultra-high ABA over-producing plants (Fig. 6.3).

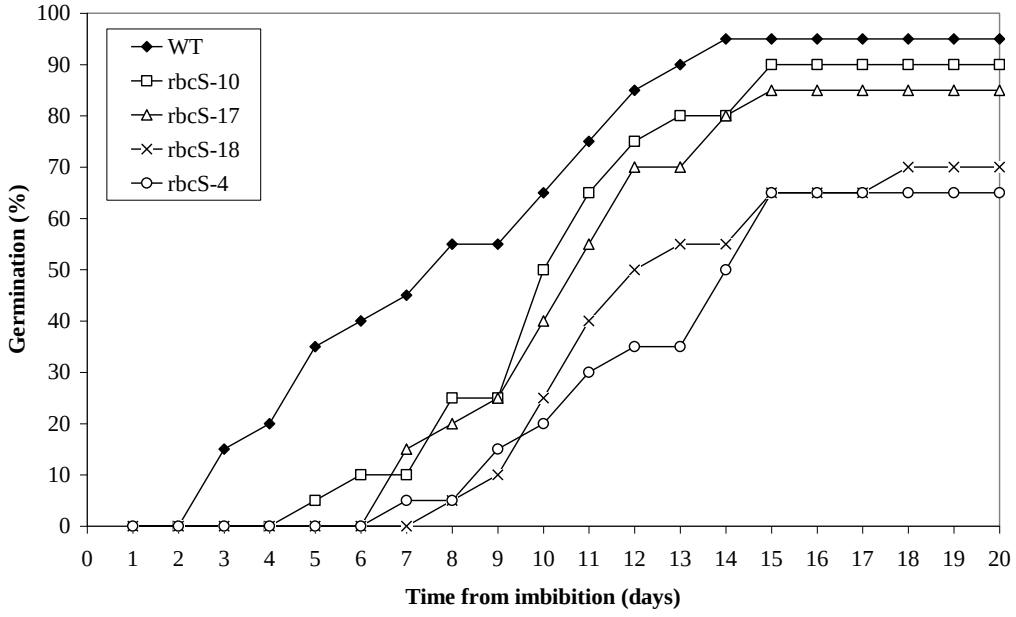
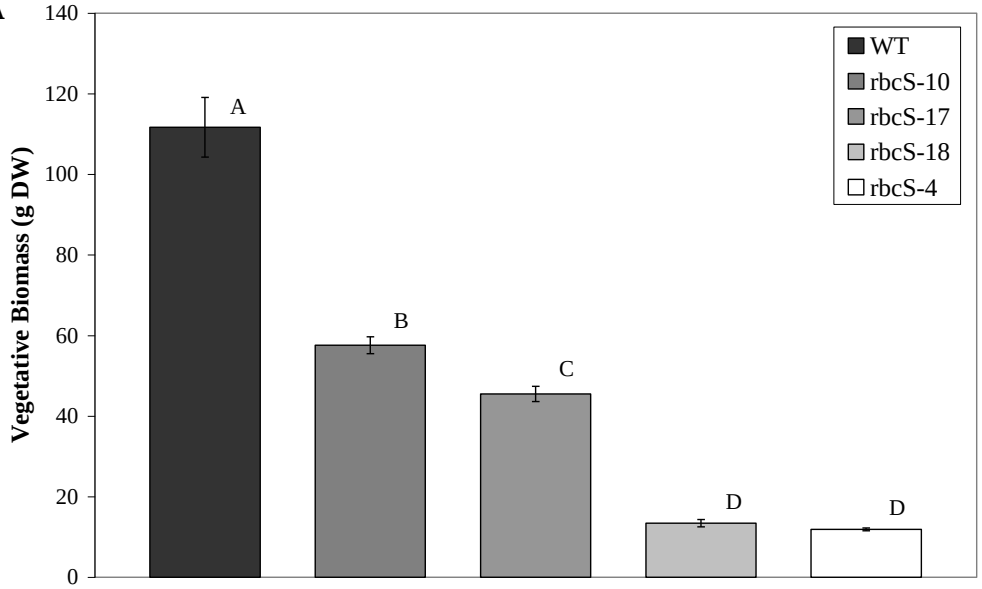
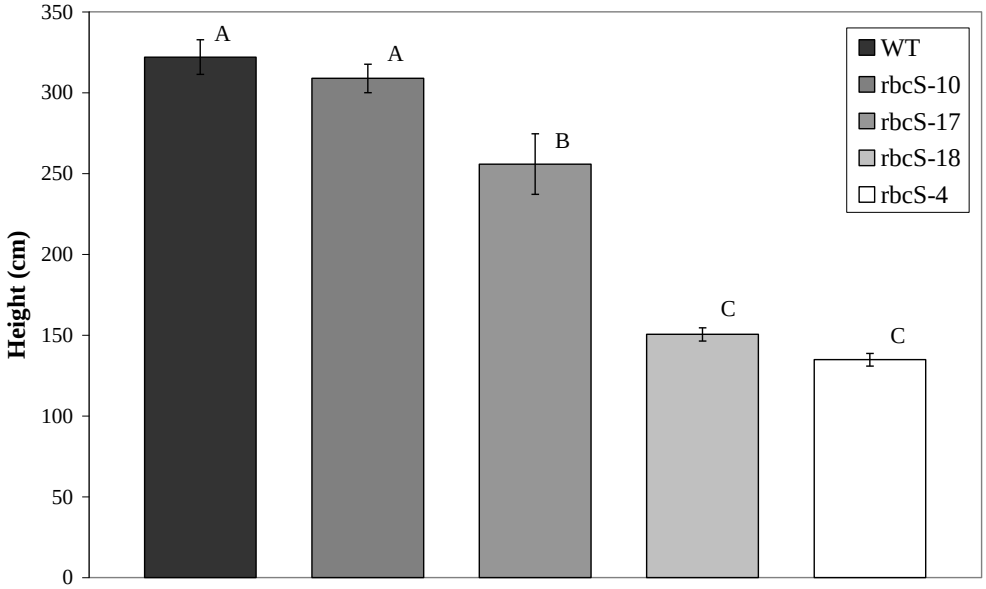


Figure 6.2. Germination of rbcS lines over a 20 day period (n=20)

A



B



C

	Vegetative DW (g)	**Height (cm)**
Fpr.	*<0.001*	*<0.001*
d.f.	12	12
s.e.d.	5.06	14.44
l.s.d.	11.02	31.47

Figure 6.3. A: Vegetative biomass production over an 18 week growth period; **B:** plant height after an 18 week growth period; **C:** statistical summary to accompany A and B; bars with differing letters indicate significant differences (P<0.05).

6.4.3 Leaf Chlorophyll Concentration

All four rbcS lines exhibited severely reduced chlorophyll contents ($P<0.001$) (Fig. 6.4B) and had leaves which visibly exhibited a yellow phenotype, which was more extreme for the first four leaves to be produced. In rbcS-4, the most extreme of the rbcS genotypes, leaves had less than half of the chlorophyll of WT leaves, with mean SPAD values of 52 and 24 respectively ($P<0.001$, s.e.d=1.34, l.s.d.=2.79). High ABA transgenic tomato plants often displayed an overguttating phenotype (Thompson *et al*., 2000a), accompanied by symptoms of interveinal flooding and chlorosis of leaves. Interveinal flooding can be visually observed as leaves display a 'glassy' appearance when the airspaces become filled with liquid. All four rbcS homozygous lines exhibited this phenotype in the first four true leaves, under conditions of normal evaporative demand. Figure 6.4A shows the general pale green appearance of the leaf and stem tissue of an rbcS-10 plant and leaf sectors exhibiting further reduction in chlorophyll concentration accompanied by interveinal flooding. In cases where interveinal flooding has occurred over more prolonged periods, chlorotic patches developed in specific sectors of the leaves. These effects were most pronounced during the early growth stages.

6.4.4 Leaf gas exchange

Under optimal growing conditions, stomatal conductance for the rbcS lines was much lower than in WT plants and was accompanied by drastically reduced assimilation rates ($P<0.001$) (Fig. 6.5). The mean stomatal conductance for all four rbcS lines was only 18 % of that for WT control plants ($P<0.001$). In comparison with the differences in vegetative growth rate, the variation between the four rbcS lines was much smaller (Fig. 6.5). There was no significant difference in assimilation rate between the four rbcS lines, although all lines differed significantly from WT plants ($P<0.001$). The mean assimilation rate for the four rbcS lines was only 23% of that for WT control plants ($P<0.001$), demonstrating that, in comparison with the more mild sp lines, the direct or indirect effects associated with the extreme accumulation of ABA in rbcS plants severely affected assimilation rate and biomass production.

A

B

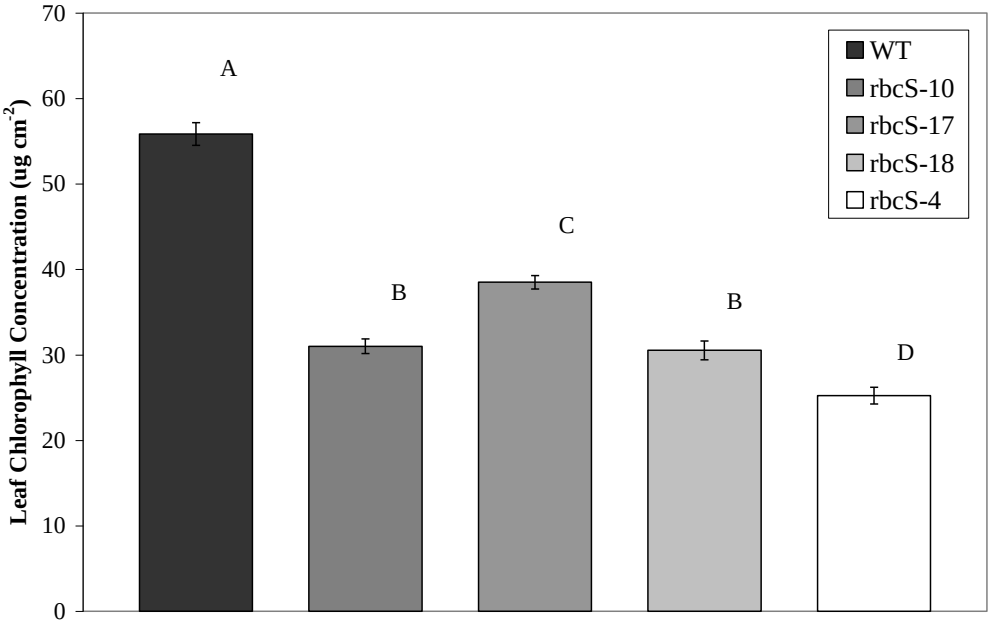
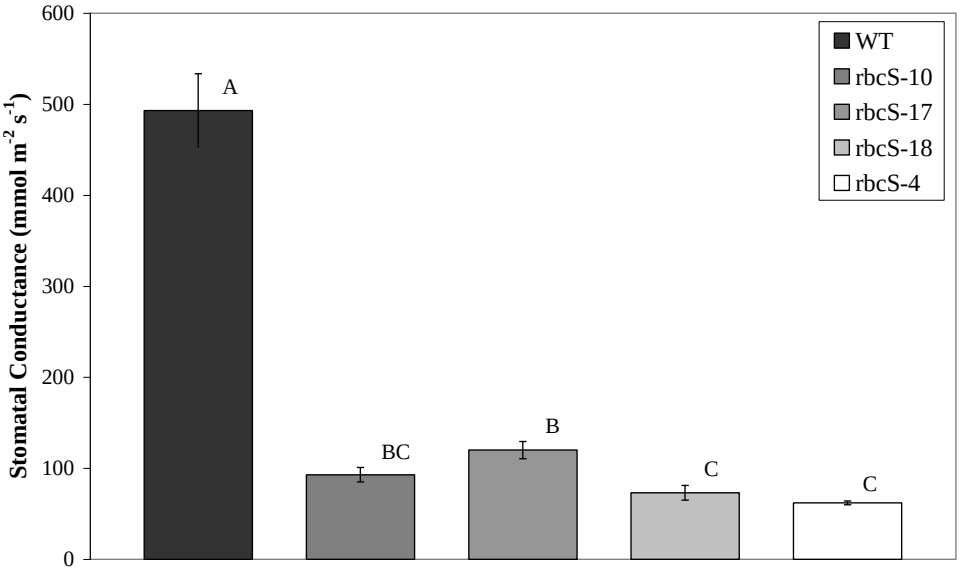
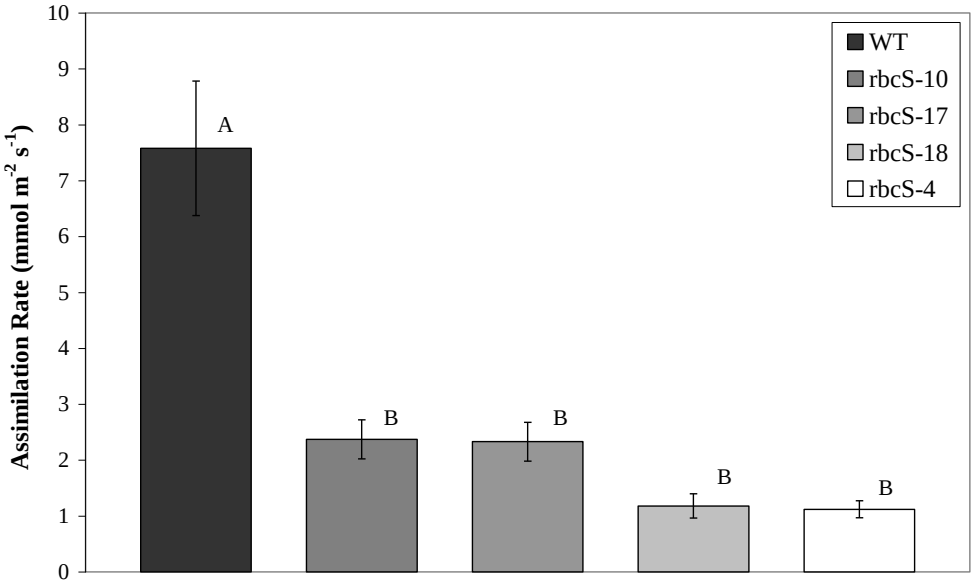


Figure 6.4. **A:** Third true leaf of an rbcS-10 plant, demonstrating interveinal leaf flooding and general reduction in chlorophyll content; **B: l**eaf chlorophyll contents for the WT and rbcS lines at 35 DAE ($P<0.001$, s.e.d=1.34, l.s.d.=2.79). Bars with different letters denote significant differences.

A



B



	Stomatal Conductance	Assimilation Rate
Fpr.	<0.001	<0.001
d.f.	20	20
s.e.d.	22.60	0.843
l.s.d.	47.14	1.759

Figure 6.5. A: Stomatal conductance and **B;** assimilation rate for WT control rbcS plants at, 119 DAE; **C:** statistical summary to accompany A and B. Bars with different letters denote significant differences.

Table 6.1. Time to first inflorescence and number of leaves produced before initiation of truss 1 (NLPI) and truss 2 in plants grown under optimal conditions for 18 weeks.

	Time to First Inflorescence (d)	**Leaves to Truss 1**	**Leaves to Truss 2**
WT	52.8	7.5	3.0
rbcS-10	75.3	10.0	6.0
rbcS-17	87.3	9.8	3.8
rbcS-18	123.5	14.0	6.5
rbcS-4	136.3	14.0	N/A
Mean	95.0	11.1	3.85
Fpr.	<0.001	<0.001	<0.001
d.f.	12	12	12
s.e.d.	1.987	0.408	0.465
l.s.d.	4.330	0.889	1.014

6.4.5 Reproductive development and fruit production of ultra-high ABA plants

The rbcS lines all showed severely impaired reproductive development compared to WT plants grown under identical conditions. NLPI and time to first anthesis were far greater in rbcS plants ($P<0.001$), with rbcS-4 plants for example taking 83 days longer than WT to initiate the first inflorescence (Table 6.1). This phenotype was not observed in the milder constitutive over-expresser lines (sp lines) in which there was no difference in NPLI, time to first anthesis and number of leaves between successive trusses (i.e. sympodial pattern) of sp lines compared to WT control plants (*cf.* Chapter 7).

Flower bud abortion was also drastically increased in all rbcS lines, with the first few inflorescences often aborting completely (Fig. 6.6A) and therefore failing to produce fruit. The rbcS-10 plants exhibited the shortest time prior to initiation of

inflorescences, but the first two inflorescences invariably failed to develop normally although the severity of this effect decreased with plant age. Figure 6.6 shows that, although trusses 1 and 2 both failed to produce fruit, the latter developed further before being aborted. During the 18 week growth period, none of the rbcS plants produced trusses that were visually similar to WT plants. Even during the later stages of growth, when fruit was finally produced by rbcS-10 and rbcS-17 plants, most flowers aborted on each truss, leaving only one or two small fruit to develop. Consistent with other biomass production data, rbcS-18 and rbcS-4 plants provided the most extreme examples of this phenotype and failed to produce any fruit during the 18 week growth period under optimal conditions.

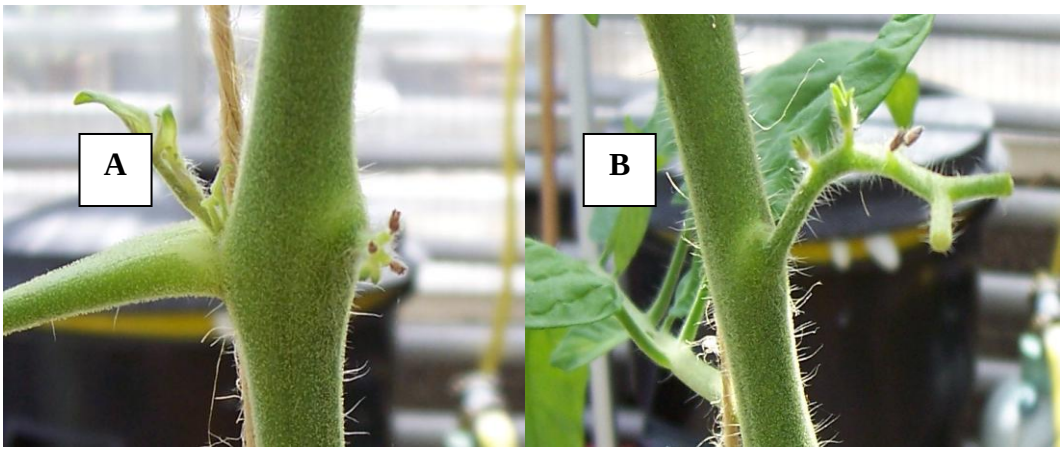


Figure 6.6. A and B: trusses 1 and 2 of an rbcS-10 plant.

6.5 DISCUSSION

6.5.1 Effect of 'ultra high' ABA accumulation on plant growth and physiology

In contrast to the high ABA transgenic lines examined previously (sp5 and sp12), which over-express the *LeNCED1* gene driven by the constitutive Gelvin super promoter, the growth and physiology of all lines transformed with the *rbcS3C*::*LeNCED1* construct showed much more extreme and undesirable phenotypes. These rbcS plants exhibited drastic reductions in biomass production and chlorophyll concentration which were maintained, albeit to a decreasing extent, throughout the growth period. The pronounced symptoms of interveinal flooding were always particularly severe in the first few leaves to be initiated, but often disappeared completely in the fully expanded leaves, emerging further up the stem on mature plants.

6.5.1.1 Ultra-high ABA accumulation dramatically restricts leaf gas exchange and biomass production

Whilst the reduction in stomatal conductance in the first available high ABA transgenic tomato lines (sp12 and sp5) was sufficient to reduce transpiration, this was not sufficiently severe to reduce assimilation rate or restrict biomass production significantly, thereby increasing WUE (Thompson *et al*., 2007a). However, in all rbcS lines, stomatal conductance and assimilation rate were severely reduced, with consequent direct or indirect negative effects on growth. The large reductions in stomatal conductance exhibited by rbcS plants meant that transpiration was also greatly reduced, with the result that from visual observation, 'ultra-high' ABA plants required much less frequent watering throughout the experimental period than WT plants. Such differences in stomatal behaviour are to be expected from the differing extent of ABA accumulation in the rbcS and sp lines. Thompson *et al*. (2007a) reported that the maximum enhancement of leaf ABA accumulation in the two sp lines was 1.6-fold, whereas dehydrated leaves of WT plants (subjected to turgor loss for 5-6 hours) exhibited a 2.8-fold increase in ABA concentration relative to unstressed leaves (Tung *et al*., 2008). In the rbcS lines, the high level of expression

achieved by the rbcS3C promoter allowed accumulation of ABA to levels closer to those typical of water-stressed WT leaves than was the case with the 'sp lines'. The greatest accumulation was observed in rbcS-18 plants which exhibited a 2.5-fold increase, compared with non-stressed WT controls (Tung *et al*., 2008; Appendix 7).

Previous experiments in which water supplies were rationed, causing WT and eventually sp12 plants to become water stressed, have revealed the extreme water saving capacity of rbcS-10 plants and demonstrated that these ultra-high ABA over-producers can attain an equivalent vegetative biomass to WT controls when water is severely restricted (Chapter 4). During the water rationing experiment (Chapter 4), the ultra-high rbcs-10 plants, which are strongly pre-adapted to water-stress, consistently conserved large amounts of soil moisture due to their significantly lower stomatal conductance, allowing turgor and hence growth to be maintained when WT plants were experiencing water-stress. Improved drought tolerance has been noted in other species which have been transformed with constructs based on the use of constitutive promoters to over-express NCED genes (Iuchi *et al*., 2001; Aswath *et al*., 2005). The constitutive over-expressers described above are, however, milder in their absolute increases in ABA accumulation, while the extreme negative impacts on growth and physiology exhibited by the rbcS lines have not been reported for other species (Iuchi *et al*., 2001; Aswath *et al*., 2005). For example Aswath *et al*., (2005) reported that the growth of transgenic bent grass plants over-expressing the cowpea *VuNCED1* gene was visually similar to that of WT control plants under non-stress conditions.

The severe reductions in assimilation rate in all rbcS lines almost certainly must have contributed to their slow growth rate and may have limited assimilate supplies to support fruit production. However, Tung *et al.* (2008) reported that the pool of xanthophyll, which not only provides substrate for NCED, but is also necessary for the formation of the light harvesting complex II, were reduced (Kuhlbrandt *et al*., 1994). The photosystem II antennae dissipate excess excitation energy as heat (Demmig-Adams, 1990) in a process known as non-photochemical quenching (NPQ); and zeaxanthin can play a key role in this process at high light intensity (Demmig-Adams and Adams, 1992). If the use of light energy is reduced, the highly reactive intermediates produced by photosynthetic light reactions combine with

molecular oxygen to produce compounds harmful to higher plant cells. As CO_2 influx is limited by restricted stomatal opening, the balance of excitation energy absorption by the chlorophyll antenna and its use in photochemistry can be severely affected (Horton *et al*., 2001), causing photooxidative damage to the photosynthetic apparatus (Johnson *et al*., 2007). Therefore, a reduction in the size of the zeaxanthin pool and other key components of the photoprotective mechanism, may reduce the photosynthetic capacity of rbcS plants, contributing to their low growth rates. This problem could possibly be alleviated by crossing rbcS plants with the transgenic tomato line, BCH12. This line over-expresses *LeBCH2*, a gene encoding the enzyme (BCH) which catalyses an earlier step in the ABA biosynthetic pathway. BCH12 plants exhibit significantly increased mean zeaxanthin, all-*trans* violaxanthin and the 9'-*cis* neoxanthin concentrations within the leaves (Balasubramanian, 2007).

6.5.1.2 *Ultra-high ABA plants exhibit reduced chlorophyll content and flooding of intercellular leaf spaces*

All rbcS lines exhibited a general reduction in leaf and shoot chlorophyll concentration, combined with sectored chlorosis and 'glassiness' associated with interveinal flooding in the younger leaves. This general and uniform reduction in leaf and stem chlorophyll concentration has not previously been reported for other ABA over-producing transgenic lines. Associated with this reduction in chlorophyll is the depletion of xanthophyll pools which ultimately provide substrate for NCED (Tung *et al*., 2008). The excessive depletion and cleavage of xanthophylls, which also function as components of light harvesting complex II, may have reduced the photosynthetic capacity of rbcS leaves. Alternatively, the large increase in leaf ABA in rbcS plants may have induced down-regulation of important photosynthetic genes, thereby reducing leaf chlorophyll concentration. Researchers have previously shown that exogenous application of ABA to the stems of tomato plants can reduce the mRNA levels of *rbcS* and *cab* genes in leaves (Bartholomew *et al*., 1991). However, similar levels of endogenous ABA induced by wilting do not appear to reduce leaf chlorophyll concentration.

As well as the phenotype of general 'yellowness' in the rbcS lines, interveinal leaf flooding and over-guttation were commonly observed. These latter symptoms are most likely to have resulted from the ABA-induced increase in root hydraulic conductivity reported previously for both sp and rbcS lines (Thompson *et al*., 2007a; Tung *et al*., 2008), in combination with reduced stomatal conductance. The sp12 and sp5 lines have both been reported to exhibit over-guttation and interveinal flooding (Thompson *et al.,* 2000a), although this occurred only under conditions of low evaporative demand and has not been observed under standard glasshouse conditions (Chapters 4 and 5). This was not the case for the rbcS lines, however, as these regularly exhibited both of these phenotypes, even under normal glasshouse conditions. In this case, the combined effects of increased root hydraulic pressure and severely reduced stomatal conductances are likely to have increased the frequency of leaf flooding, even during conditions of normal evaporative demand, with prolonged periods of this phenomenon causing patches of interveinal chlorosis, particularly in the lower leaves. Over-guttation was most frequently observed at dawn in the glasshouse, but may also deliberately be triggered by transferring plants from low to high humidity environments.

6.5.2 Use of a tissue specific promoter (rbcS3C) to aviod ABA-induced seed dormancy

ABA is essential for induction of seed dormancy (King *et al*., 1976) and ABA-deficient mutants often show reduced seed dormancy in addition to severely decreased desiccation tolerance (Karssen *et al*., 1983; Ooms *et al*., 1993). One of the few negative effects of mild over-production of ABA in the sp lines of tomato was the increase in seed dormancy (Chapter 7; Thompson *et al*., 2000a). In sp12, the mildest line, this disadvantage can easily be offset by simply sowing seeds a few days earlier than those of the WT genotype. This would not be considered a serious drawback for commercial growers, as sowing protocols could be adjusted accordingly. However, the problem of excessive dormancy is not so easily solved for seed of the slightly less mild sp5 line, which typically shows higher values for WUE than sp12 (Thompson *et al*., 2007a). To achieve uniform germination of this line, treatment with norflurazon, a herbicide which blocks carotenoid, and hence

ABA synthesis, by inhibiting phytoene desaturase, is required (Smith, 1997). However, this herbicide induces photobleaching of the emerging cotyledons when seeds are treated at high concentrations or for prolonged periods. This can result in death when most of the photosynthetic area is bleached and even when less extensive areas are affected, there may still be a lack of uniform development of emerged seedlings. For this reason an alternative, light-regulated (*rbcS3C*) promoter was used to drive the *LeNCED1* expression, with the objective of creating ABA over-producing lines in which seed dormancy is not increased to the levels requiring the use of norflorazon to induce germination. The *rbcS3C* gene is only transcribed in photosynthetically active organs, including the leaves of mature plants and cotyledons of seedlings grown in light (Wanner and Gruissem, 1991). This enables the creation of rbcS lines showing ultra-high ABA accumulation in green shoots, but which would be relatively unaffected by the predicted increase in seed dormancy, if equivalent ultra-high ABA levels could be produced using constructs based on a constitutive promoter.

The use of the tissue-specific *rbcS3C* promoter, which has low expression in non-photosynthetic tissue (Gittins *et al*., 2000), successfully reduced seed dormancy in all homozygous rbcS lines, with only a 2-6 day delay in germination. This slight delay is easily compensated for by adjusting sowing dates. In addition to suggesting that the tissue-specificity of the rbcS3C promoter accounted for the observed reduction in seed dormancy, Tung *et al*. (2008) proposed that, as rbcS3C mRNA is not detectable in tomato fruit (Sugita and Gruissem, 1987), it is unlikely that the *rbcS3C*::*LeNCED1* transgene significantly increases ABA biosynthesis in the maternal fruit tissue and therefore does not affect seed development and germination.

Tung *et al*. (2008) reported a 'lollipop' phenotype when rbcS lines were germinated in petri dishes, resulting from the reduced ability of the expanding cotyledons to escape from the testa. They hypothesised that this phenomenon probably resulted because increased embryonic ABA biosynthesis during embryogenesis affected development of the testa. However, this phenotype was not observed when seeds were sown in compost due to the combined effect of a softening of the testa by the moist compost and removal of the softened testa from the cotyledons as the hypocotyl extended as a result of the resistance provided by the 1 cm layer of

compacted compost at the surface of the pots. This allowed the cotyledons to emerge and expand normally, negating the need for droplets of water to be applied to facilitate testa softening (Tung *et al*., 2008).

6.5.3 Effect of 'ultra high' ABA concentrations on reproduction

'Ultra-high' ABA lines containing the *rbcS3C*::*LeNCED1* construct exhibited a severely altered pattern of reproductive development, with a significantly longer vegetative period before the first flower truss was produced, as well as a much greater frequency of abortion of whole trusses or individual flowers. This altered flowering phenotype was not observed in the milder transgenic ABA over-producing tomato lines (sp5 and sp12). Deswarte *et al*. (in prep.) reported that flowering was delayed when Arabadopsis was transformed using the same *rbcS3C*::*LeNCED1* construct as in the tomato lines. However, this delay amounted to 3-4 days compared to 22-83 d in the equivalent rbcS lines of tomato.

The effect of high ABA levels on the initiation of flowering in tomato appears to be consistent with the nutrient diversion hypothesis (Sachs and Hackett, 1969), which postulates that the quantity of assimilate available to the apex during the sensitive period must reach a defined threshold before inflorescence initiation can take place. The greatly reduced stomatal conductances and extremely slow growth rates of the rbcS tomato lines, especially during the early phases of growth, may perhaps provide a direct 'assimilate-based' cause for the observed substantial increase in NLPI in these lines. This hypothesis could also be used to explain the significant increase in flower bud abortion in the rbcS tomato lines as low assimilate availability during flower development stimulates the abortion of flower buds (Heuvelink, 2005).

6.5.4 Utility of ultra-high ABA genotypes and optimal levels of tissue-specific ABA over-production

Use of the *rbcS3C*::*LeNCED1* construct has shown that ultra-high levels of ABA over-production may be achieved in transgenic tomato lines by manipulating a single key ABA biosynthetic gene. In the leaves of these transgenic rbcS lines,

LeNCED1 mRNA levels are very similar to those typically found in dehydrated WT plants (Tung *et al*., 2008). The effects of long-term ultra-high ABA accumulation include interveinal flooding, reduced chlorophyll content, impaired cotyledon release from the testa, drastically reduced growth rates and significantly restricted leaf gas exchange. Whilst such symptoms are undesirable in commercial tomato production systems, the ability of ultra-high ABA lines to survive on limited water supplies may be useful in situations where production of even limited quantities of biomass can be beneficial. For example, in climates where WT plants could not survive prolonged drought, enhanced ABA lines may improve the ability of plants to sustain growth under conditions of low soil moisture content until seasonal rainfall was received.

In the well-watered treatments, which are more comparable to optimal agricultural conditions, a drastic biomass penalty for the substantial water savings in the rbcS lines was observed (Fig 6.3). Interestingly, this was not the case in transgenic *Arabidopsis* plants over-expressing *LeNCED1* using the same tomato promoter (rbcS). The use of identical rbcS3C::*LeNCED1* constructs to transform *Arabidopsis* resulted in transgenic plants with ABA accumulation levels similar to those of the sp5 and sp12 lines of tomato. The rbcS transgenic lines of *Arabidopsis* exhibited a significant increase in WUE for both gravimetric (WUE_p) and instantaneous gas exchange (WUE_{iGE}) measurements (Deswarte *et al*., in prep.). *Arabidopsis* lines also showed a much reduced delay in flowering compared to transgenic tomato plants. This trend was consistent for all comparisons of the effects of the construct in tomato and *Arabidopsis*, in which the strength of over-expression and the associated phenotypes differ greatly. Transgene expression was lower in the rbcS *Arabidopsis* lines than in the rbcS tomato lines (Tung *et al*., 2008; Deswarte *et al*., in prep.), meaning that the same rbcS3C::*LeNCED1* construct was capable of producing much greater increases in ABA content in tomato. The "lollipop" phenotype described by Tung *et al*. (2008), whereby the cotyledons fail to emerge from the testa, was not observed in *Arabidopsis*.

Use of the rbcS3C tomato promoter in *Arabidopsis* resulted in plants with more optimal levels of ABA over-production, possibly due to the imperfect compatibility between the *Arabidopsis trans* acting factors and the *cis* elements of the tomato promoter (Deswarte *et al*., in prep.). Use of the *Arabidopsis* RuBisCO small sub-unit

promoter (AtrbcS) to drive NCED transgene expression in tomato may therefore achieve optimal ABA over-production whilst maintaining appropriate tissue specificity to overcome the increased seed dormancy problems of transgenic sp lines.

7 WATER USE EFFICIENCY OF DOUBLE GENE TRANSGENIC LINES

7.1 INTRODUCTION

7.1.1 β-carotene hydroxylase

It is well documented that NCED catalyses a key regulatory step in ABA biosynthesis (Qin and Zeevaart, 1999; Thompson *et al*., 2000A), but less is known about the regulation of earlier steps in the pathway. The enzyme β-carotene hydroxylase (BCH) catalyses the reaction in which β-carotene is hydroxylated on both rings to produce the first xanthophyll in the ABA pathway, all-*trans*-zeaxanthin (Cunningham and Grant, 1998; Hirschberg, 2001). The ABA biosynthetic pathway is discussed more fully in Chapter 1.

Sun *et al.* (1996) first identified BCH cDNA in *Arabidopsis thaliana*, and BCH genes have subsequently been identified in many species including the Solanaceous species, tomato (Hirschberg, 1998), pepper (Bouvier *et al*., 1998) and tobacco (Götz *et al*., 2002). BCH is encoded by two genes in tomato (Hirschberg, 2001; Galapaz *et al*., 2006), *CrtR-b1* and *CrtR-b2* (alternatively known as *BCH-1* and *BCH-2*), which appear to be involved in various physiological and developmental processes. *CrtR-b1* is highly expressed in green tissue, resulting in the formation of an all-*trans*-zeaxanthin pool which functions in photoprotection, whereas *CrtR-b2* is activated in maturing flowers to produce yellow xanthophyll pigments which attract pollinating insects (Hirschberg, 2001; Galapaz *et al*., 2006). This second gene has recently been shown to be strongly expressed in both leaves and roots in response to water-stress, suggesting a possible additional function for *CrtR-b2* in producing the β-xanthophylls required to sustain ABA biosynthesis (Taylor *et al*., 2005; Sonneveld *et al*., in preparation). When a construct encoding BCH was over-expressed in transgenic *Arabidopsis* plants, a two-fold increase in the level of violaxanthin in the leaves was found, with a consequent increase in tolerance to light and temperature stress (Davison *et al*., 2002). Similarly, tomato plants transformed with a CaMV35S-driven *LeCrtR-b2* (BCH) construct also showed a twofold increase in all-*trans*-violaxanthin in transgenic tomato leaves (Taylor *et al*., 2005).

7.1.2 Over-expression of *LeNCED1* depletes the xanthophyll precursor pool

Plants over-expressing the *LeNCED1* transgene exhibit significantly improved WUE (Chapter 5; Thompson *et al*., 2007a), although it is possible that the degree of ABA over-production in these plants may not have reached a level which would provide optimal stomatal behaviour to achieve maximum improvement in WUE. However, there appears to be a limit on the extent to which ABA can be increased via constitutive over-expression of *LeNCED1* alone. When tomato plants were transformed with constructs using the rbcS3C promoter to drive *LeNCED1* over-expression, many of the transgenic plants were found to have very high levels of ABA (Tung *et al*., 2008; Appendix 7). This 'ultra-high' level of ABA over-production was accompanied by several undesirable phenotypes, including highly restricted stomatal conductances and severely reduced growth rates, possibly because the high levels of NCED activity caused excessive depletion of xanthophyll precursor pools.

As discussed above, the milder sp::*LeNCED1* over-expressing plants (sp12 and sp5) show more efficient use of water without major negative impacts on biomass production. These plants contain reduced quantities of xanthophyll precursors, with lower leaf concentrations of zeaxanthin, all-*trans* violaxanthin, 9'-*cis* neoxanthin and β-carotene compared to WT plants (Balasubramanian, 2007). This could be because increased expression of *LeNCED1* increases demand for xanthophyll precursors, with the result that endogenous levels of *LeBCH2* mRNA are insufficient to maintain precursor pool sizes. These plants have occasionally been found to exhibit mild symptoms of leaf chlorosis, although this is associated with interveinal leaf flooding and was observed under only conditions of high humidity. Transformation of potato with the same *LeNCED1* construct induced a more extreme photobleaching phenotype which was attributed to depletion of the xanthophyll pool (Symonds, 2002).

Several studies have highlighted the importance of BCH in the synthesis of β-xanthophylls. When transgenic *Arabidopsis* plants constitutively over-expressed an *AtBCH* construct, the xanthophyll pool size doubled (Davison *et al*., 2002). In

tobacco plants transformed with a triple-op inducible promoter, there was a 33% increase in β-xanthophyll levels after a 24 hour period of tetracycline induction of BCH mRNA (Paul Royle, unpublished data). The over-expression of a *LeBCH2* construct in tomato significantly increased mean zeaxanthin, all-*trans*-violaxanthin and the 9′-*cis* neoxanthin concentrations within the leaves (Balasubramanian, 2007); this study also revealed that up-regulation of BCH alone increased conversion of β-carotene to ABA (Section 7.1.3). In these plants, the size of the β-carotene pool was consistently unaffected, demonstrating that maintenance of the large β-carotene pool in leaves is tightly regulated, probably via a feedback mechanism (Taylor *et al*., 2005). Pogson and Rissler (2000) showed that when plants were transformed with an antisense BCH construct, the pool of β-xanthophylls was significantly decreased.

Over-production of an enzyme such as BCH, which is important in the control of substrate supply for NCED, may provide a solution to the problem of reduced xanthophyll precursor pools and thereby allow further accumulation of ABA under unstressed conditions.

7.1.3 Simultaneous over-expression of two ABA biosynthetic genes

In single construct transgenic tomato genotypes, over-expression of *LeNCED1* (lines sp12 and sp5) significantly increased leaf ABA accumulation (Thompson *et al*., 2000). In BCH12 plants, over-expression of *LeBCH2* alone increased ABA content in unstressed leaves by 38% compared to WT controls (Appendix 8). These plants also exhibited reduced stomatal conductances and transpiration rates, suggesting a possible effect on plant water use efficiency when BCH alone is over-expressed (Balasubramanian, 2007). Creating plants which combine over-expression of *LeBCH2* and *LeNCED1* might increase substrate availability, thereby sustaining an increased flux through the ABA biosynthetic pathway and maintaining long-term elevation of ABA accumulation. To produce a useful additional improvement in WUE, any double construct 'high ABA' transgenic lines would have to maintain higher ABA concentrations than single gene lines, but not to the extent of the 'ultra-high' rbcS lines (Tung *et al*., 2008), to avoid any negative effects on plant growth and physiology.

To create transgenic lines which simultaneously over-express *LeBCH2* and *LeNCED1*, a homozygous *LeBCH2* over-expressing line was crossed with the two homozygous sp lines (sp12 and sp5) to create two double gene lines, G28 (BCH12/sp12) and G29 (BCH12/sp5). Details of the procedures involved are given in Chapter 3. G28 and G29 both exhibit substantially increased seed dormancy compared to the parental lines (BCH12, sp12 and sp5) and will not germinate without application of the herbicide norflurazon (Chapter 9). This observation suggests the existence of an additive or synergistic effect of up-regulating two ABA biosynthetic genes.

The *LeBCH2* and *LeNCED1* transgenes significantly increased leaf ABA content in the BCH12 and sp12 genotypes respectively compared to WT plants, while their simultaneous over-expression in the double transgenic G28 line resulted in the highest leaf and root ABA contents (Balasubramanian, 2007; Appendix 8). The direct effect of the transgenes on roots was investigated by measuring ABA concentration in isolated root cultures of WT, BCH12, sp12 and G28. Although the roots of BCH12 showed no detectable increase in ABA, roots of sp12 exhibited a three-fold increase in ABA compared to the wild-type. When both transgenes were combined, ABA concentration in the roots of line G28 was increased by over 10-fold (Balasubramanian, 2007; Appendix 8). These observations suggest that the combined over-expression of the *LeBCH2* and *LeNCED1* transgenes has a synergistic effect on ABA content in both leaves and roots. The increased supply of xanthophyll precursors caused by up-regulation of BCH appears to provide additional substrate for conversion to xanthoxin by NCED, so permitting greater increases in ABA than can be achieved by over-expression of *LeNCED1* alone.

7.2 AIMS

In transgenic tomato plants over-expressing *LeNCED1* alone, it is possible that the sustainable level of ABA over-production is limited by the availability of ABA precursors. Creating plants with simultaneous over-expression of *LeBCH2* and *LeNCED1* provides greater substrate availability to sustain an increased flux through the ABA biosynthetic pathway. Over-expression of the *LeBCH2* or *LeNCED1* transgenes independently increased leaf ABA content relative to WT plants, whereas their interaction in the double transgenic (G28) line resulted in further increases in leaf and root ABA contents. The extent to which this additional increase affects growth, productivity and WUE has yet to be evaluated.

The experiments reported here were designed to investigate whether the additive/synergistic effect of over-expressing two ABA biosynthetic genes provided further improvements in WUE compared to single transgenic genotypes over-expressing only one of these genes. The implications of further increases in ABA over-production on plant physiology, growth and reproductive development were also analysed.

7.3 MATERIALS AND METHODS

7.3.1 General Experimental Design

Seeds were sown using to the appropriate protocol for each genotype to synchronise development at the end of the seedling establishment phase (Chapter 3). Twelve uniform plants of each line were selected at the four leaf stage for inclusion in the experiment; six of these were destructively harvested for each line to determine dry weight at the start of the experiment. The six remaining replicates of each line were re-potted into 7 litre diameter pots and arranged in a randomised block design in the glasshouse. The compost (Levington M3) was then watered until saturated. Once excess water had drained from the pots, each was weighed to ascertain its weight at field capacity. Uvi ground cover disks (Growing Technologies, Derby, UK) were placed on the surface of the compost to minimise evaporation. A blank pot which did not contain a plant was included within each

block; these and all other pots were watered to field capacity at the start of the experiment and re-weighed every day. Although the compost in pots containing plants was covered with UVi disks to minimise evaporation, the blank pots allowed accurate measurements of, and correction for, daily weight loss by evaporation from the compost. Daily weight loss from the blank pots was subtracted from the corresponding values for pots containing plants to obtain daily measurements of transpiration.

All pots were weighed daily to determine the quantity of water used and supplied with an equivalent volume of water to return the compost to field capacity. Daily transpiration rates were summed to determine total water loss plant^{-1} during the experimental period. All plants were then harvested and above-ground dry weight, plant height and the length of each leaf were recorded. Mean biomass for each line at the beginning of the experiment was subtracted from the corresponding value at final harvest to determine biomass accumulation during the experimental period. Transpiration efficiency (TE_p) was calculated for all plants.

7.3.2 Experiment 1 - WT, BCH12, sp12 and G28

The duration of this gravimetric experiment was altered slightly from the preliminary TE_p experiment described in Chapter 5, as the experimental period was increased to 30 days. Gas exchange measurements were made for the terminal leaflet of the youngest fully expanded leaf of all plants on days 28, 29 and 30 using a CIRAS-2 Infrared Gas Analyser and 2.5 cm^2 Parkinson leaf cuvette (PP Systems, Hitchin, Herts, UK) . On day 30, leaf greenness of the youngest fully expanded leaf was determined using a Minolta SPAD-502 meter. SPAD values were then converted to leaf chlorophyll concentration (μg cm^{-2}) using the calibration presented in Chapter 3.

7.3.3 Experiment 2 – WT, BCH12, sp5 and G29

The design of Experiment 2 was identical to Experiment 1 except that its duration was reduced to 25 days because the plants became too large to transport comfortably between their position in the experimental design and the balance during the latter

stages of Experiment 1. On day 25, chlorophyll concentration in the youngest fully expanded leaf was determined as for experiment 1.

Results from the destructive harvest in Experiment 1 suggested that the variable stage of reproductive development at harvest introduced inconsistencies in the dry weight data obtained as fruit production had just begun, and some plants had greater truss weights than others at harvest. Flower trusses were therefore removed from all plants, in Experiment 2 to prevent fruit set and standardise the stage of plant development at harvest. A separate set of plants grown alongside those used for Experiment 2 for were analysed to characterise reproductive development. These were selected at the same time as those in the main gravimetric experiment and were arranged in an identical randomised block design, with six replicate plants for each line (WT, BCH12, sp5 and G29). Plants were grown under identical environmental conditions to those in Experiment 2 and were monitored daily for signs of the initiation of inflorescences and first anthesis of each flower truss. Time to initiation of the first inflorescence, number of leaves preceding the first inflorescence (NLPI), and the number of leaves between each truss on the sympodial stem were recorded for all plants.

7.3.4 Experiment 3 - WT, BCH12, sp12, sp5, G28 and G29

As Experiments 1 and 2 were conducted at different times of year, the inevitable differences in environmental conditions were reflected by differences between experiments in *TEp* values for WT plants. Whilst some comparisons can legitimately be made between the two data sets, a larger gravimetric *TEp* experiment was designed to include all three 'single' and both 'double' gene lines (BCH12, sp12, sp5, G28 and G29) and WT controls. The experimental period was again 25 days and flower trusses were removed from all plants to prevent complications associated with fruit set.

7.3.5 Data Analysis

Analysis of variance (ANOVA) for a randomised block design was carried out for data, using Genstat 8th edition (Lawes Agricultural Trust, Rothamstead, Herts, UK).

7.4 RESULTS

7.4.1 Experiment 1 - WT, BCH12, sp12 and G28

7.4.1.1 Gravimetric Measurements of Transpiration

The lines were ranked WT>BCH12>sp12>G28 for mean daily transpiration over the 30 day experimental period, with values of 506, 451, 436 and 381 ml d^{-1}, respectively (Fig. 7.1). WT plants generally transpired most rapidly while G28 plants transpired least water. The difference between BCH12 and sp12 was less pronounced, with BCH12 plants only transpiring significantly more than sp12 on 7 individual days ($P<0.05$). The effect of over-expression of the two ABA biosynthetic genes was additive as daily mean transpiration by G28 plants was lower than that of either of the single gene over-expressers ($P<0.001$).

Total transpiration over the 30 day experimental period is shown in Figure 7.1B. WT plants transpired more water than all 'high ABA' lines, whereas G28 plants transpired least (25% less water than WT; $P<0.001$). This reduction in transpiration equates to a saving on irrigation of 4.11 kg H_2O $plant^{-1}$ during the experimental period. The effect of the over-expression of both genes was additive as the G28 plants used less water than the single gene over-expressers (BCH12 and sp12; $P<0.001$). This difference was not attributable to variation in plant size when the experiment commenced because the four genotypes did not differ significantly in terms of their initial dry weight.

Table 7.1. Stomatal conductance (g_s) on Days 28-30 of the experimental period and accompanying ANOVA summary (n=6).

	Stomatal Conductance (mmol m^{-2} s^{-1})		
	Day 28	**Day 29**	**Day 30**
WT	363	315	388
BCH12	281	289	306
Sp12	257	262	283
G28	230	280	315
Fpr.	0.063	0.379	0.116
s.e.d.	45.4	29.6	42.1
l.s.d.	98.9	63.2	90.0

7.4.1.2 Instantaneous Gas Exchange

The gas exchange measurements made on Days 28-30 showed no significant difference in stomatal conductance between lines (P>0.05, Table 7.1). On day 28, the lines were ranked in the same order as for total water use, although this difference was not significant (P=0.063). It is important to note that PPFD values were low on all three dates when measurements were made, despite the provision of supplementary lighting.

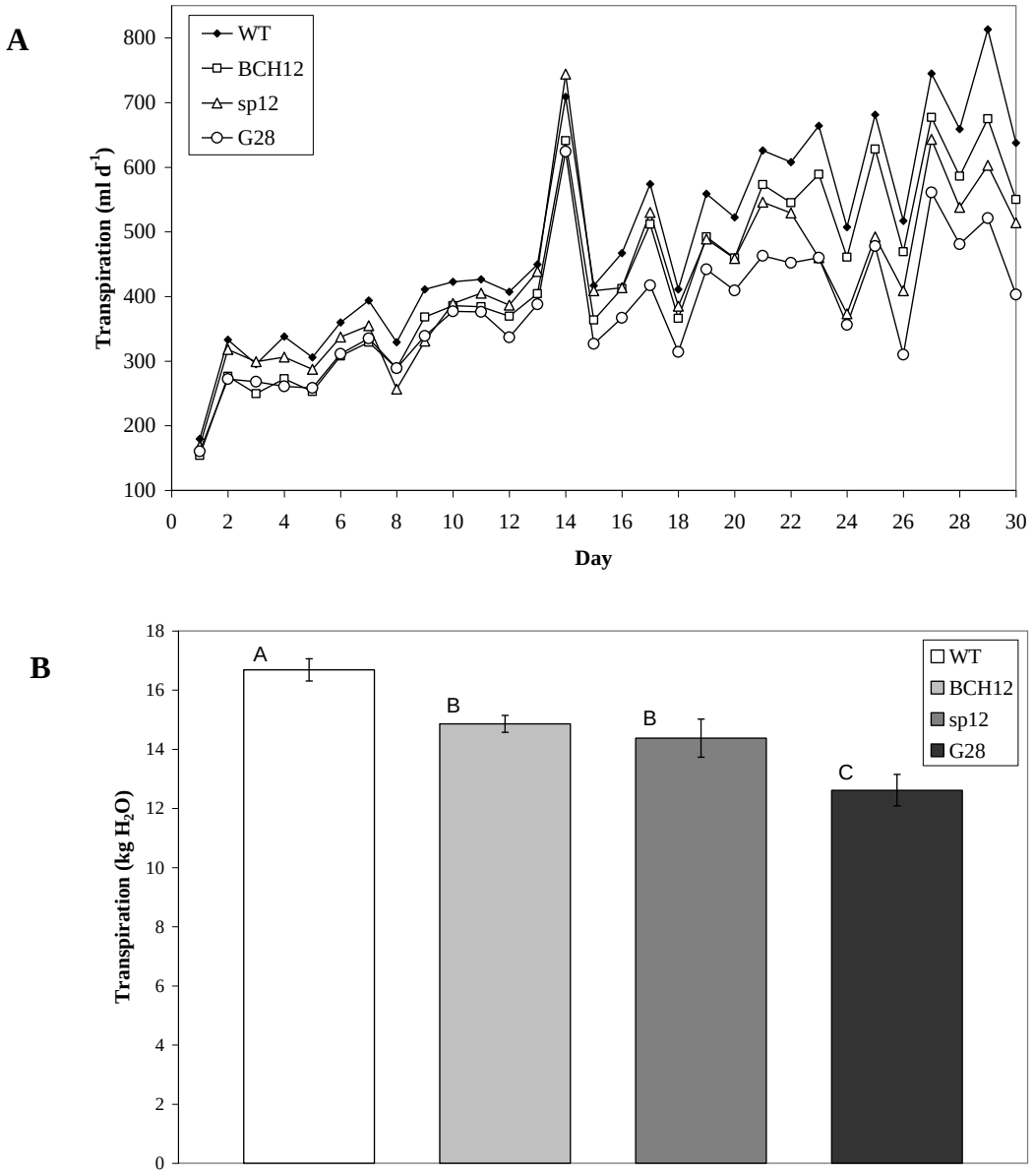
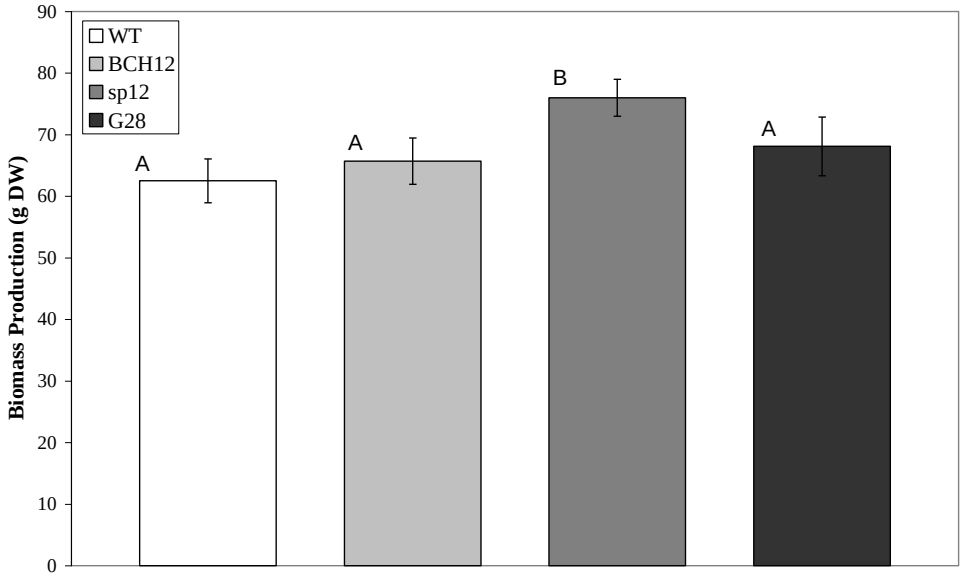
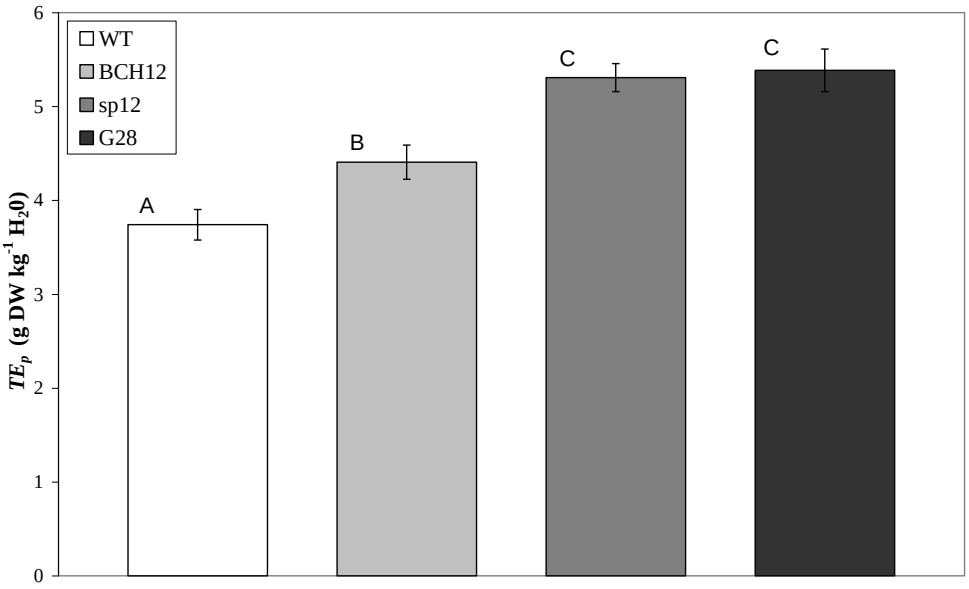


Figure 7.1. A: Mean daily transpiration measured gravimetrically for WT, BCH12, sp12 and G28 plants during the 30 day experimental period; **B:** Total transpiration during the experimental period. Differing letters associated with the histograms indicate significant differences (P<0.05), error bars represent standard error of the mean.

A



B



C

	Biomass Accumulation (g DW)	**Transpiration (kg H_2O)**	***TE_p*** **(g DW kg^{-1} H_2O)**
F pr.	0.003	*<0.001*	*<0.001*
s.e.d	2.92	0.446	0.128
l.s.d	6.27	0.957	0.275

Figure 7.2. A: Biomass accumulation and **B:** mean TE_p during the 30 day experimental period; **C:** statistical summary from AVOVA (n=6). Differing letters associated with the histograms indicate significant differences (P<0.05), error bars represent standard error of the mean.

Table 7.2. Mean plant height, leaf length and chlorophyll concentration with accompanying ANOVA summary (n=6).

	Plant height (cm)	**Leaf length (cm)**	**Leaf chlorophyll concentration (µg cm^{-2})**
WT	182.7	37.0	57.3
BCH12	185.7	49.0	55.5
Sp12	165.5	44.8	57.0
G28	183.1	42.26	56.7
Fpr.	*0.004*	*<0.001*	*0.716*
d.f.	23	23	23
s.e.d.	4.920	0.775	1.312
l.s.d.	10.56	1.662	2.919

7.4.1.3 Biomass production

The sp12 plants exhibited greater biomass production during the experimental period than the other three lines (P<0.05, Fig. 7.2A), perhaps due to the timing of harvest when fruit production had just begun and sp12 had a greater truss weight than the other genotypes examined (P<0.001; Appendix 3). Plant height did not differ significantly between WT, BCH12 and G28, although plants of the sp12 line were shorter (P<0.05). This height difference was not reflected by biomass, as sp12 produced more biomass than the other lines (P<0.05), although the three 'high ABA' lines had longer leaves than WT plants (P<0.001). The SPAD measurements showed no difference in chlorophyll content between the four lines (P>0.05, Table 7.2).

7.4.1.4 Transpiration Efficiency

Consistent with the findings of Experiment 1, WT plants had a lower TE_p values than all three 'high ABA' lines (P<0.001, Fig. 7.2B). Lines G28 and sp12 also had a greater TE_p than BCH12, although there was no difference between the two single gene lines due to the greater biomass accumulation of sp12. Although G28 plants transpired less water than the single gene over-expressers, no significant effect of over-expression of two ABA biosynthetic genes was apparent for the seasonal mean TE_p values. However, the ranking of transpiration efficiency for the three 'high ABA' genotypes was as expected from their ABA over-production levels (G28>sp12>BCH12).

7.4.2 Experiment 2 - WT, BCH12, sp5 and G29

7.4.2.1 Gravimetric Measurements of Transpiration

WT plants again exhibited the greatest daily transpiration rate throughout the 25 day experimental period, with values consistently being lowest in G29 plants ($P<0.001$, Fig. 7.4). The interaction between sp5 and BCH12 was similar to that for sp12 and BCH12 (Fig. 7.1A), as the two single gene transgenic lines generally used similar quantities of water (Fig. 7.3). Mean daily transpiration for WT plants was greater than for all 'high ABA' lines ($P<0.001$). Interestingly, transpiration was lower in G29 than in BCH12 and sp5 ($P<0.001$), demonstrating an additive effect of the over-expression of the two ABA biosynthetic genes. In terms of daily transpiration rate the lines were therefore ranked WT>BCH12>sp5>G29, with mean values of 671.8, 588.9, 580.4 and 485.0 ml d^{-1}, respectively.

As in previous experiments, WT plants transpired more water during the experimental period than all three 'high ABA' lines ($P<0.001$, Fig. 7.4). There was an additive effect of over-expression of two genes, as G29 used less water over 25 days than either of the single gene over-expressers ($P<0.001$). G29 plants used 27% less water than WT plants, which translates to a saving of 4.58 kg $plant^{-1}$ of irrigation water during the 25 day experimental period.

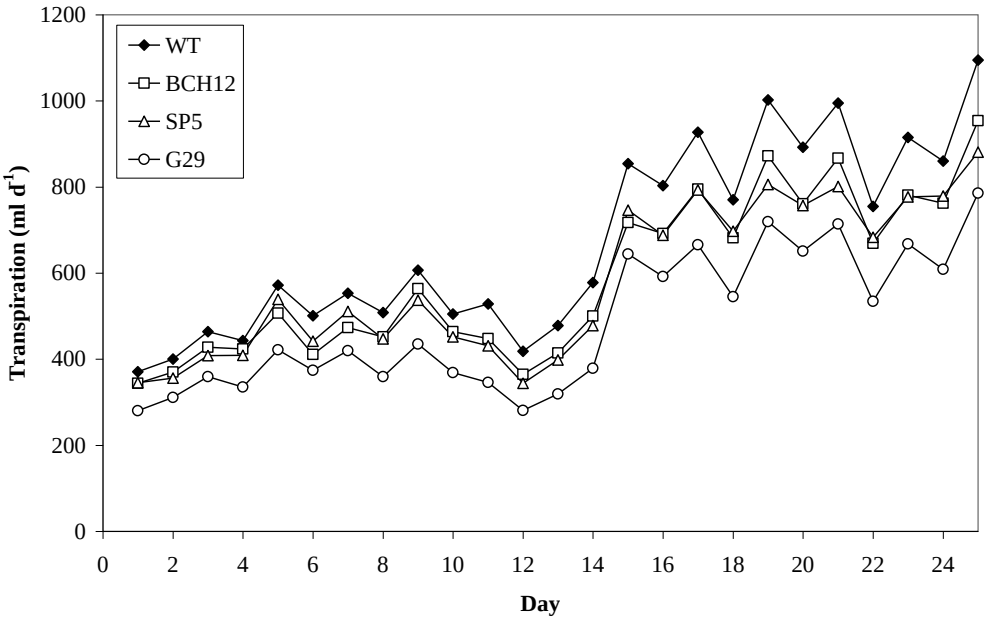


Figure 7.3. Daily transpiration measured gravimetrically for WT, BCH12, sp5 and G29 plants during the 25 day experimental period.

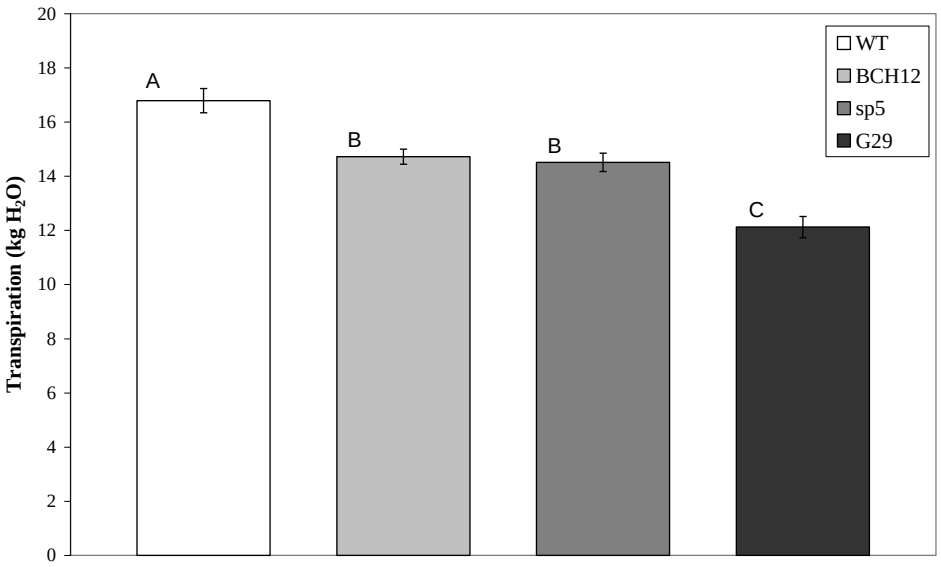


Figure 7.4. Total transpiration during the 25 day experimental period. Differing letters associated with the histograms indicate significant differences (P<0.05), error bars represent standard error of the mean.

Table 7.3. Mean plant height, leaf length and leaf chlorophyll concentration, with accompanying ANOVA summary (n=6).

	Height (cm)	**Leaf length (cm)**	**Leaf chlorophyll concentration (µg cm^{-2})**
WT	151.2	38.0	58.3
BCH12	154.5	38.3	57.1
sp5	153.2	49.5	57.1
G29	160.0	39.4	56.3
Fpr.	*0.328*	*<0.001*	*0.350*
d.f.	23	23	23
s.e.d.	4.62	1.488	0.9082
l.s.d.	10.44	3.367	1.935

7.4.2.2 Biomass Production

The destructive harvest of half of the plants when the experiment began confirmed there was no difference in initial dry weight between the four lines (P>0.05; Fig. 7.5), although sp5 produced a greater biomass during the 25 day experimental period than the other three lines (P<0.05). This observation is consistent with Experiment 1, in which plants over-expressing *LeNCED1* (sp12)

produced the greatest amount of biomass (P<0.001). Biomass production did not differ significantly between the other three lines.

The results for leaf length support previous findings that sp lines, particularly sp5, tend to have longer leaves in comparison with WT controls. Mean leaf length for sp5 was 10 cm longer than for the other lines examined (P<0.001; Table 7.3). This observation supports that of Thompson *et al*. (2007), who reported that petiole length was greater in sp5, relative to WT plants. However, it is important to note that the double gene over-expresser (G29) did not also have significantly longer leaves, despite being derived from a cross involving the sp5 transgenic line (Table 7.3). No differences in plant height or leaf chlorophyll content were detected between lines (Table 7.3), suggesting there were no obvious negative effects of ABA over-production on the growth of the 'high ABA' lines which were visually identical apart from the longer leaf phenotype observed in sp lines.

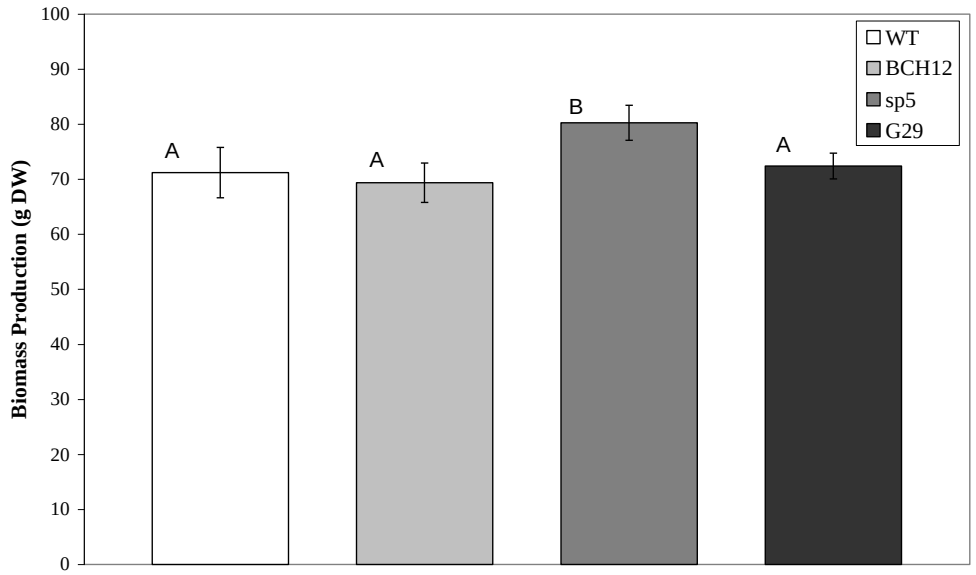
7.4.2.3 Transpiration Efficiency

Although the three 'high ABA' lines required less irrigation, there was no evidence of any negative impact on biomass production. This therefore translated into an improved TE_p for all 'high ABA' lines relative to WT plants. Unlike Experiment 2, the improved water saving capacity of the double gene line compared to the single gene over-expressers was significant for TE_p (P<0.001); thus, BCH12, sp5 and G29 showed improvements in TE_p of 11, 30 and 41% respectively relative to WT plants.

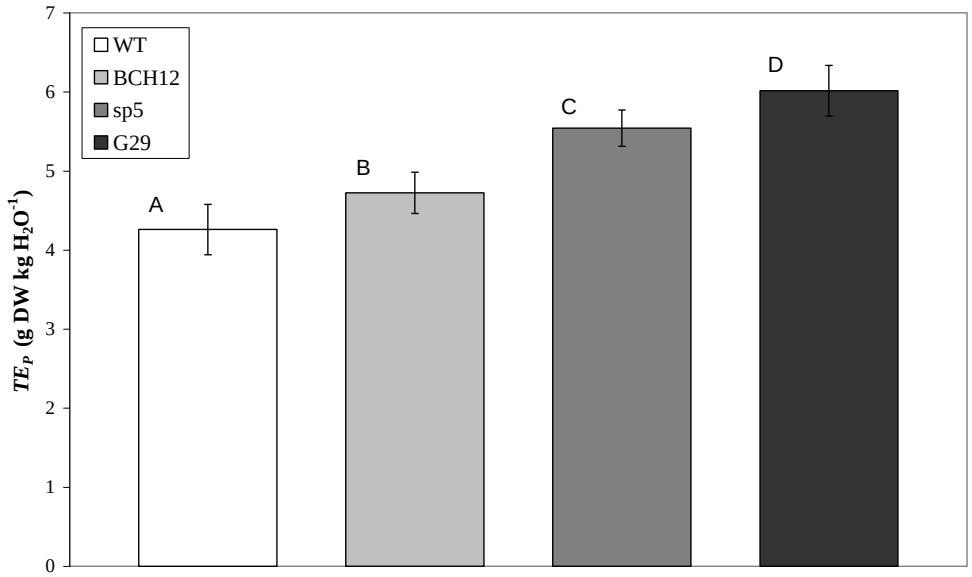
7.4.2.4 Reproductive Development

There was no significant difference in time to initiation of the first inflorescence or the number of leaves preceding the first inflorescence (NLPI) between WT, BCH12, sp5 or G29 plants (P>0.05). The WT plants developed in units comprising three leaves, followed by an inflorescence, a common pattern in many tomato cultivars. All three 'high ABA' lines also exhibited a similar developmental pattern to WT plants.

A



B



C

	Biomass Accumulation (g DW)	**Transpiration (kg H_2O)**	***TE_p*** **(g DW kg^{-1} H_2O)**
F pr.	0.006	*<0.001*	*<0.001*
s.e.d	2.727	0.354	0.212
l.s.d	5.813	0.755	0.453

Figure 7.5. A: Biomass accumulation and **B:** mean TE_p during the 25 day experimental period; **C:** statistical summary from AVOVA (n=6). Differing letters associated with the histograms indicate significant differences (P<0.05), error bars represent standard error of the mean.

Table 7.4. Time to first anthesis and number of leaves to trusses 1 and 2 (n=6).

	Time to First Anthesis (d)	Leaves to Truss 1	Leaves to Truss 2
WT	60.5	8.2	3.33
BCH12	60.0	8.3	3.33
Sp5	58.5	7.3	3.17
G29	60.0	7.3	3.17
Fpr.	*0.494*	*0.130*	*3.250*
s.e.d.	1.34	1.47	0.272
l.s.d.	2.85	1.08	0.58

7.4.3 Experiment 3 - WT, BCH12, sp12, sp5, G28 and G29

7.4.3.1 Gravimetric Measurement of Transpiration

All high ABA lines transpired less water during the 25 day experimental period than WT controls ($P<0.001$; Fig. 7.6). The quantity of water transpired did not differ significantly between BCH12 and sp12 plants, whereas sp5, the single-gene line with the greatest level of ABA over-production, used less water than the other two single-gene lines (BCH12 and sp12; $P<0.001$). Both double-gene lines transpired less than all other lines ($P<0.001$), with G29 transpiring less than G28 ($P<0.001$). G28 and G29 plants transpired 25 and 32% less water respectively than WT controls, similar to Experiments 1 and 2, in which the corresponding savings were 25 and 27% respectively. The difference in water use was not due to a variation in plant size, as neither initial mean dry weight or biomass accumulation differed significantly between (Appendix 6).

7.4.3.2 Biomass Production

Whilst marked differences in the volume of water transpired were observed during the experimental period, biomass production did not differ between lines (Fig. 7.7A), and as mentioned previously, there was no difference in plant dry weight between lines at the start of the experimental period (Appendix 6).

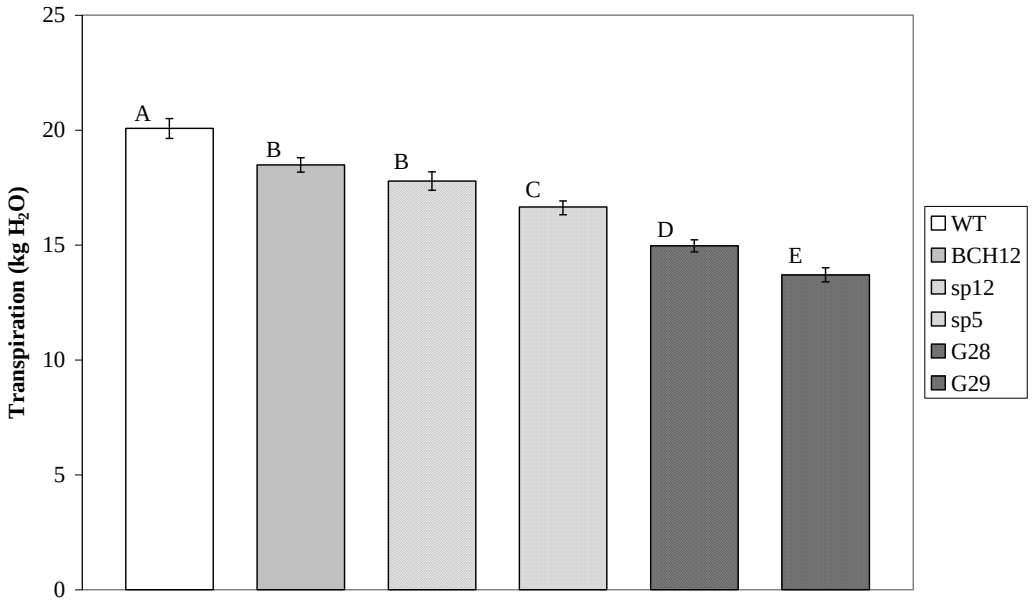
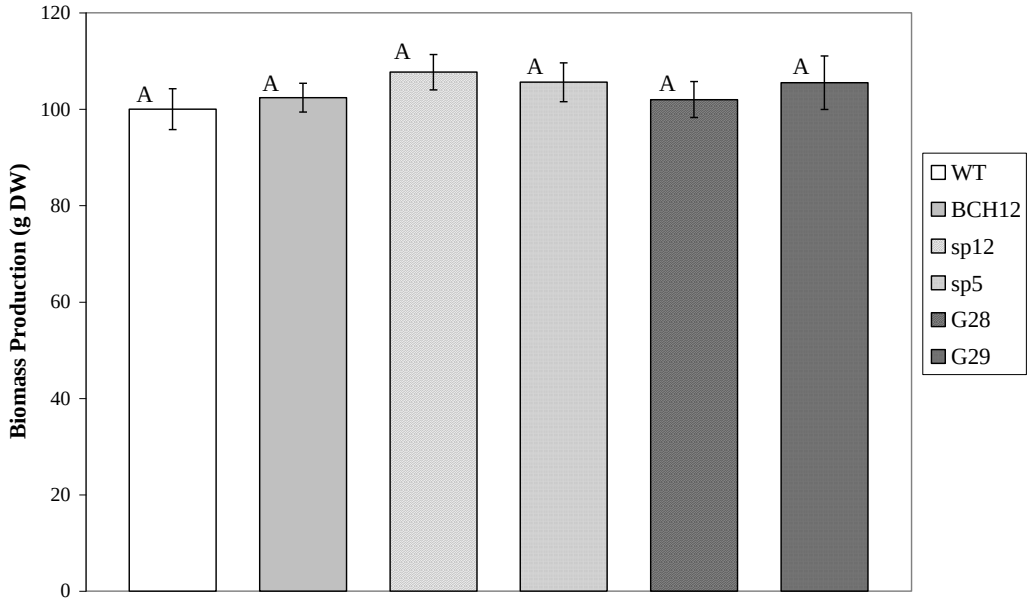


Figure 7.6. Total transpiration during the 25 day experimental period. Differing letters associated with the histograms indicate significant differences (P<0.05), error bars represent standard error of the mean.

7.4.3.3 Transpiration Efficiency

All high ABA lines exhibited greater *TEp* values than WT controls (P<0.001), with mean values ranging from 4.98 g DW kg^{-1} H_2O for WT to 7.68 g DW kg^{-1} H_2O for G29 (Fig. 7.7). The ranking of lines in terms of TE_p was as predicted from the level of ABA over-production, as the values for single-gene lines were consistently greater than for WT controls. As with the single gene gravimetric experiment reported in Chapter 5, there was no significant difference between the two sp lines, although these were consistently ranked sp5>sp12 for TE_p, as predicted from the level of ABA over-production. There was an additive effect of over expressing two ABA biosynthetic genes, as the double-gene lines (G28 and G29) exhibited the greatest efficiencies, thereby providing 37 and 54% improvements in TE_p respectively relative to WT controls.

A



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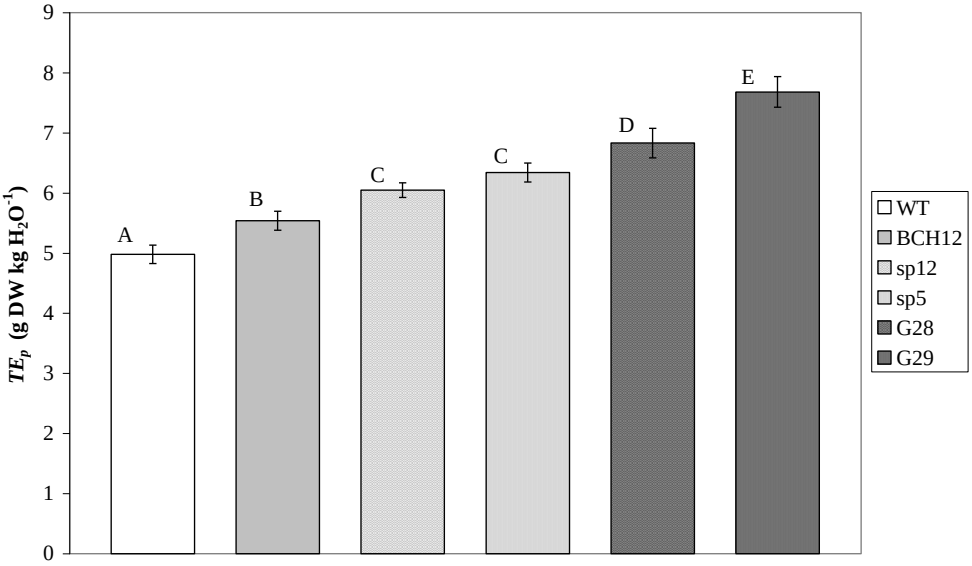


C

	Biomass Accumulation (g DW)	**Transpiration (kg H_2O)**	***TE_p*** **(g DW kg^{-1} H_2O)**
F pr.	0.409	*<0.001*	*<0.001*
s.e.d	3.93	0.344	0.214
l.s.d	8.10	0.708	0.441

Figure 7.7. A: Biomass production and **B:** mean TE_p during the 25 day experimental period; **C:** Statistical summary from AVOVA (n=6). Differing letters associated with the histograms indicate significant differences (P<0.05), error bars represent standard error of the mean.

7.5 DISCUSSION

7.5.1 Further increases in ABA over-production do not negatively affect plant growth, physiology and reproductive development

7.5.1.1 Biomass Production

ABA has been reported to have negative impacts on plant growth in some experiments (e.g. Creelman *et al*., 1990), but more recent studies have challenged the notion that ABA always acts as a growth inhibitor (Sharp and LeNoble, 2002). Chapters 4 and 5 demonstrated that there was no negative implications of mild ABA over-production on growth, as biomass production in sp plants was consistently similar to or exceeded that of WT controls under both well-watered and sub-optimal irrigation conditions, in marked contrast to the 'ultra-high' ABA rbcS lines (Chapter 6; Tung *et al*., 2008) in which growth rates and leaf gas exchange were significantly reduced. It was therefore possible that a further increase in ABA, beyond that shown by the mild sp lines, would have similar negative implications for growth as in the 'ultra-high' ABA over-producing plants. However, destructive harvests at the end of all three gravimetric water use experiments reported in this chapter revealed that the double transgene 'high ABA' lines, which over-produce ABA to a greater extent than in the previously analysed sp lines (Chapters 4 and 5; Appendix 8), produced an equivalent biomass to WT controls. This observation suggests that the level of ABA accumulation in these plants was insufficient to substantially alter assimilation rate, which would in turn affect long-term biomass production in the double transgene 'high ABA' lines.

7.5.1.2 Seed Germination

Simultaneous over-expression of *LeNCED1* and *LeBCH2* induced severe effects on seed germination. In lines G28 and G29, dormancy was greater than when either of the transgenes was individually over-expressed. Both lines showed no germination under standard germination conditions (Chapter 9) and required application of norflurazon to inhibit ABA biosynthesis (Gamble and Mullet, 1986) and break dormancy. The prolonged seedling establishment phase observed in the single gene sp lines was slightly more severe in the 'double transgene' lines, although not as pronounced as that observed for seed dormancy. Seed germination and seedling

establishment in all 'high ABA' lines is characterised and discussed more fully in Chapter 9.

7.5.1.3 Reproductive Development

No significant differences in the time to initiation of the first inflorescence or the number of leaves preceding the first inflorescence (NLPI) were detected between the WT, BCH12, sp5 and G29 lines. In general, NLPI for tomato is at least 6-8 leaves (Heuvelink, 2004), while the duration of the sensitive period which determines the position on the stem where the first inflorescence develops is *ca*. 10 days from the onset of cotyledon expansion (Dieleman and Heuvelink, 1992).

After initiation of the first flower truss, most tomato cultivars develop in 'units' consisting of three leaves followed by an inflorescence, which build up the sympodial stem (Heuvelink, 2004). The single and double gene 'high ABA' lines both followed this pattern, with no difference in the number of leaves between inflorescences, in contrast to the rbcS lines which exhibited a severely altered pattern of reproductive development (Chapter 6). The vegetative period before the first flower truss was produced was between 22 and 83 days longer in rbcS plants than in WT plants. There was also a much greater frequency of abortion of whole trusses or individual flowers in these 'ultra-high' ABA plants. Such extreme effects on reproductive behaviour were not observed when *Arabidopsis* was transformed using the same *rbcS3C*::*LeNCED1* construct that was used to produce the tomato rbcS lines: flowering in Arabidopsis was delayed by only 3-4 days compared to WT controls (Deswarte, in prep.). Transgene expression was lower in the rbcS *Arabidopsis* lines than in the rbcS tomato lines, probably because the promoter was less active in a heterologous host, and therefore the construct has a less severe effect than in tomato.

7.5.1.4 Chlorophyll Concentration

The 'ultra-high' ABA rbcS lines exhibit a general reduction in leaf and shoot chlorophyll concentration, combined with sectored chlorosis and 'glassiness' associated with interveinal flooding in the younger leaves. This has not been observed in the milder sp lines, although both sp and rbcS plants may exhibit depletion of the xanthophyll pools which ultimately provide substrate for NCED

(Balasubramanian, 2007; Tung *et al*., 2008). As well as providing substrate for NCED, the various xanthophyll pools are also essential for photosynthetic and photoprotective mechanisms, such as the formation of the light harvesting complex II (Kuhlbrandt *et al*., 1994). Zeaxanthin has a key role in NPQ and other adaptations to stress caused by high light intensity (Demmig-Adams and Adams, 1992; Johnson *et al*., 2008). No significant differences in chlorophyll concentration were observed between the single gene, double gene and WT control plants in the present studies, indicating that the further increase in ABA accumulation in lines G28 and G29 had no negative implications for this parameter.

7.5.2 Over-expression of *LeBCH2* increases TE_p

Preliminary physiological observations of the BCH12 line suggested that the up-regulation of the BCH enzyme alone may have a significant effect on water use characteristics. Instantaneous gas exchange measurements showed that BCH12 plants exhibited reduced stomatal opening and transpiration rates relative to WT control plants (Balasubramanian, 2007). In all three gravimetric experiments reported here, BCH12 plants transpired significantly less water during the experimental period than WT control plants but accumulated a biomass similar to that of WT plants, so providing a significant improvement in TE_p relative to WT plants. These findings provide further evidence that increases in the conversion of β-carotene to all-*trans*-zeaxanthin via the up-regulation of BCH may significantly affect stomatal behaviour. The improvement in TE_p was lower than in plants over-expressing *LeNCED1,* which have consistently shown improved water use efficiency (Chapters 4 and 5; Thompson *et al*., 2007a). This might have been predicted on the basis that NCED is widely assumed to be the major rate-limiting step in the ABA biosynthetic pathway (Qin and Zeevaart, 1999; Thompson *et al*., 2000). However, the independent effect of BCH alone on TE_p would not have been predicted.

7.5.3 Combined over-expression of two ABA biosynthetic genes allows further improvement in *TEp*

Whilst plants over-expressing BCH or NCED transgenes showed an increase in TE_p relative to WT plants, the two 'double transgene' lines (G28 and G29)

consistently showed the greatest improvement. In Experiment 3, these lines exhibited 25 and 27% savings in total transpiration over the 25 day experimental period, which translated into 37 and 54% increases in TE_p respectively, thereby demonstrating that it is possible to increase ABA production above that in 'single trangene' sp lines to produce plants whose stomatal behaviour more closely approaches that required for optimal improvement in TE_p in the context of agricultural crop production.

The effect of combining the over-expression of the two ABA biosynthetic transgenes (*LeNCED1* and *LeBCH2*) appears to have an additive effect on TE_p, whereas this is not the case for leaf ABA concentration and seed germination, for which the simultaneous effect of the two transgenes appeared to be synergistic rather than additive. The additive and synergistic effects of over-expressing two ABA biosynthetic transgenes are discussed more extensively in Chapter 10.

8 THE USE OF 'HIGH ABA' LINES AS ROOTSTOCKS

8.1 INTRODUCTION

8.1.1 Genetically modified crops

The results presented in Chapter 7 showed that simultaneous over-expression of two ABA biosynthetic genes (*BCH* and *NCED*) in transgenic tomato plants provided 25 and 27 % savings in total transpiration over a 25 day experimental period, which translated into improvements in TE_p of 37 and 54 % respectively. As mature tomato plants may use 2-3 l H_2O $plant^{-1}$ d^{-1} (OMAFRA, 2003), reductions in irrigation of up to 25% during the growing season would bring potentially substantial agronomic benefits. However, these 'high ABA' lines are genetically modified and, whilst this technology may be used in countries which permit the growth of GM crops, they are not currently acceptable in UK agriculture.

GM technology has been controversial in international terms for many years (Gaskell, 2004), although GM varieties of crops such as cotton and maize developed in the USA are currently used for large-scale commercial production in many countries (Fukuda-Parr, 2006). In the UK, however, continuing market uncertainty over the perceived risks associated with GM crops and related products led to their withdrawal from supermarket shelves in the late 1990s (Horlick-Jones *et al*., 2006). Since then, public opinion has not altered significantly, meaning that if 'high ABA' technology is to be exploited commercially in the UK, alternative approaches must be developed.

8.1.2 Rootstocks in Crop Production

Rootstocks are commonly used in crop production for various purposes, ranging from control of soil-borne diseases to reductions in tree height to facilitate harvesting of fruit. For example, the species *Lagenaria siceraria* has been used to provide rootstock for the production of watermelon since the 1930s, to alleviate *Fusarium* wilt disease (Yamakawa, 1982). Since the 1920s, rootstocks have been used for the field production of tomato to reduce soil-borne diseases caused by pathogens such as *Fusarium oxysporum* (Csiznszky *et al*., 2005). More recently, it

has been reported that in soil-less glasshouse production, grafting may improve tolerance of drought (Bhatt *et al*., 2002), salinity (Fernandez-Garcia *et al*., 2002) and temperature (Rivero *et al*., 2002) stress. The use of tomato rootstocks is therefore now common in glasshouse production, with the most widely used rootstock cultivar being Maxifort (De Ruiter Seeds; Heijens, 2004).

8.1.3 The use of 'high ABA' rootstocks

8.1.3.1 Root-to-shoot signaling

Plants can perceive a lack of soil moisture in the rooting environment and communicate this information to the shoot in a process often described as root-to-shoot communication and is discussed in Chapter 1. The use of ABA-deficient mutants and WT plants in reciprocal grafting experiments has allowed evaluation of the extent to which root-sourced ABA influences shoot phenotype. When ABA-deficient rootstocks are grafted onto WT scions (WT/mutant), the WT shoot phenotype is generally maintained by scion ABA biosynthesis, indicating that the lack of root biosynthesis has no significant effect on growth or leaf area (Chen *et al*., 2002; Holbrook *et al*., 2002; Dodd *et al*., 2009), leaf gas exchange (Tal *et al*., 1970; Fambrini *et al*., 1995; Holbrook *et al*., 2002; Dodd *et al*., 2009) or shoot/leaf ABA concentration (Chen *et al*., 2002). However, the reciprocal mutant/WT (scion/rootstock) grafts have provided less consistent results, as some mutant scion phenotypes of mutant/WT plants showed no differences relative to mutant/mutant self-grafts (Holbrook *et al*., 2002). However, in most studies, there appeared to be some degree of partial phenotypic reversion of various aspects of the mutant phenotypes, including reduced transpiration rate (Chen *et al*., 2002), increased leaf water potential (Fambrini *et al*., 1995; Dodd *et al*., 2009) and leaf ABA concentration (Fambrini *et al*., 1995; Chen *et al*., 2002). Dodd *et al*. (2009) reported that the stomatal conductance of individual leaves appeared to be reduced in mutant/WT plants, but there was no difference in whole plant transpiration rate between mutant/WT and mutant/mutant plants. These authors suggested that a threshold shoot ABA concentration may be required to prevent excessive transpiration of the ABA-deficient scion, and that insufficient ABA was produced by the WT rootstock to attain this level.

8.1.3.2 Biosynthesis of ABA in the root system

Photosynthesis is a primary function of green tissue and carotenoids/xanthophylls serve as accessory pigments in the photosynthetic apparatus, having important roles in both light harvesting and photoprotection (Niyogi *et al*., 2000; Davison *et al*., 2002; Cuttris and Pogson, 2004). As a result, photosynthetic tissues have a substantial capacity to produce carotenoids/xanthophylls, whereas these light-associated functions are not required in tissues which are incapable of photosynthetic activity. For this reason, carotenoid/xanthophyll concentrations are much lower in non-photosynthetic tissue such as roots than in leaves and green fruits (Fraser *et al*., 1994). For example, carotenoid/xanthophyll concentrations in tomato roots may be as low as 0.27 % of those typically found in leaves grown in the light (Parry and Horgan, 1992).

Accumulation of ABA in roots in response to dehydration stress (Hartung and Davies, 1991; Griffiths *et al*., 1996) is likely to be one of the principal components involved in long-range signaling in plants (Davies *et al*., 2005), although the precise role of root-synthesised ABA in this signalling mechanism is unclear. Dehydration of isolated root segments has been shown to increase their ABA concentrations in several crop species (e.g. Cornish and Zeevaart, 1985; Zhang and Davies, 1987). Genuinely root-synthesised ABA could potentially be exploited by using 'high ABA' rootstocks to provide a strong root-sourced ABA signal under well-watered conditions, which may exceed the threshold required to reduce stomatal conductance in the shoots and improve whole plant WUE.

The lower abundance of carotenoids in roots than in shoots (Parry and Horgan, 1992) means that substantially increasing ABA production in unstressed roots is likely to be more difficult than in green tissue (Taylor *et al*., 2005). Nevertheless, in isolated root cultures of tomato lines which constitutively over-express *LeNCED1*, ABA concentrations were 3.8-fold greater than in WT controls (Thompson *et al*., 2007b). Tetracyline-based induction of an *LeNCED1* encoding transgene in tobacco roots also increased ABA concentration, suggesting that NCED catalyses a key rate-limiting step for ABA biosynthesis in roots (Thompson *et al*., 2007b). However, it is possible that, when *LeNCED1* is up-regulated in roots, the supply of precursors may become rate-limiting for ABA production (Taylor *et al*., 2005). Whilst NCED is one

of the rate-limiting enzymes for ABA biosynthesis in roots, other steps are more restrictive to the flux of carotenoid/xanthophyll precursors through the ABA biosynthetic pathway in roots as compared to shoots (Thompson *et al*., 2007b).

8.1.3.3 Over-expression of phytoene synthase increases carotenoid levels

The first committed step in carotenoid biosynthesis is the dimerisation of geranylgeranyl pyrophosphate (GGPP) to form the colourless C_{40} compound phytoene, a reaction catalysed by phytoene synthase (PSY; Hirschberg, 2000). This reaction is an important regulatory step in carotenoid synthesis and has been manipulated to increase carotenoid accumulation in many species, most notably to produce so-called 'golden rice' with enhanced nutritional quality (Ye *et al*., 2000). In tomato fruit, expression levels of *PSY* were found to be closely correlated with carotenoid content during ripening (Giuliano *et al*., 1993). Antisense expression of a *PSY* gene in tobacco plants reduced both carotenoid and chlorophyll concentrations in the leaves (Busch *et al*., 2002). The role of various carotenoids/xanthophylls in the ABA biosynthetic pathway was discussed more fully in Chapter 1.

In tomato, there are two *PSY* genes, *PSY1* (the *R* gene), which is strongly expressed in the chromoplasts of mature fruit and flowers (Bartley *et al*., 1992; Fray and Grierson, 1993), and *PSY2*, which is expressed in the chloroplasts of green photosynthetic tissues e.g. unripe fruit, leaves and stems (Bartley and Scolnik, 1993). In transgenic tomato plants, the strong constitutive over-expression of *PSY1* driven by the 35S promoter resulted in plants rich in carotenoids, with elevated phytoene, ζ-carotene and lycopene levels in unripe fruit and reduced chlorophyll concentration in leaves (Fray *et al*., 1995). As GGPP is required for the synthesis of gibberellic acid, phytol and carotenoids, it was suggested that competition between these pathways results in plants with a dwarfism phenotype due to gibberellin and chlorophyll deficiencies (Fray *et al*., 1995). These transgenic tomato plants also display increased root pigmentation associated with increased lycopene and β-carotene contents, indicating that PSY catalyses a key rate-limiting step in carotenoid biosynthesis in roots (Taylor *et al*., 2005).

The *LePSY1* gene was chosen to be constitutively over-expressed alongside *LeNCED1* in transgenic tomato plants (Jones, 2007), with the objective of increasing

the flux of carotenoid precursors through the pathway to prevent NCED from becoming limited by substrate availability, thereby increasing ABA biosynthesis in the roots. The impact of this double gene approach on root-synthesised ABA concentration was analysed using isolated root cultures. When *PSY1* was over-expressed alone, ABA biosynthesis in the roots increased significantly relative to WT controls (Jones, 2007). Over-expression of *LeNCED1* alone in the roots produced larger increases in ABA (Thompson *et al*., 2007b), whilst simultaneous up-regulation of both genes increased ABA concentration still further (Jones, 2007). The latter increase was more than additive, relative to the degree of ABA over-production in each of the parental lines (Jones, 2007). Similar effects have been found in transgenic tomato lines that simultaneously over-express both *LeBCH2* and *LeNCED1* (G28, *cf*. Chapter 7), where the direct effect of combining the transgenes on root ABA concentration was analysed using isolated root cultures. The influence of the two transgenes in the roots increased ABA concentrations by over 10-fold relative to WT controls, again indicating a synergistic effect of over-expressing two ABA biosynthetic genes (Balasubramanian, 2007).

The 'double transgene' lines (G28 and G29), described fully in Chapter 7, showed substantially improved transpiration efficiencies. Whilst these plants exhibited dramatically increased ABA concentrations in isolated roots, it is unclear whether root stocks of this genotype would be sufficient to influence transpiration when grafted to WT scions. If the root-to-shoot ABA signal provided by these 'double transgene' root stocks is insufficient, there is potential to further enhance ABA production in the roots as the flux of precursors through the biosynthetic pathway could be further increased by combining the three ABA biosynthetic genes discussed above to produce tomato plants in which ABA over-production in the roots is further enhanced. As the PSY, BCH and NCED enzymes all catalyse key steps in ABA production in roots, the combined over-expression of all three transgenes would allow more substantial increases in root-sourced ABA production under well-watered conditions. It is possible that these 'triple transgene' lines may exhibit some of the negative symptoms of 'ultra high' ABA accumulation observed in rbcS plants (*cf*. Chapter 6), and also the dwarfism characteristics observed in parental *PSY* over-expressing lines (Fray *et al*., 1995). These phenotypic abnormalities would make them unsuitable for use in crop production systems, although they may have

sufficiently high rates of root ABA production to enhance whole plant water use efficiency when grafted onto WT scions.

8.2 AIMS

The aim of the work reported here was to develop rootstocks which produce sufficient ABA to restrict the loss of water from scions to which they have been grafted. This involved analysis to determine whether either of two 'high ABA' lines produce sufficient root-sourced ABA to achieve this objective.

As the G29 line, which over-expresses both the *LeBCH2* and *LeNCED1* transgenes, provided the greatest improvement in WUE in whole plants, it is possible that, by grafting this 'high ABA' line as rootstock onto WT scions, the increased ABA production in the roots brought about by the two transgenes may reduce whole plant water use. A reciprocal grafting experiment was therefore conducted to determine the extent to which 'high ABA' production in either the roots or the shoot affects whole plant WUE. However, it was also possible that the roots of G29 would not provide a sufficiently strong ABA signal to the WT scion to improve whole plant water use efficiency. As root tissue has a limited supply of carotenoid precursors, two 'double transgene' lines were crossed to further increase precursor flux through the pathway, thus creating triple over-expressing F_1 lines (3G) which were homozygous for the sp5 *NCED1* transgene insert and heterozygous for the *BCH* and *PSY* T-DNA insertion loci. A gravimetric TE_p experiment was designed to establish whether the additional PSY gene, which functions in the first committed step in the carotenoid biosynthesis pathway, permits additional ABA biosynthesis in the roots, so providing a stronger root-to-shoot ABA signal.

8.3 MATERIALS AND METHODS

8.3.1 General Grafting Protocol

Plants of equal size were selected for the appropriate scion/rootstock combination, ensuring that stems were of equal diameter using calipers. The stems of plants designated for use as rootstock were severed at an angle of 30^{o} midway between the stem base and cotyledons. A 2 cm length of latex tube with the appropriate inner diameter was fitted over the rootstock stem until the cut surface of the stem was mid-way along the tube. The stems of plants designated as scions were then severed as described above and fitted into the latex tube, ensuring that the cut surfaces of the scion and rootstock were in complete contact. Plants were then placed in a propagator and covered with a lid with closed vents. When the shoots had recovered from the initial wilt, relative humidity in the propagator was gradually reduced by opening the vents slightly further on successive days. The grafts required *c*. 10 days for a sound union to form. This was adjudged to have occurred when the latex tube bulged, at which point it was carefully removed using a sharp, single-sided razor blade. The plants were then grown under standard glasshouse conditions.

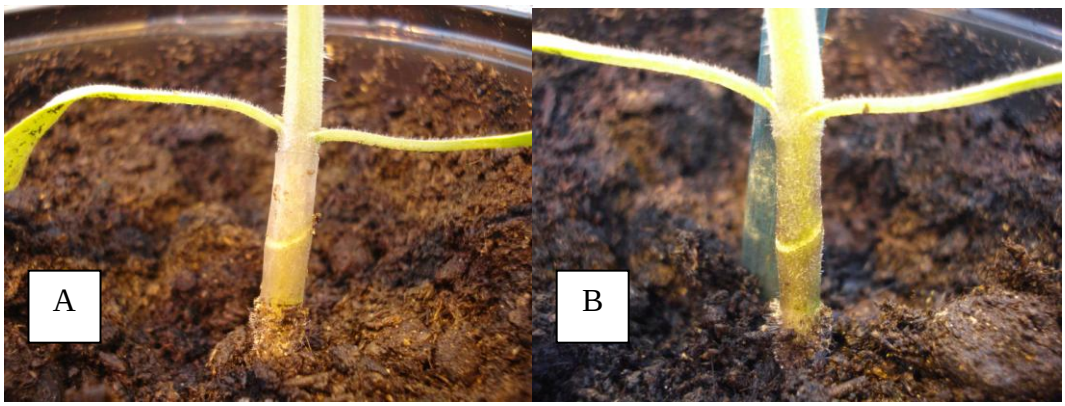


Figure 8.1. Photographs demonstrating the formation of graft union: **A**, graft union supported by the latex tube; and **B**, after removing the tube.

8.3.2 General Gravimetric TE_p Experimental Design

The compost (Levington M3) was watered until saturated and, once excess water had drained from the pots, each was weighed to ascertain its weight at field capacity. Uvi ground cover disks (Growing Technologies, Derby, UK) were placed on the surface of the compost to minimise evaporation. A blank pot which did not contain a plant was included in each block. All pots were watered to field capacity at the start of the experiment, weighed daily to determine the quantity of water used, and supplied with an equivalent volume of water to return the compost to field capacity. Although the compost in pots containing plants was covered with Uvi disks to minimise evaporation, the blank pots allowed accurate measurements of, and correction for, daily weight loss by evaporation from the compost. Daily weight loss from the blank pots was subtracted from the corresponding values for pots containing plants to obtain daily measurements of transpiration.

Daily transpiration rates (adjusted for evaporation from the compost) were summed to determine total water loss plant^{-1} during the experimental period. All plants were then harvested and above-ground dry weight, plant height and the length of each leaf were recorded. Mean biomass for each line at the beginning of the experiment was subtracted from the corresponding value at final harvest to determine biomass accumulation during the experimental period. Transpiration efficiency (TE_p) was calculated for all plants.

8.3.3 G29 reciprocal grafting experiment

Sixty G29 seeds were sown according to the appropriate sowing protocol (*cf.* Chapter 3). As the stem diameters of WT and G29 plants needed to be identical for successful graft unions to form, four batches of 20 WT seeds were sown after the G29 seedlings had emerged to achieve synchronised development at the stage when three true leaves had been formed. At this point, seedlings with uniform stem diameters (ideally 2.5 mm) were chosen to form 25 replicate grafts for each scion/rootstock combination i.e. WT/WT, WT/G29, G29/WT and G29/G29 (scion/ rootstock). The general grafting protocol described in Section 4.2.1 was followed.

The most uniform 12 plants for each scion/rootstock combination were selected at the four fully expanded leaf stage for inclusion in the experiment. Six representative plants of each scion/rootstock combination were destructively harvested to determine mean dry weight at the start of the 25 d experimental period. The six remaining replicates of each scion/rootstock combination were re-potted into 7 l pots and arranged in a randomised block design in the glasshouse.

8.3.4 Creation of 'triple transgene' lines

8.3.4.1 F_1 Generation

To create a 'triple transgene' line (3G), the constitutively over-expressing *LeNCED1* and *LeBCH2* and *LePSY1* two 'double transgene' lines were crossed. The homozygous G29 line (*cf*. Chapter 7) constitutively over-expresses *LeNCED1* and *LeBCH2* whereas MJ8 constitutively over-expresses *LeNCED1* (homozygous for the same sp5 T-DNA insertion loci as G29) and *LePSY1* (segregating Z171D4A line; Fray *et al*., 1995). Nine individual MJ8 plants (A-I), grown from seed supplied by Matthew Jones (Jones, 2007) were crossed with G29. Twenty seed from the MJ8H x G29 cross (F_1 generation) were germinated using a 1.0 mg l^{-1} norflurazon treatment for five or six days. This treatment was chosen as it represented a 1 or 2 day increase over the usual 4 day 1.0 mg l^{-1} norflurazon treatment which provided successful germination of the parental G29 seed. The F_1 plants produced were labelled H14-H22 and were homozygous for the sp::*LeNCED1* construct of the sp5 line, heterozygous for the 35S:: *LeBCH2* construct of the BCH12 line, and had an unknown copy number of the 35S::*LePSY1* construct.

8.3.4.2 F_2 Generation

Five F_1 plants were self-fertilised to provide seed of the F_2 generation (H-15, H-16, H-18, H-20, H-22). The plants chosen represented the full range of heights among the original eight plant F_1 generation (H14-22). Seed from the chosen five lines was sown in Petri dishes containing 1.0 mg l^{-1} norflurazon and left until the radicle had started to emerge. Seed was then washed and sown according the standard protocol (*cf*. Chapter 3) to ensure the sowing protocol did not select against seeds with exceptionally high or low ABA concentrations and hence intensity of seed

dormancy. Plants from the F_2 generation were scored for the severity of the *PSY* over-expression phenotype according to the severity of chlorosis at the shoot apex and colour of unripe fruit (*cf*. Fig. 8.2). Gas exchange measurements were made using a CIRAS-1 Infrared Gas Analyser (PP Systems, Hitchin, Herts, UK) coupled with a 2.5 cm^2 Parkinson leaf cuvette after 12 weeks of growth. Three replicate measurements were taken at one hour intervals between 10.30 am and 1.30 pm for each 3G F_2 generation plant, as well as for the WT and G29 controls.

8.3.5 F1 'triple transgene' (3G) rootstock experiment

A gravimetric TE_p experiment was designed to test whether TE_p differed between WT scions with three different rootstock types (WT, PC and 3G). As well as using WT rootstock as a control to test whether the 3G rootstock affected shoot phenotype when grafted to WT scions, a 'perfect control' (PC) was created (Table 8.1) to analyse any additional effect of the third transgene (PSY1). The 3G F_1 plants were homozygous for the sp5 T-DNA insertion locus of the *LeNCED1* construct and heterozygous for the BCH12 T-DNA insertion locus of the *LeBCH2* construct. A separate transgenic tomato line was therefore created by crossing of sp5 with G29 to provide a line which was homozygous for the sp5 T-DNA insertion locus of the *LeNCED1* construct and heterozygous for the BCH12 T-DNA insertion locus of the *LeBCH2* construct (line PC). So the PC line was identical to the 3G line apart from the absence of the PSY1 transgene.

Twenty-five triple F_1 seed were sown in 1.0 mg l^{-1} norflurazon for four days before being washed and sown according to the standard protocol. When the triple F_1 seedlings had produced the first true leaf, three batches of 20 PC seeds were sown at three day intervals using the standard protocol for sp5 seed. When the PC seedlings had produced the first true leaf, three batches of 40 WT seeds were sown at three day intervals to ensure seedlings could be synchronised in terms of size at the time of grafting. The grafting protocol described previously was followed. Due to the limited availability of F_1 seed and non-uniform germination and seedling growth, only 11 F_1 seedlings were available for grafting to produce WT/3G plants, compared to 20 replicate grafts for the WT/WT and WT/PC combinations. Eight plants of sufficiently uniform size were available for the WT/3G scion/rootstock combination when the experiment began. The experiment was designed to have five replicates of

each scion/rootstock combination. In addition, three replicates were used for destructive harvest immediately before the experiment commenced to determine the initial mean plant biomass for each scion/rootstock combination. Gas exchange measurements were made on Days 10, 11 and 12 of the 30 d experimental period. At final harvest (Day 30), the shoot (scion) was severed *c*. 2 mm below the cotyledons. A delivery tube was attached to the cut stump and xylem sap exudate was allowed to drip into a centrifuge tube, which was kept in darkness and surrounded by ice. Exudate emerging from the cut stump during the first hour was discarded, after which it was collected for the following two hours and subsequently frozen at -70 °C for ABA analysis. ABA was determined by radioimmunoassay, as described by Mulholland *et al.* (2003).

Table 8.1. Description of transgenic tomato lines. Letters denote transgene over-expression; N = *LeNCED1*, B = *LeBCH2*, P = *LePSY1* and + = WT gene. The notation "NN/BB/PP" represents a homozygous line at these three transgene loci. + is also used to indicate the absence of a transgene on one chromosome at each of the loci.

Line	**Type of Line**	**Parental Lines**	**Number of transgene T-DNA insertion loci**	**Level of zygosity at transgene over-expression loci**		
				LeNCED1	***LeBCH2***	***LePSY1***
WT (Tm2a)	-	Tm2a x Tm2a	0	++	++	++
BCH12	'single transgene'	BCH12 x BCH12	1	++	BB	++
sp5	'single transgene'	sp5 x sp5	1	NN	++	++
PSY (Z-line)	'single transgene'	PSY x PSY (Z-line)	1	++	++	PP
G29	'double transgene'	sp5 x BCH12	2	NN	BB	++
MJ8	'double transgene'	PSY (Z-line) x BCH12	2	NN	++	P+
PC	'double transgene'	sp5 x G29	2	NN	B+	++
3G	'triple transgene'	G29 x MJ8	3	NN	B+	P+

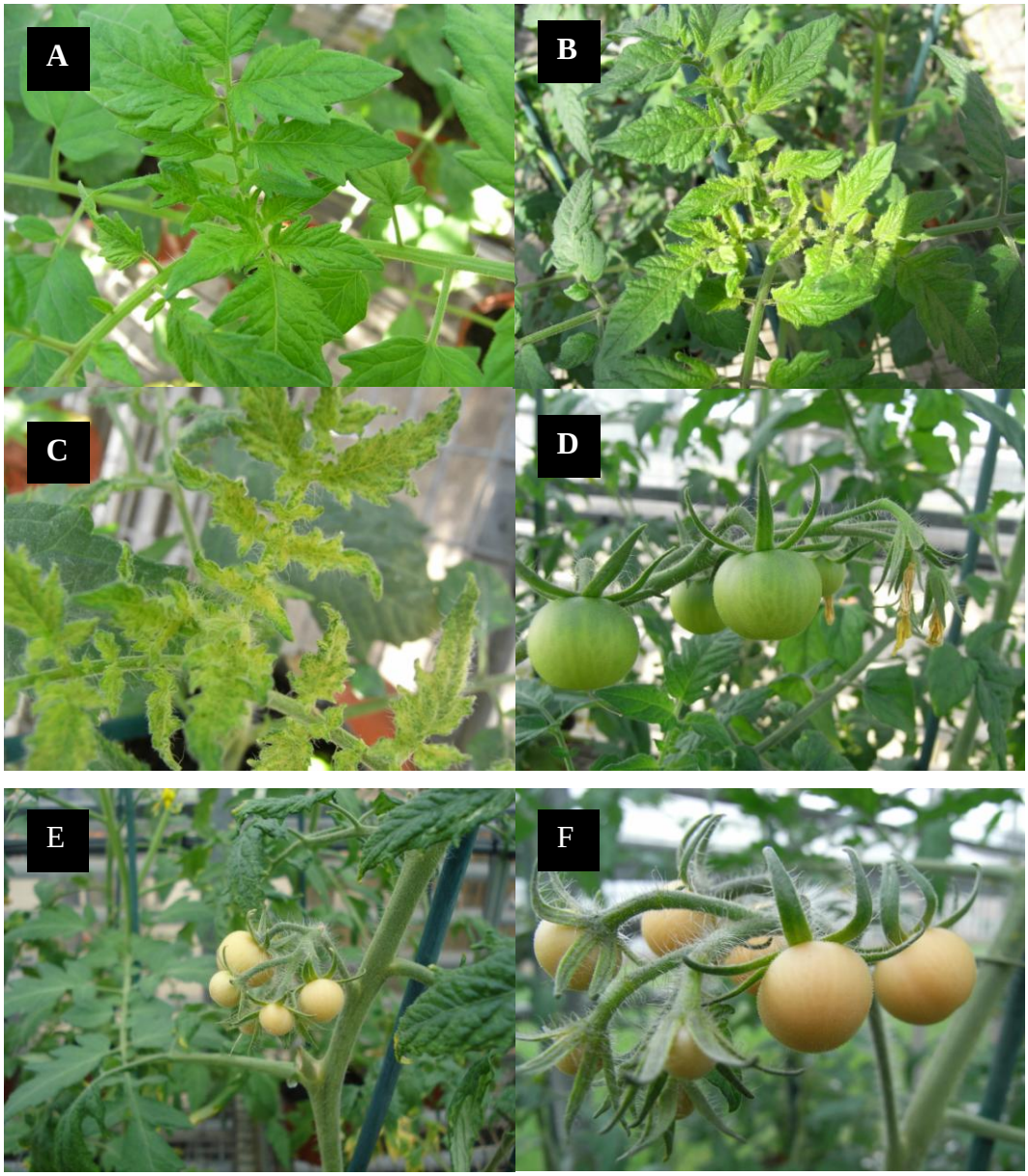


Figure 8.2. Shoot apex of plants showing: **A**, no chlorosis; **B**, mild chlorosis; and **C**, severe chlorosis and immature fruit colour of 3G F_2 plants; **D**, Green (WT phenotype); **E**, Pale Yellow (intermediate phenotype); and **F**, Peach (*PSY* over-expression phenotype). The contribution of Miss C. White in the above photography is gratefully achnowledged.

8.4 RESULTS

8.4.1 G29 reciprocal grafting experiment

Biomass production during the experimental period was greater in plants with G29 scions (G29/WT and G29/G29) than in those with WT scions (WT/G29 and WT/WT) (Fig. 8.3), regardless of rootstock type ($P<0.001$). G29 self-grafts (G29/G29) transpired less water than all other scion/graft combinations ($P<0.05$; Fig. 8.3), and used 11 % less water than WT self-grafts (WT/WT). This difference is smaller than observed in previous whole plant gravimetric TE_p experiments (Chapter 7), in which G29 used 30 % less water over a 25 d period. The less profligate use of water by WT plants in the present experiment was possibly an affect of the differing times of year experiments were conducted, rather than any difference in stomatal characteristics between G29/G29 self-grafts and intact G29 plants.

TE_p was greater in the G29 self-grafts than in all other scion/graft combinations ($P<0.001$), and was 25 % higher than in WT self-grafts (Fig. 8.4). The G29 rootstock had no effect on TE_p in WT/G29 plants as there was no significant difference between these and WT self-grafts. However, G29 scions grafted to WT roots did have an effect, as TE_p was significantly greater for G29/WT plants than for plants with WT scions (WT/G29 and WT/WT; $P<0.001$). The G29/G29 plants also had significantly greater TE_p than G29/WT ($P<0.001$), showing that, although the 'high ABA' scion had an effect on its own, the addition of 'high ABA' rootstock provided a further improvement in TE_p.

8.4.2 F_1 'triple transgene' rootstock experiment

8.4.2.1 Gas Exchange

Measurements of mean stomatal conductance on three consecutive days during the experimental period revealed no significant difference between scions grafted onto different rootstocks (Fig. 8.3), as was also the case for measurements made on specific sampling dates (data not shown). However, despite the absence of significant differences between grafting treatments, plants were consistently ranked in the order WT>PC>3G for stomatal conductance on all measurement dates.

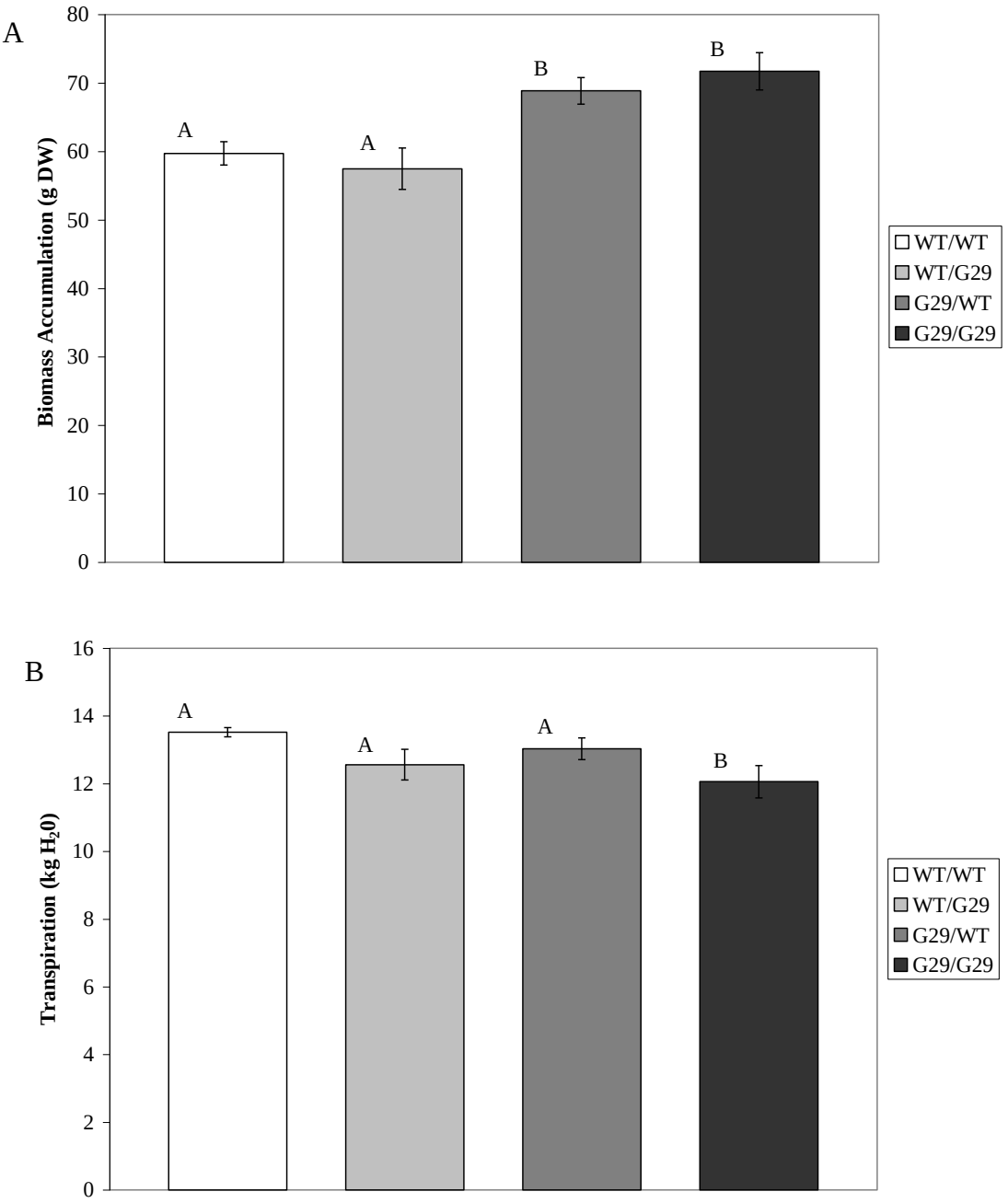
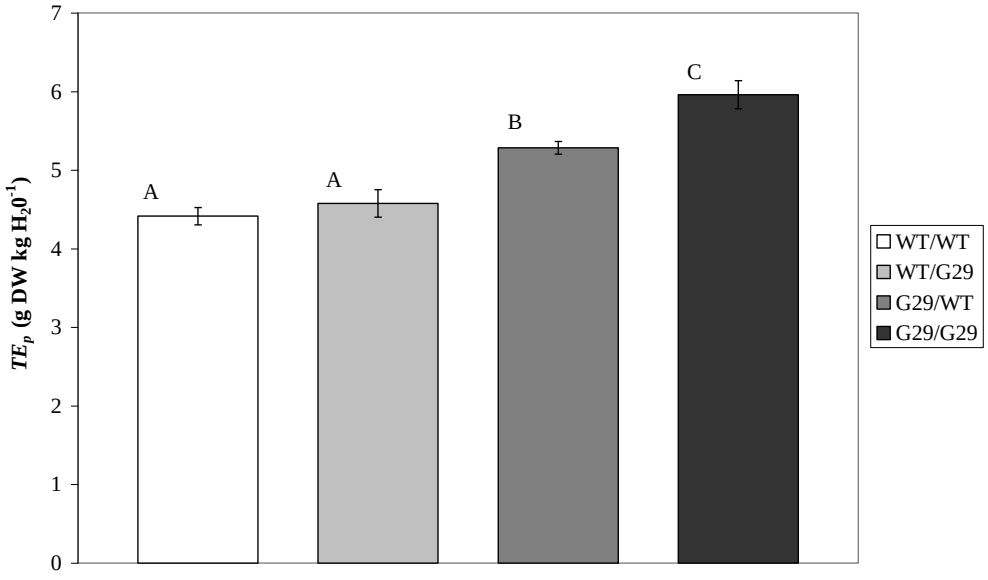


Figure 8.3. A, Biomass accumulation and **B**, total transpiration during the experimental period;. Differing letters associated with the histograms indicate significant differences ($P<0.05$); error bars represent standard errors of the mean (n=6).

A



B

	Biomass Accumulation (g DW plant^{-1})	**Transpiration (kg H_2O plant^{-1})**	***TE_p*** **(g DW kg^{-1} H_2O)**
F pr.	*<0.001*	*<0.05*	*<0.001*
s.e.d	3.30	0.425	0.206
l.s.d	7.03	0.96	0.439

Figure 8.4. **A**, Mean TE_p during the experimental period; **B,** Statistical summary from AVOVA (n=6). Differing letters associated with the histograms indicate significant differences (P<0.05); error bars represent standard errors of the mean.

8.4.2.2 Transpiration Efficiency

WT scions with 3G rootstocks (WT/3G) transpired less water than WT/WT self-grafts over the 30 d experimental period (P<0.05; Fig. 8.5). This difference did not result from variation in plant size at the start of the experiment as biomass for all three scion/rootstock combinations did not differ significantly at the initial harvest (Appendix 8). However, WT/PC plants were intermediate and did not differ significantly either from WT/WT or WT/3G plants in the total quantity of water transpired (Fig. 8.5). There was no significant difference in biomass production between WT scions grafted onto each rootstock type (Fig. 8.6), although the difference in TE_p between each rootstock type approached significance (P=0.067). It was also observed that, consistent with total transpiration and stomatal conductance, the plants ranked in the order 3G>PC>WT for TE_p.

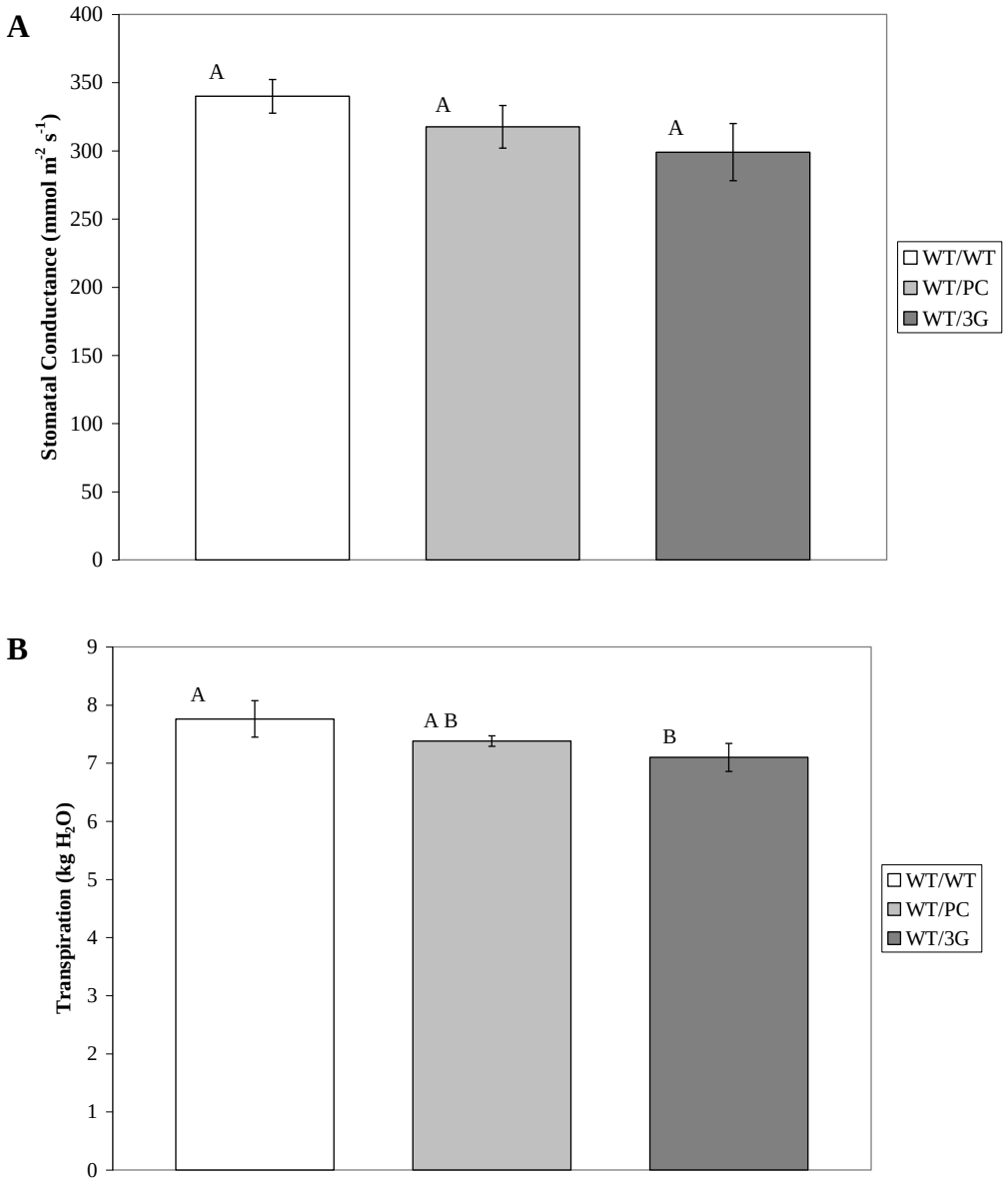
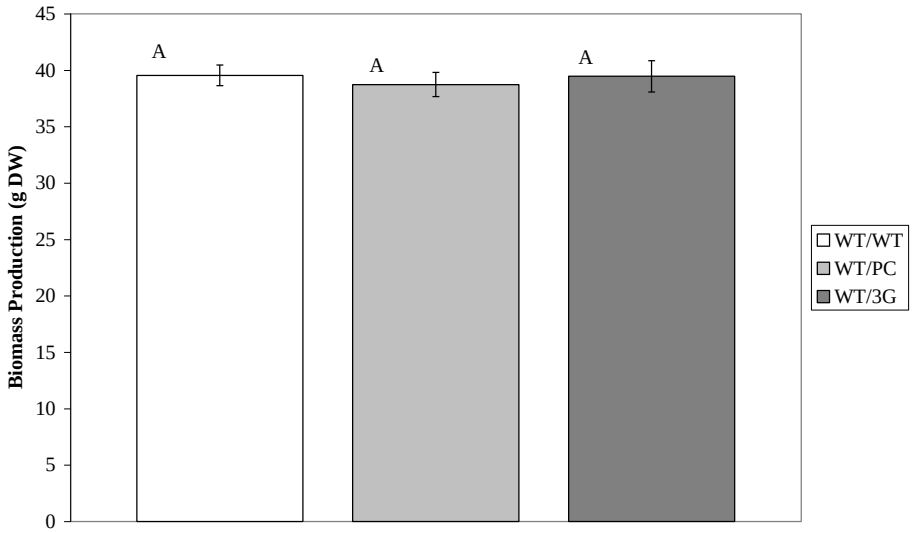
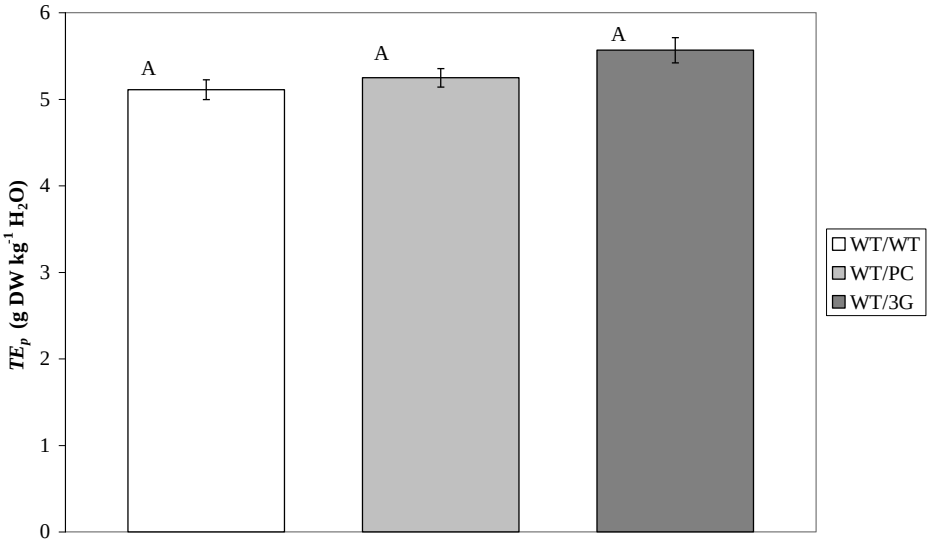


Figure 8.5. **A**, mean stomatal conductance measured on days 10-12; and **B**, total transpiration during the experimental period. Differing letters associated with the histograms indicate significant differences ($P<0.05$); error bars represent standard errors of the mean (n=5).

A



B



C

	Biomass Accumulation (g DW plant^{-1})	**Transpiration (kg H_2O plant^{-1})**	***TE_p*** **(g DW kg^{-1} H_2O)**
F pr.	0.243	0.048	0.067
s.e.d	0.484	0.220	0.169
l.s.d	1.117	0.507	0.389

Figure 8.6. A. biomass accumulation; **B**, mean TE_p during the experimental period; and **C**, statistical summary from AVOVA (n=5). Differing letters associated with the histograms indicate significant differences (P<0.05); error bars represent standard errors of the mean.

8.4.2.3 Xylem Sap ABA Concentration

The ABA concentration of xylem sap exuding from detopped plants was analysed by radioimmunoassay (Fig. 8.7). WT/PC and WT/3G plants both had greater sap ABA concentrations that WT/WT plants ($P<0.05$), but there was no detectable difference between the two transgenic rootstock types. In comparison with WT controls, PC and 3G rootstocks provided 5.9 and 6.2-fold increases in sap ABA concentration respectively.

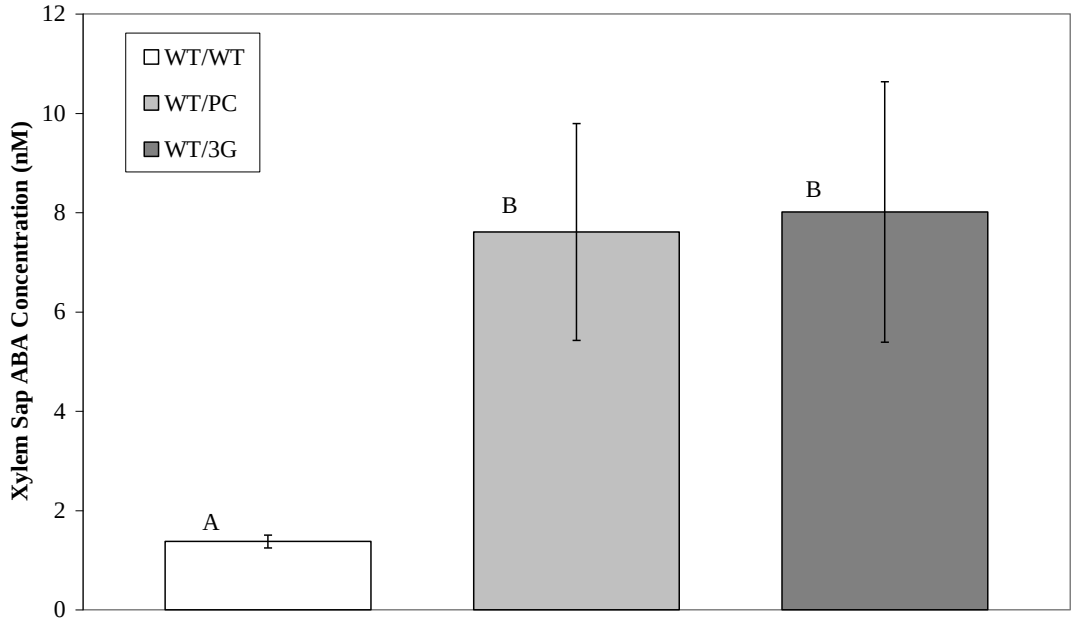


Figure 8.7. ABA concentration in xylem sap exuding from stumps of detopped plants. Differing letters associated with the histograms indicate significant differences ($P<0.05$); error bars represent standard errors of the mean (n=5, l.s.d: 4.1). The contribution of H.W. Hilton (Warwick-HRI) in performing the ABA assay is gratefully acknowledged.

8.4.3 The F_2 'triple transgene' population

8.4.3.1 Scoring of the PSY Over-expression Phenotype

The colour of immature fruit was classified as green, intermediate (pale yellow) or peach; in all cases, most plants in the F_2 generation exhibited the intermediate unripe fruit type. Scoring of apex chlorosis followed a similar pattern, with the majority of plants falling into the 'mild chlorosis' category. Overall in the case of both immature fruit and apex chlorosis parameters, there appeared to be a ratio of *c*. 1:3:1 for severe, intermediate and symptom-free plants (Fig. 8.8).

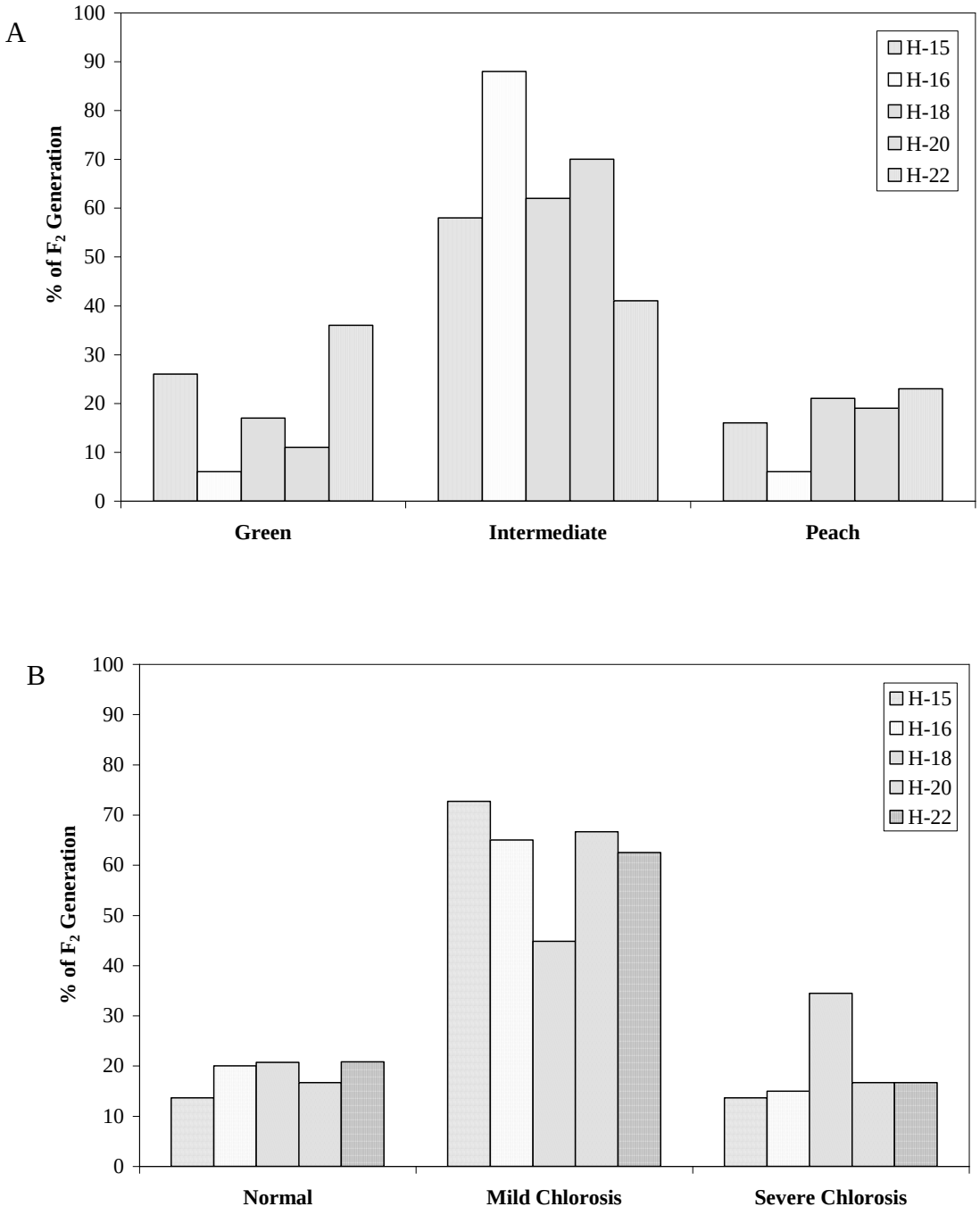


Figure 8.8. **A**, Unripe fruit colour; and **B**, severity of shoot apex chlorosis for 3G F_2 plants expressed as a percentage of total plant number for each of the five H-F_2 generations. *cf.* Figure 8.2 for definitions of each classification.

8.4.3.2 Stomatal Conductance

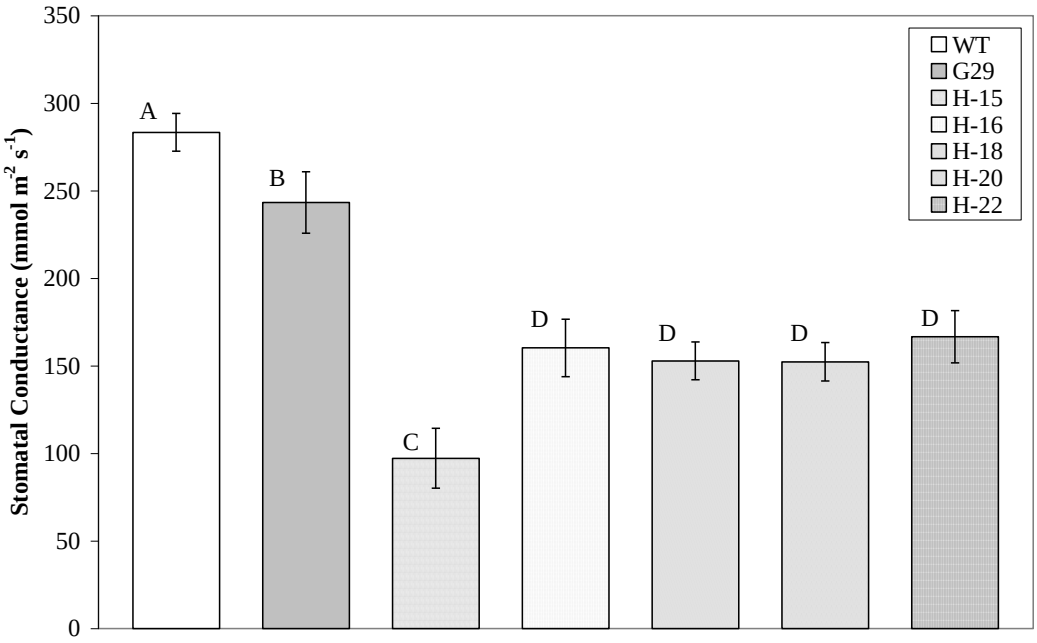


Figure 8.9. Mean stomatal conductance for all five H-F_2 generations and the G29 and WT controls (n=6, P<0.001, l.s.d: 34.94). Differing letters associated with the histograms indicate significant differences (P<0.05); error bars represent standard errors of the mean.

Stomatal conductance was greater in WT plants than in all 'triple transgene' H-lines and G29 control plants (P<0.001), and was also greater in G29 plants than in the five H-lines (P<0.001). No significant difference was found between lines H-16, -18, -20 and -22, although line H-15 had a lower conductance than all other lines (P<0.001). Mean stomatal conductance for all 'triple transgene' F_2 plants was 49 and 40 % of that for WT and G29 respectively.

8.5 DISCUSSION

8.5.1 Rootstocks over-expressing *LeNCED1* and *LeBCH2* do not affect whole plant TE_p when grafted to WT scions

The reciprocal grafting experiment using 'double transgene' (G29) and WT plants revealed that 'high ABA' rootstocks alone did not produce sufficient root-synthesised ABA to affect the stomatal behaviour of WT scions and influence TE_p significantly (Fig. 8.4). In contrast, when 'high ABA' shoots were grafted onto WT rootstocks (G29/WT) there was a significant effect on TE_p relative to WT self-grafts. It should also be noted that a further improvement in TE_p was obtained when the 'high ABA' line was self-grafted, demonstrating an additive effect of combining ABA over-production in the root and shoot.

The importance of root-to-shoot signalling is well established (Davies *et al.*, 2005). In response to reduced soil water availability, changes in stomatal behaviour in the shoot may occur in the absence of any change in leaf water status. As mentioned in the introduction to this chapter, this has previously been demonstrated using split-root (Blackman and Davies, 1985) and soil pressure chamber (Passioura, 1988) techniques. It is also clear that ABA influences stomatal behaviour (Jones and Mansfield, 1970) as, when ABA is fed into the transpiration stream, it is imported to guard cell walls in a process which is correlated with subsequent reductions in stomatal conductance (Zhang and Outlaw, 2001). There is therefore potential to influence shoot stomatal behaviour by using rootstocks which over-produce ABA, although the level of over-production appears to be insufficient in the case of G29.

When *LeNCED1* alone is over-expressed in transgenic tomato plants, a 3.8-fold increase in root ABA concentration has been reported (Thompson *et al*., 2007b). However, when rootstocks of this line (sp5) were grafted onto WT scions, there was no significant effect on stomatal behaviour. Thompson *et al*. (2007b) also demonstrated that the significantly decreased stomatal conductance characteristic of sp5 plants is largely due to shoot-sourced ABA. When the direct effect of combining two transgenes on ABA concentration in the roots was analysed using isolated root cultures, transgenic tomato lines which simultaneously over-express *LeBCH2* and *LeNCED1* produced more than 10-fold more root-synthesised ABA than WT controls

(Balasubramanian, 2007). The reciprocal grafting experiment reported here indicates that the extent of this increase in ABA production is insufficient to influence stomatal behaviour in WT shoots.

When ABA-deficient mutant and WT plants are reciprocally grafted, the extent to which root-synthesised ABA influences shoot phenotype can be analysed. The greater importance of shoot-synthesised ABA relative to root-synthesised ABA was demonstrated by the fact that ABA-deficient rootstocks (*flacca* and *sitiens*) had little effect on WT shoot phenotype (Chen *et al*., 2002; Holbrook *et al*., 2002; Dodd *et al*., 2009). However, when reciprocal grafts were performed (mutant/WT), the ABA produced by the WT rootstocks partially reversed a number of ABA-deficient phenotypes (Chen *et al*., 2002; Holbrook *et al*., 2002; Dodd *et al*., 2009), although these effects were by no means consistent across all studies. It can be concluded that root-synthesised ABA may influence shoot phenotype, although not to the extent induced by shoot-sourced ABA. When drawing conclusions from studies using the tomato mutants *flacca* and *sitiens* as ABA-deficient rootstocks, it is important to note that these ABA-deficient roots may nevertheless initiate an ABA signal following the movement of ABA precursors to the shoot. In both mutants, the biosynthesis of ABA is blocked at the last step in the pathway, during which *cis*-ABA-aldehyde is converted to *cis*-ABA (Taylor *et al*., 1988). When the ABA-specific aldehyde oxidase step is blocked, this leads to accumulation of *trans*-ABA alcohol (Linforth *et al*., 1987), which may be transported to the shoot and converted into biologically active *cis*-ABA. This issue could be avoided by using an alternative ABA-deficient mutant such as *notabilis*, which is blocked earlier in the ABA biosynthetic pathway, at the step catalysed by NCED (Burbridge *et al*., 1999; Thompson *et al*., 2004). There was a significant difference in biomass production during the gravimetric experiment between different scion/rootstock combinations which appeared to be an effect of scion genotype, whereby plants with G29 shoots produced a greater biomass than those with WT shoots.

8.5.2 The use of 'triple transgene' lines as rootstocks

8.5.2.1 Effect of F_1 rootstock on whole plant TE_p

When the line that provided the greatest improvement in TE_p as a whole plant (G29) was grafted to WT scions, whole plant water use characteristics were not significantly affected (Fig. 8.4). The increase in root-synthesised ABA resulting from the two transgenes designed to over-express *NCED1*and *LeBCH2* was therefore insufficient to influence the stomatal behaviour of the WT scion. This was also observed in the alternative 'double transgene' line (MJ8) which over-expresses *LeNCED1* and *LePSY1*, which, when grafted as rootstocks to WT scions, did not significantly affect any gas-exchange parameters (Jones, 2007). It is likely that, while the step catalysed by NCED in the ABA biosynthetic pathway is rate-limiting for ABA biosynthesis in shoots, previous steps are more restrictive to the flux of precursors through this pathway in roots (Thompson *et al*., 2007b).

When two ABA biosynthetic genes were over-expressed, increased production of ABA was observed in roots, with *LeNCED1*/*LeBCH2* and *LeNCED1/LePSY1* respectively showing *c*. 5 and 10-fold increases relative to WT controls (Balasubramanian, 2007; Jones, 2007). As discussed previously, neither of these' double transgene' combinations provides a sufficiently strong root-sourced ABA signal when used as rootstocks to affect stomatal conductance significantly in WT scions. Roots of the *LeNCED1/LePSY1* line had a large pool of β-carotene, suggesting that the increased activity of the PSY enzyme was producing more carotenoid precursors of ABA; however, due to a bottleneck at the step catalysed by BCH, the increase in carotenoid supply was not fully reflected by increased ABA biosynthesis. The two 'double transgene' lines were therefore crossed to create a 'triple transgene' line (3G) which constitutively over-expressed *LeBCH2*, *LePSY1* and *LeNCED1*, which are all regulate key steps in the biosynthesis of ABA and its carotenoid precursors.

The gravimetric rootstock experiment described here can only be considered as a preliminary evaluation of the potential use of the 3G line. As the creation of a fully homozygous 3G line was still at a preliminary stage, only F_1 seed was available when this experiment was performed. Whilst the seed was homozygous for the sp5

T-DNA insertion locus of the *LeNCED1* construct, it was heterozygous for the BCH12 T-DNA insertion locus of the *LeBCH2* construct and had an unknown number of *LePSY1* transgene copies. However, creation of the 'perfect control' line (PC) allowed evaluation of whether the presence of the *LePSY1* construct affected the ability of the rootstock to influence stomatal behaviour in the WT scion.

Gas exchange measurements on three consecutive days showed there was no difference between rootstock types, although these were consistently ranked in the order WT>PC>3G in terms of stomatal conductance. There was also no effect of rootstock on biomass production, in contrast to the G29 reciprocal grafting experiment, which showed greater biomass production in plants with 'high ABA' scions. There was, however, one significant effect associated with the up-regulation of all three genes as the WT/ 3G plants transpired significantly less water over the 30 d period than WT self-grafts (Fig 8.5). However, the difference in TE_p between the three rootstock types was not quite significant (P=0.067), although these were ranked in the order 3G>PC>WT. As limited F_1 seed was available for this experiment, replication was less than ideal.

Analysis of exudate from detopped plants revealed that xylem sap ABA concentration was 5.9- and 6.2-fold greater for PC and 3G rootstocks than for WT rootstock. This observation is consistent with expectation as Thompson *et al*. (2007b) reported that xylem sap ABA concentration in line sp5, which over-expresses *LeNCED1* alone, was 3.5-fold greater than in WT plants. What was not expected was that, although the 3G rootstocks appeared to produce slightly higher ABA concentrations than PC, the difference was not significant. However, it should be noted that there was substantial variation in the values for the individual replicates of 3G, possibly because the plants used were from the F_1 generation, whose variable copy number would provide potential variation in the strength of *LePSY1* over-expression.

Hydraulic conductivity is a term used to describe the ease with which water moves across membranes and through the apoplast (Taiz and Zeiger, 2006). Root hydraulic conductivity can be increased to compensate for reduced soil water availability, and this process has been reported to be associated with increases in root ABA

concentration (Jeshke *et al*., 1997; Vysotskaya *et al*., 2004). Thus, application of exogenous ABA to root systems increased their hydraulic conductivity (Lp_r; Hose, 2000). Transgenic 'high ABA' tomato plants exhibited increased Lp_r values (Thompson *et al*., 2007b), whereas Lp_r was decreased in ABA-deficient mutants (Tal and Nevo, 1973). The hydraulic conductance of roots is probably regulated by ABA-induced changes in the activity and abundance of aquaporins (Hose *et al*., 2000; Seimens and Zwiazek, 2004). The higher ABA concentrations in PC and 3G roots may cause aquaporins to accumulate (Aroca *et al*., 2006), thus increasing Lp_r. The rate of sap flow may therefore vary between rootstock types and a more accurate measurement of ABA delivery rate from roots to shoot could perhaps have been calculated if exudation rate had also been measured.

ABA appears to be constantly re-circulated between roots and shoots (Hartung *et al*., 2002) and, whilst all rootstocks were grafted to the same scion type, a more accurate quantification of the ability of the 'triple transgene' rootstocks to over-produce ABA is required. It is possible that high levels of ABA production in the roots are not detectable using xylem exudate as loading of ABA into the xylem sap could become saturated. Growing isolated root cultures and measuring ABA concentration directly would provide the most accurate measure of the ability of these roots to synthesise ABA independently of the shoots.

8.5.2.2 Phenotype of the F_2 generation

Each of the eight individual H-F_1 plants accumulated substantial quantities of lycopene in their roots (C. White, pers. comm), a compound not found in roots which do not over-express *LePSY1* (e.g. WT and G29 roots). The presence of lycopene confirmed that the H-F_1 population contained at least one copy of the T-DNA insertion locus, leading to over-expression of *LePSY1*. Five F_1 plants were chosen to be taken on to the F_2 generation and the entire population was scored for the severity of the *LePSY1* over-expression phenotype and preliminary gas exchange measurements were conducted.

Each of the five H-F_2 lines had significantly lower stomatal conductances than the WT and G29 controls (Fig. 8.9), indicating that the presence of the *LePSY1* transgene

significantly affected stomatal behaviour. Scoring chlorosis of the shoot apex and immature fruit colour revealed that segregation of both parameters occurred in a 1:3:1 ratio. The 'peach colour' phenotype of plants over-producing *LePSY1* was first reported by Fray *et al*. (1995), who found that lycopene was prematurely produced by immature fruit. When over-expression of *LePSY1* was combined with *LeNCED1* in MJ8 plants (Table 8.1), the resulting line also exhibited these two characteristic phenotypes. As well as significantly increased levels of phytoene, ζ-carotene and lycopene, immature fruit over-expressing *LePSY1* also exhibit premature depletion of chlorophyll, perhaps due to utilisation of GGPP (the substrate for PSY) in the carotenoid synthesis pathway at the expense of phytol and hence chlorophyll synthesis (Fraser *et al*., 2007). The MJ8 parental line was not homozygous for the *LePSY1* transgene T-DNA insertion loci and H-F_1 plants did not exhibit either of the shoot apex or immature fruit phenotypes, consistent with the lack of homozygosity at all of the *LePSY1* transgene insertion loci. The 'peach' fruit and chlorosis of the shoot apex phenotypes were found once again in the F_2 population (Fig. 8.8). The phenotypes associated with *LePSY1* over-expression, described above were combined with analysis of carotenoid concentration in the roots to select individual lines to be taken on to the F_3 generation. These plants will eventually be analysed for homozygosity of the *LeBCH2* and *LePSY1* transgenes, with the aim of producing a true breeding and uniform 'triple transgene' line with homozygous over-expression of all three transgenes (ongoing work performed by C.White).

The combination of the impact of reduced chlorophyll content on growth rates and the general 'dwarf' phenotype associated with over-expression of *LePSY1* (Fray *et al*., 1995) mean it is unlikely that 'triple transgene' plants homozygous for over-expression of the *LePSY1* transgene would provide an agronomic advantage, despite their potential to improve whole plant water use efficiency. Whilst preliminary gas exchange measurements indicated that addition of the third ABA biosynthesis gene reduced stomatal conductance relative to plants over-expressing two genes (G29), it is likely that the reduced growth rate would impact on overall TE_p and the general chlorosis could cause pleiotropic effects that might affect stomatal conductance. However, preliminary analysis of the 3G F_1 plants as rootstocks suggested a potentially beneficial effect on whole plant water use when grafted to WT scions. In this preliminary experiment, lack of uniformity and the limited number of replicates

made it less likely that significant effects would be detected. When a uniform triple homozygous 3G line is available, in which the over-expression of all three transgenes is maximised, a highly replicated gravimetric TE_p experiment which included both 'double transgene' parental lines (MJ8 and G29) as controls would be more likely to reveal whether combining the over-expression of *LeNCED1*, *LeBCH2* and *LePSY1* transgenes in rootstocks will generate a sufficiently strong root-sourced ABA signal to affect stomatal behaviour in the WT scion.

9 SEED GERMINATION AND SEEDLING ESTABLISHMENT OF 'HIGH ABA' LINES

9.1 INTRODUCTION

9.1.1 The role of ABA in the regulation of seed germination and dormancy

Germination may be defined as the transition period between the resting seed and the time of visible radicle emergence (Bewley and Black, 1994). It occurs due to chemical weakening of the endosperm by hydrolytic enzymes, the production of which is, at least in part, controlled by the embryo. Seed dormancy is an adaptive trait that may improve survival of the next generation by optimising the distribution of germination over time (Kermode, 2005) and geographical distance (Taiz and Zeiger, 2006). Dormancy is defined as the inability of an intact and viable seed to complete germination under optimal conditions (Bewley, 1997). During the dormancy phase, seeds do not germinate as they are incapable of producing the enzymes that degrade cell walls (Groot *et al*., 1988). This mechanism synchronises germination with seasonal cycles to avoid unfavourable weather and ensure survival and establishment of seedlings. Most seeds are therefore able to survive during periods when conditions are unfavourable for germination.

ABA is known to be important in the induction of seed dormancy (King *et al*., 1976), as has been demonstrated using ABA-deficient mutants which typically show reduced dormancy and display severely decreased desiccation tolerance and longevity (Karssen *et al*., 1983; Ooms *et al*., 1993). Evidence for the extent of the contribution of ABA biosynthesis and catabolism to dormancy has also been gained from transgenic studies. For example, over-production of ABA resulting from over-expression of ABA biosynthetic genes produced seeds with slightly greater dormancy than the wild type in *Nicotiana plumbaginifolia*, a diploid tobacco relative (Frey *et al*., 1999), and dormancy has been induced in otherwise completely non-dormant tomato seeds (Thompson *et al*., 2000). Application of exogenous ABA to *Zea mays* and tomato seeds also prevents germination of the embryo (Neill *et al*., 1987; Hilhorst *et al*., 1998).

Fluridone and norflurazon inhibit carotenoid production and therefore prevent ABA biosynthesis (Gamble and Mullet 1986). Methods involving the application of inhibitors of ABA biosynthesis have demonstrated that *de novo* ABA synthesis in dormant seeds is involved in the maintenance of dormancy (Debeaujon *et al*., 2000). When applied to dormant *Nicotiana* seeds, fluridone breaks dormancy, demonstrating the importance of *de novo* ABA synthesis (Grappin *et al*., 2000). The role of ABA in seed development, germination and dormancy is discussed more fully in Chapter 1.

9.1.2 Seed germination in a crop production environment

Whilst seed dormancy provides an evolutionary advantage, ensuring survival of subsequent generations by distributing progeny over space and time, crop production systems require reliable and uniform germination. Total percentage germination and uniformity of germination are both important characteristics when considering the production of tomato crops (Heuvelink, 2005) and germination is closely related to the characteristics of the seed. Thus, if fruit is harvested and seed is subsequently extracted before full maturity, seed viability and germination are both reduced (Kerr, 1963). Seed size also influences germination, as smaller seeds are often associated with more rapid germination, due to the reduced thickness of the endosperm. In other species, larger seeds have been shown to have a greater ability to survive environmental stress, such as drought (e.g. Leishman and Westoby, 1994). Environmental factors such as temperature strongly influence seed germination, and the optimum for germination in tomato is between 20 and 25^{o}C (Thompson, 1974). Tomato seeds will germinate at soil water potentials ranging from slightly above permanent wilting point to field capacity, with the optimal moisture content being between 50 and 75% of field capacity (Heuvelink, 2005).

In crop production systems, seedling survival and establishment are important to maximise crop productivity. Following germination of tomato seeds, a period of vegetative growth precedes a coincident phase of vegetative and reproductive growth (Picken *et al*., 1986). The seedling or 'establishment' phase is one of the most sensitive stages in the life cycle of plants and is often strongly influenced by the available storage reserves within the seed (Santos and Buckeridge, 2004). As the

cotyledons are the main photosynthetic organs during the early stages of seedling growth (Hussey, 1962), emergence of fully viable cotyledons of optimal size is crucial to the rapid and successful establishment of tomato seedlings.

9.1.3 Increased seed dormancy and reduced seedling growth in tomato plants over-producing ABA

Thompson *et al*. (2000) first reported increased seed dormancy in transgenic tomato plants over-expressing *LeNCED1* using constructs involving a constitutive promoter (Gelvin superpromoter). Thus, final percentage seed germination in lines sp12 (previously reported as D9) and sp5 was respectively 54 and 15% compared to WT final percentages of typically 90-100%. This dormancy effect was overcome by application of norflurazon, which restored percentage in both sp lines to that of WT seeds. Norflurazon is a herbicide which blocks carotenoid, and hence ABA synthesis (Smith, 1997), and its mode of action is via the inhibition of the enzyme phytoene desaturase which catalyses the desaturation of phytoene to phytofluene in the carotenoid-biosynthesis pathway (Tomlin, 1997). This observation suggested that over-expression of *LeNCED1* in the embryo increased ABA synthesis and the consequent increase in the ABA content within the seed was responsible for inducing the prolonged dormancy. This was confirmed by measurements of seed ABA content which showed that both sp lines (sp12 and sp5) contained threefold more ABA than in WT seeds (Thompson *et al*., 2000).

The sp lines described above were subsequently shown to be more efficient in their use of water, with no negative consequences on long-term growth rate (*cf*. Chapters 4 and 5). The only adverse effect on growth of constitutively over-producing ABA occurred during the propagation phase, when sp12 and sp5 plants exhibited significantly delayed germination and seedling establishment (Thompson *et al.,* 2000). It was therefore desirable that over-expression of the *LeNCED1* gene is restricted to tissues in which increased ABA over-production is beneficial. An alternative, light-regulated (tomato *rbcS3C*) promoter (Carrasco *et al*., 1993; Gittins *et al*., 2000) was therefore chosen to drive *LeNCED1* expression, with the primary objective of eliminating undesirable seed dormancy. The use of the tissue-specific *rbcS3C* promoter, which exhibits low expression in non-photosynthetic tissues

(Gittins *et al*., 2000), successfully reduced seed dormancy in all homozygous rbcS lines, with only a 2-6 day delay in germination (*cf*. Chapter 6). However, Tung *et al*. (2008) reported a 'lollipop' phenotype when rbcS lines were germinated in petri dishes, resulting from the reduced ability of the expanding cotyledons to escape from the testa. This phenotype was also noted in sp plants when seed was germinated using the same method (Symonds, 2002).

9.2 AIMS

Transgenic tomato plants which constitutively over-produce ABA have water saving abilities that are of potential agricultural benefit (Chapters 4 and 5; Thompson *et al.*, 2007a). It was vital that reliable protocols were developed to synchronise the initial germination and subsequent growth stages in order to accurately characterise the growth and water use of these plants. To further enhance the water saving ability of 'high-ABA' plants, the simultaneous up-regulation of two ABA biosynthetic genes in transgenic tomato lines has resulted in transgenic plants that over-produce ABA to a greater extent than those based on a single gene. This provided further enhancement of the water saving properties of these plants, but increased the problem of prolonged seed dormancy. The trials reported here involved developing germination protocols, precisely tailored for each 'high ABA' line. For the more extreme 'high ABA' lines, this involved determining the optimal application of norflurazon required to break the increased dormancy without reducing the photosynthetic area of the cotyledons through bleaching. An alternative germination method was also developed to minimise the occurrence of the 'lollipop' phenotype reported by Tung *et al*. (2008).

A separate growth trial was designed to characterise the extent to which the seedling establishment phase is prolonged in 'high ABA' plants. This involved examining differences in the growth and physiology of young tomato plants showing a range of ABA over-production levels and determination of the point at which the growth rate increased to match that of WT, as observed in older 'high ABA' plants (*cf*. Chapters 4, 5 and 7).

9.3 MATERIALS AND METHODS

9.3.1 Germination Trials

9.3.1.1 Petri dish germination trial of single gene lines

Sixty seeds from each genotype (WT, sp12, sp5 and rbcS-10) were germinated on sterile filter paper in 9 cm petri dishes at 24^{o}C, kept moist using distilled water and scored for percentage germination on a daily basis. Seeds were considered to have completed germination upon the emergence of the radicle.

9.3.1.2 Norflurazon application preliminary trial (sp5)

Treatment with norflurazon involved applying 1.0, 0.5 or 0.1 mg l^{-1} solutions for one, two or three days, with 20 replicate seeds sown for each concentration x duration treatment combination. Once the treatment had been completed, the seeds were thoroughly washed in distilled water to remove any residual norflurazon before being germinated on sterile filter paper in 9 cm Petri dishes at 24^{o}C; the seeds were kept moist using distilled water and scored for percentage germination on a daily basis. Seeds were considered to have completed germination upon the emergence of the radicle.

9.3.1.3 Norflurazon application trial (WT, sp5 and G28)

This experiment involved applying 1.0, 0.5 or 0.1 mg l^{-1} norflurazon solutions for one or three days, with 10 replicate seeds sown for each concentration x duration treatment combination. Treatment took place in 9 cm Petri dishes on sterile filter paper at 24^{o}C.

The preliminary trials and general observations of the germination of 'high ABA' seeds indicated that the seed testa remained relatively hard following germination, trapping the emerging cotyledons. Although it was possible to remove the testa with forceps after softening it with distilled water in some cases, the failure of the cotyledons to emerge generally prevented further seedling development. Therefore for this experiment, the seeds were washed thoroughly in distilled water following norflurazon treatment to remove any residual norflurazon and planted in Levington F2s compost in individual 9 cm pots at a depth of 1 cm. The compost was carefully

compacted to ensure close contact with the seed and prevent any possibility of desiccation. The compost provided a source of abrasion, allowing the seed coat to soften and be removed before it could dry and harden. The seeds were kept under standard glasshouse conditions (*cf*. Chapter 3) and germination was recorded daily; germination was judged to have occurred when both cotyledons had fully emerged from the testa. To maintain high humidity in the air space above the compost and facilitate the softening of the testa, each pot was covered with the lid of a 9 cm Petri dish (Fig. 9.1). These were removed following emergence of the hypocotyl hook to avoid hindering seedling development, allow the cotyledons to emerge into the atmospheric environment and enable scoring of the extent of cotyledon bleaching. Once the cotyledons had expanded, the plants were kept well-watered under glasshouse conditions and the extent of bleaching of the emerged cotyledons was scored. The classification system was based upon assigning plants to one of four categories: no bleaching, mild bleaching, severe bleaching or death.

Figure 9.1. Petri dish lids were used to maintain high humidity until the hypocotyl hook emerged from the compost (left) and photobleaching of the emerged cotyledons following treatment of the seeds with high concentrations of norflurazon and/or for prolonged periods (right).

9.3.2 Seedling Establishment Phase Growth Trial (WT, BCH12, sp12, sp5, G28, G29)

9.3.2.1 Experimental design

This experiment was designed to characterise the differences in growth rate between the three single gene and two double gene 'high ABA' lines and the WT control line during seedling establishment. Plants were grown for 10, 20, 30 or 40 days, with a destructive harvest being carried out after each growth period. Ten

replicates of each genotype were harvested at each harvest date. Above ground dry weight, plant height and individual leaf lengths (including cotyledons) were recorded at each destructive harvest.

9.3.2.2 Plant Material

To ensure that seed germination occurred on the same day for all genotypes, it was necessary to stagger the sowing of 'high ABA' seeds. As BCH12 seeds show no increase in dormancy, they could be sown at the same time as WT seed. The seeds of sp12 germinate with no requirement for norflurazon treatment, but germination is on average five days later than that of WT seeds (Fig. 9.2). The standard norflurazon treatments for sp5, G28 and G29 seed were altered slightly, as it was essential for this experiment that no cotyledon bleaching was induced as a result of the herbicide pre-treatment. The usual protocol for the lines was as follows: sp5 seeds treated with 0.5 mg 1^{-1} norflurazon solution for one day, G28 with 1.0 mg 1^{-1} for three days, and the most dormant line, G29, treated with the same 1.0 mg 1^{-1} norflurazon solution for four days. This allowed reliable germination in the longer term growth trials described in Chapters 5 and 7, in which plant development needed to be synchronised at the four leaf stage and slight patches of chlorophyll loss were too small to affect long term growth rates. As the 0.5 mg. l^{-1} dose applied for 24 h to sp5 seeds had not previously caused cotyledon bleaching, this was not altered. To achieve germination of the double gene G28 and G29 lines without inducing bleaching of the cotyledons, the norflurazon application rate was reduced to 1.0 mg l^{-1} for two and three days respectively for G28 and G29 seeds.

As this experiment required 40 seeds of each line to germinate on the same day and the reduction in the usual norflurazon dose for G28 and G29 would reduce uniformity of germination, large numbers of seeds (150-300) were sown in 25 cm pots containing Levington M3 compost (Scott's Professional, Suffolk, UK). Pot size was increased from the 9 cm pots used in the standard sowing protocol as the volume of compost would not have been great enough to sustain 40 day old plants and it was undesirable to have to re-pot the plants mid-way through the experimental period. On Day 1 of the sowing schedule, 300 G28 and 300 G29 seeds were sown, followed by 200 sp5 seeds on Day 2. On Day 3, 200 sp12 seeds were sown (without norflurazon treatment) and 150 BCH12 and 150 WT seeds were sown on Day 8. The

date when the cotyledon hook emerged was recorded for all pots and seedlings were judged to be ready for inclusion in the experiment when both cotyledons had opened i.e. were 90^{o} to the hypocotyl axis. When the experiment commenced, 40 seedlings of each line (WT, BCH12, sp12, sp5, G28, G29) were selected after ensuring that they had all germinated on the same day.

9.4 RESULTS

9.4.1 Petri dish germination trial of single gene lines (WT, sp12, sp5 and rbcS-10)

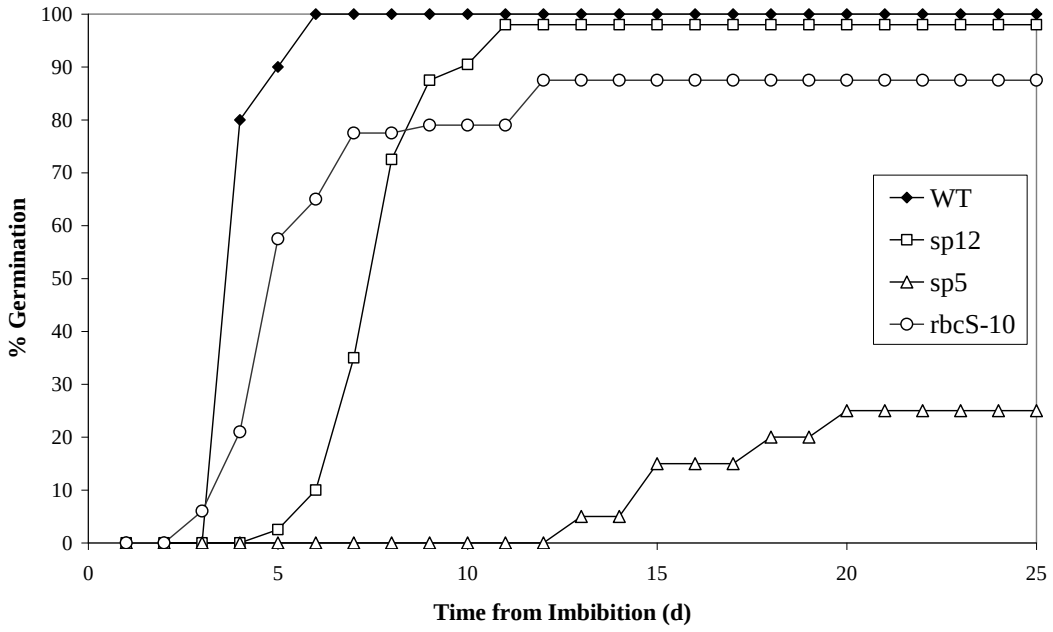


Figure 9.2. Percentage germination of WT, sp12, sp5 and rbcS10 genotypes over a 25 day period after imbibition (n = 60 seeds).

This initial assessment of germination for the three single gene 'high ABA' lines supported the findings of Thompson *et al.* (2000a) that high ABA levels prolonged dormancy, thus inhibiting germination (Fig. 9.2). As sp5 overproduces ABA to a greater extent then sp12, the effect was more pronounced in the former. The sp12 seeds reached 98% germination five days after WT Ailsa Craig seeds achieved 100% germination. There was no germination of sp5 seeds until 12 days after imbibition and a total of only 25 % germination was achieved after 20 days, demonstrating that seed germination in this genotype was both inhibited and non-uniform. The rbcS-10 line was transformed using the RuBisCO small-subunit (SSU)

promoter with the primary objective of reducing the problems of increased seed dormancy (*cf*. Chapter 6). The rbcS-10 seeds exhibited a slightly less uniform germination pattern than WT and sp12, although 87.5 % germination was achieved 12 days after imbibition.

9.4.2 Norflurazon application preliminary trial (sp5)

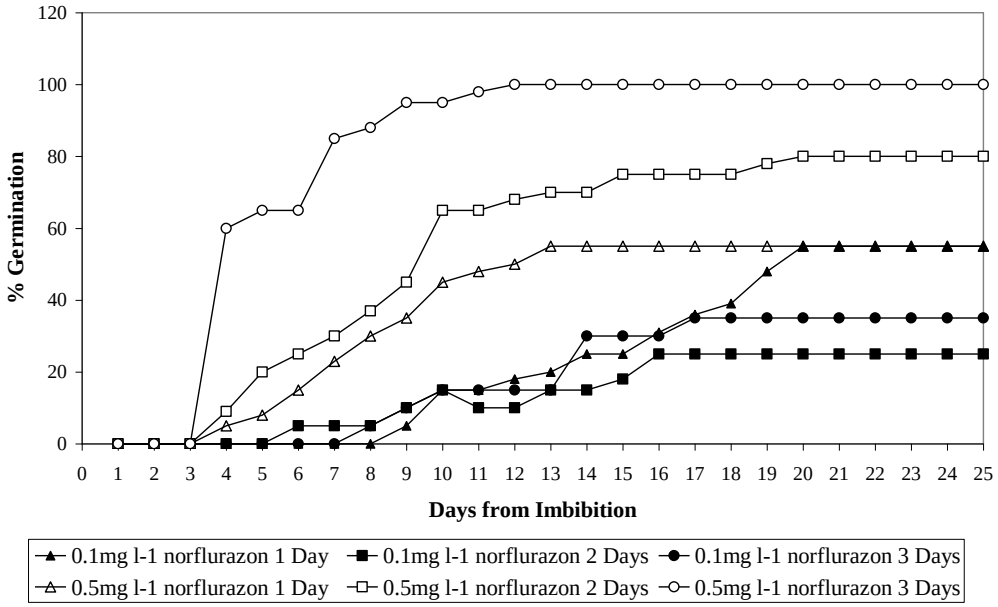


Figure 9.3. Percentage germination of sp5 seeds treated with 0.5 or 0.1 mg l^{-1} norflurazon for 1, 2 or 3 days (n=20).

The sp5 seeds treated with 0.1 mg l^{-1} norflurazon showed a slight increase in germination rate but overall percentage germination in the two and 3 day treatments was not increased greatly relative to seed which had not been treated with norflurazon (Fig. 9.2; 25 and 35 % respectively). Surprisingly, when 0.1 mg l^{-1}, norflurazon was used, the one day duration treatment showed the greatest increase in germination, to a total of 55 %, 20 days after imbibition. By contrast, the 0.5 mg l^{-1} treatments produce significant increases in total percentage germination and the values increased with the duration of treatment. Treatment with 0.5 mg l^{-1} norflurazon for 1, 2 and 3 days achieved 55, 80 and 100 % germination respectively. Whilst application of the higher concentration of norflurazon for the longest duration improved uniformity and achieved 100% germination after 12 days, the cotyledons emerging from the testa were visibly bleached, as was also the case for the 0.5 mg l^{-1} norflurazon in the two day treatment, albeit to a lesser extent.

9.4.3 Norflurazon application trial (WT, sp5 and G28)

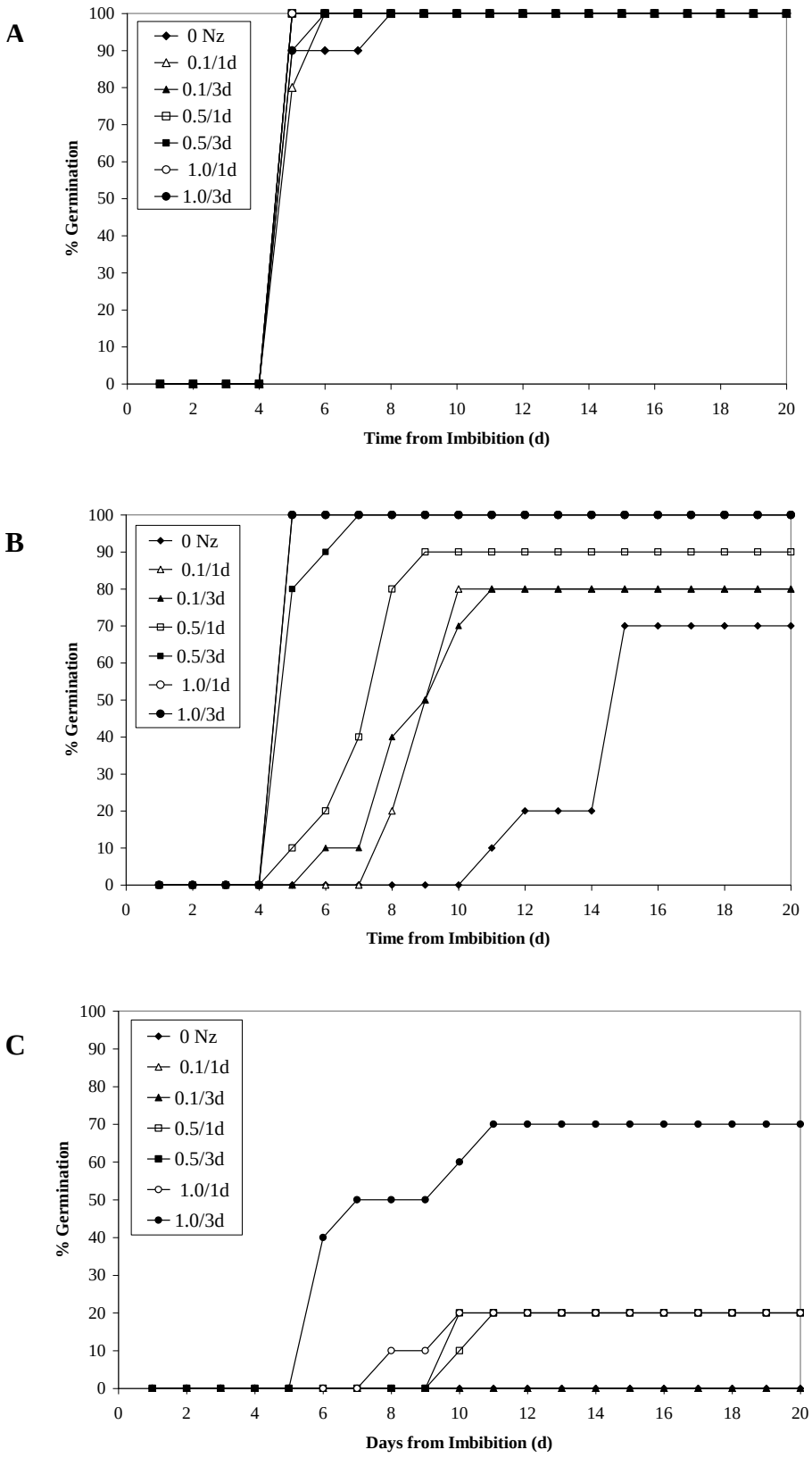


Figure 9.4. Timecourses of germination for **A**: WT, **B**: sp5 and **C**: G28 seeds, under various norflurazon (Nz) treatments (n = 10).

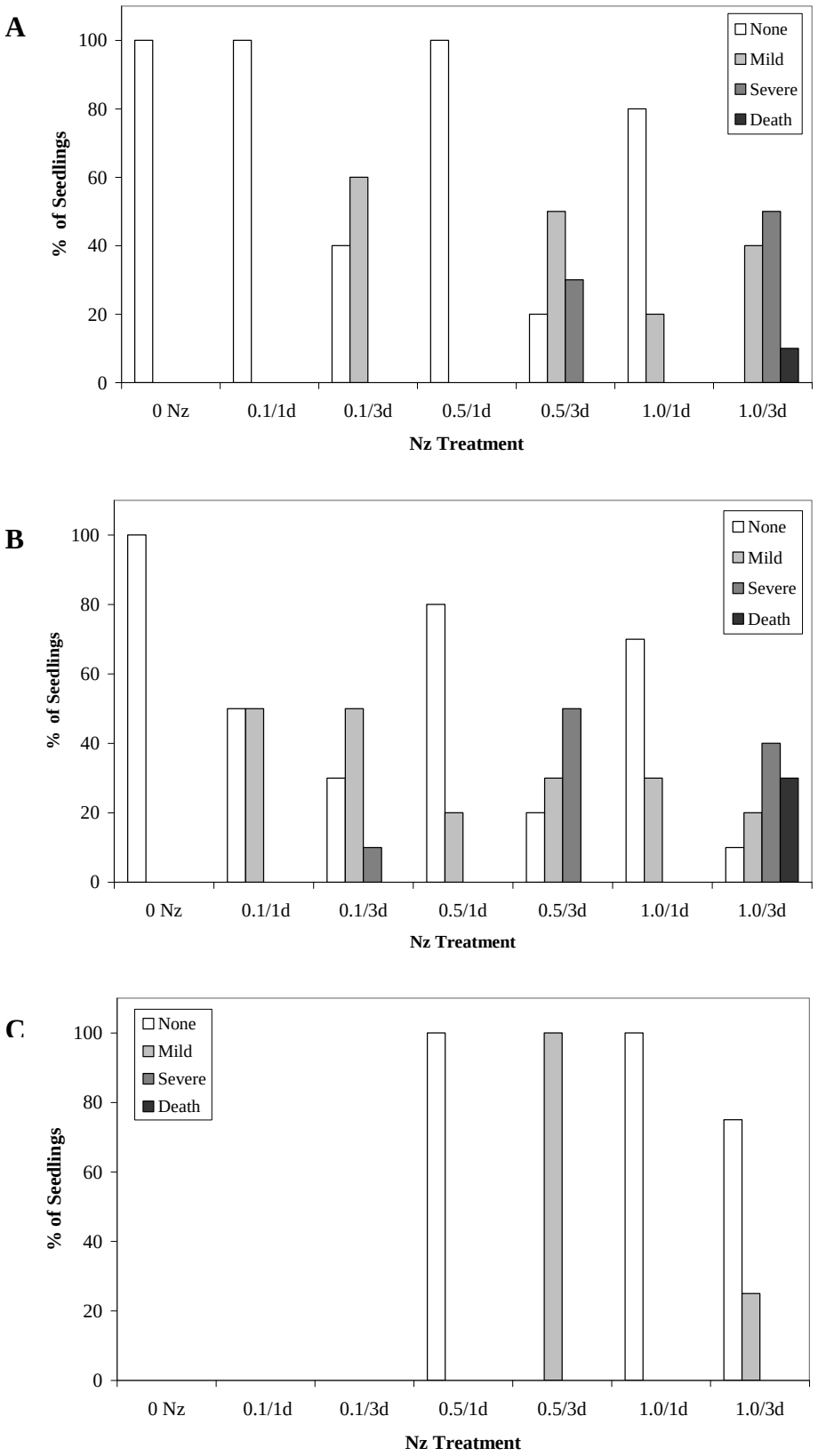


Figure 9.5. Level of photobleaching and necrosis in newly emerged cotyledons of **A**: WT, **B**: sp5 and **C**: G28 seedlings, shown as a % of total seedling number in each treatment x genotype combination (n = 10). When no data are shown, treatment with Nz failed to induce germination.

9.4.3.1 Germination rate and uniformity

WT seeds germinated quickly and uniformly, with 100% germination being achieved in all treatments by Day 8 (Fig. 9.4). With such rapid germination without the application of norflurazon, there was therefore no marked effect of any of the norflurazon treatments. The sp5 seeds reached 70% germination by day 15, which is greater than in the initial germination trial (Fig. 9.2). Unlike the WT seed, treatment with norflurazon increased the speed and uniformity of germination of sp5 seed. The 1.0 mg l^{-1} norflurazon treatment induced 100% germination within five days of imbibition, and no difference was found between the 1 and 3 day treatment durations as all seeds germinated by day 5. Although the 0.5 mg l^{-1} treatments showed slightly less uniformity in the breaking of dormancy, by days 7 and 9, 90 and 100% germination was achieved in the 1 and 3 day treatments respectively.

The 'double transgene' G28 seed showed no germination in the absence of norflurazon; indeed, neither of the 0.1 mg l^{-1} treatments was sufficient to break the strong dormancy characteristic of this line. When seeds were imbibed in the 0.1 mg l^{-1} norflurazon for one or three days, only 20 % total germination was achieved after 11 and 10 days respectively. The only treatment that had any potential to provide uniform germination of these severely dormant G28 seeds was the 1.0 mg l^{-1} for three days treatment, which produced 70 % total germination by day 11.

9.4.3.2 Cotyledon Bleaching

Increasing the concentration and duration of norflurazon treatment generally induced more severe bleaching and in some cases caused the death of emerging seedlings of all three genotypes. However, the severity of this effect differed between genotypes since WT and sp5 seedlings showed greater sensitivity to the norflurazon, with the 0.1 mg l^{-1} treatments causing mild bleaching, even though this concentration of norflurazon was not strong enough to break the dormancy of G28 seeds. Duration of treatment appeared to have a greater effect than norflurazon concentration; for example, treatment with 0.1 mg l^{-1} for three days caused more severe bleaching than treatment with 0.5 mg l^{-1} for 1 day in both WT and sp5. The highest dosage of norflurazon (1.0 mg l^{-1} for three days) induced seedling death in WT and sp5 (Figs. 9.5 A and B respectively), although this treatment only caused mild bleaching in 20 % of the seedlings in the G28 genotype (Fig. 9.5C).

9.4.4 Seedling Establishment Phase Growth Trial (WT, BCH12, sp12, sp5, G28, G29)

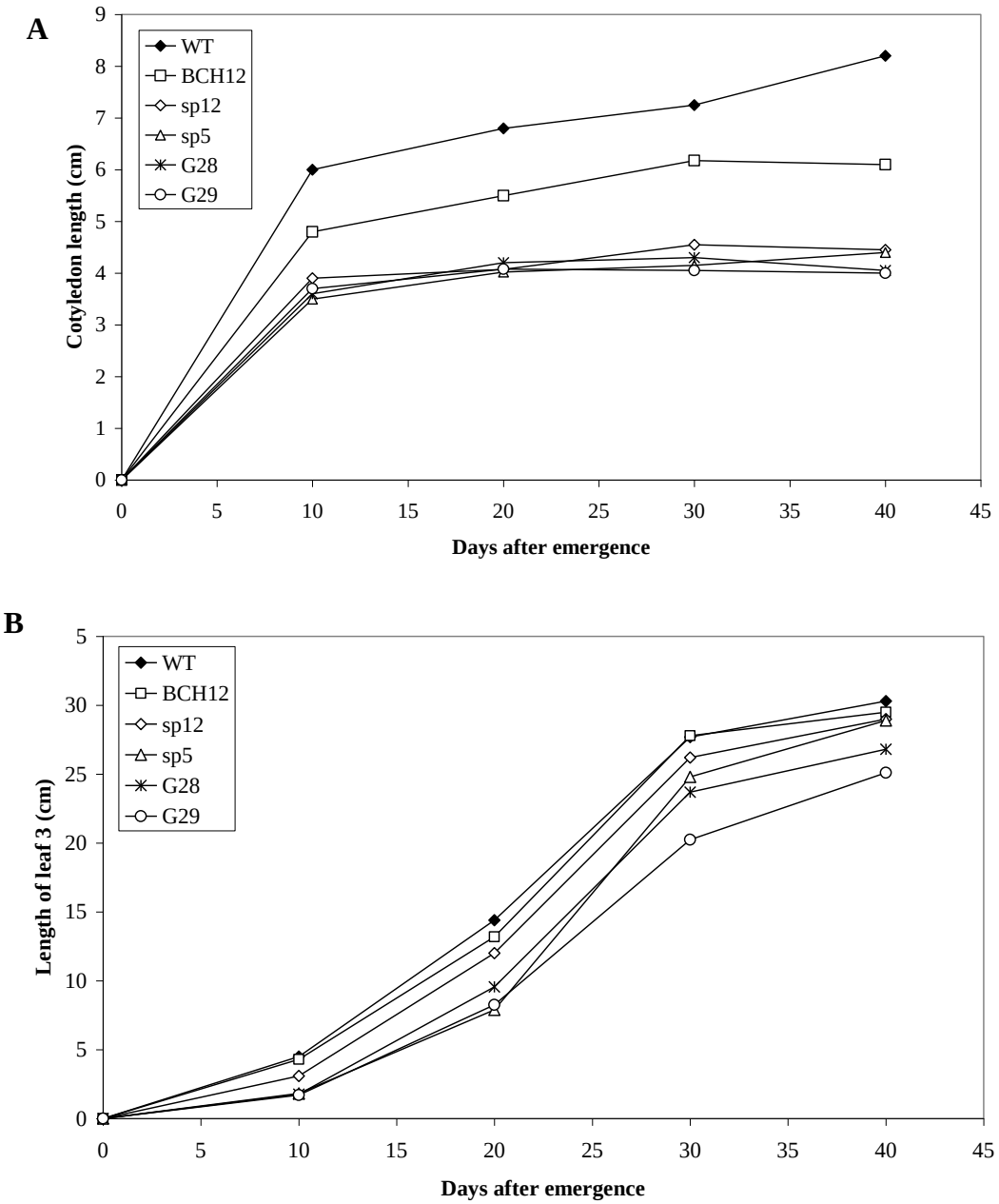


Figure 9.6. **A:** Cotyledon length and **B:** the length of leaf 3 at 10, 20, 30 and 40 days after emergence (DAE). See Table 9.2 for statistical summary.

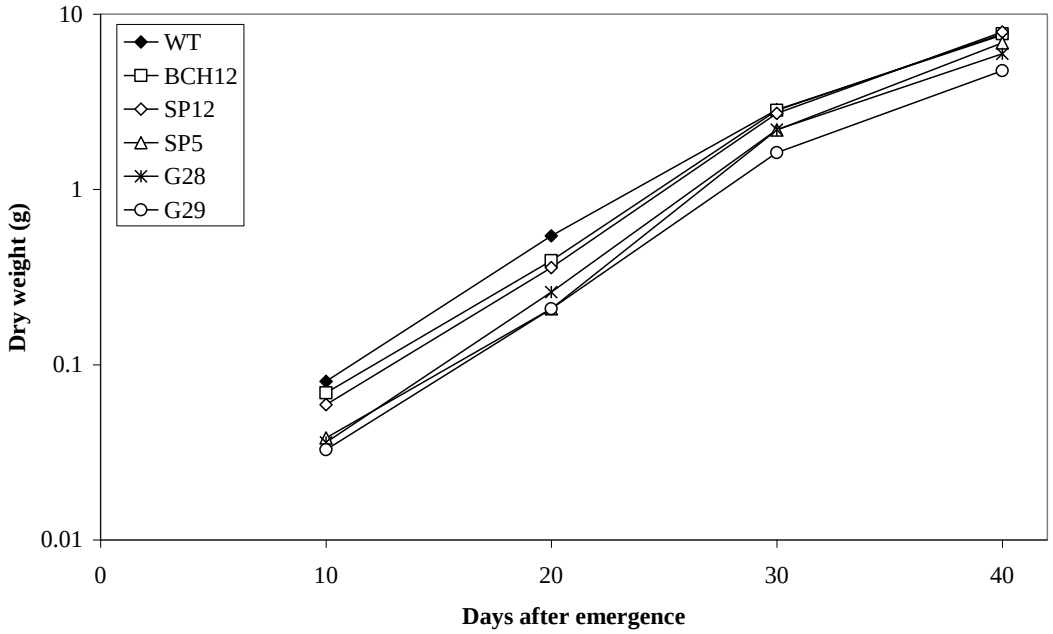


Figure 9.7. Shoot dry weight at 10, 20, 30 and 40 days after emergence (DAE). Dry weight data is plotted on a log scale. See Table 9.2 for statistical summary.

Table 9.2. Statistical summary to accompany Figures 9.6 and 9.7 (n=10).

	Cotyledon Length		**Length of leaf 3**		**Dry weight**	
	Fpr.	LSD (5%)	*Fpr.*	LSD (5%)	*Fpr.*	LSD (5%)
10 DAE	*<0.001*	0.456	*<0.001*	0.876	*<0.001*	0.013
20 DAE	*<0.001*	0.411	*<0.001*	2.097	*<0.001*	0.073
30 DAE	*<0.001*	0.442	*<0.001*	3.030	*<0.001*	0.297
40 DAE	*<0.001*	0.421	*<0.001*	1.731	*<0.001*	0.496

9.4.4.1 Cotyledon length

At all four harvest dates (10, 20 30 and 40 DAE), the cotyledon length was significantly longer for WT plants than for all 'high ABA' lines ($P<0.001$; Fig. 9.6A). Cotyledon length was also consistently greater for BCH12 seedlings than for the other 'high ABA' lines (sp12, sp5, G28 and G29). There was little variation in cotyledon length between sp12, sp5, G28 and G29 plants at all harvests, and no significant difference was detected between the four lines ($P>0.05$). At 10 DAE, WT plants had a mean cotyledon length of 6 cm compared to 4.8 cm for BCH12 and a combined mean of 3.6 cm for the other 'high ABA' lines. At 40 DAE, the mean cotyledon length of WT was 8.2 cm, compared to 6.1 cm for BCH12 and a combined

mean of 4.2 cm for the other 'high ABA' lines. There was therefore a marked difference in cotyledon expansion rate between WT and BCH12 and the other 'high ABA' lines. Whilst the mean length of WT cotyledons increased by 2.2 cm over the 30 days between the first (10 DAE) and last (40 DAE) harvests, the corresponding increase for BCH12 plants was 1.3 cm and the combined mean for the other 'high ABA' lines was only 0.6 cm.

9.4.4.2 Leaf Length

The length of leaf 3 is shown (Fig. 9.6B, log scale) as this was the youngest leaf present in all plants at all harvests (i.e. by 10 DAE some plants had not yet produced leaf 4). At 10 DAE, mean leaf length was greater in WT and BCH12 than in all other lines ($P<0.001$). sp12 plants had significantly longer leaves than sp5, G28 and G29 ($P<0.001$), but there no significant difference was detected between the latter three lines. At 20 DAE, WT plants still had significantly greater leaf length than the 'high ABA' lines ($P<0.001$), but there was no longer a significant difference between sp12 and BCH12. By 30 DAE, mean leaf length in WT plants was greater than the corresponding values for G28 and G29 ($P<0.001$), but did not differ significantly from the other lines; leaf length in G29 was significantly smaller than for all other lines. Leaf length again did not differ significantly between WT, BCH12, sp12 and sp5 at 40 DAE, for which the values were greater than for the G28 and G29 lines ($P<0.001$).

9.4.4.3 Shoot Biomass Production

After 10 and 20 days of growth (10 and 20 DAE), the quantity of biomass produced varied greatly between lines, and mean dry weight (DW) was greatest in WT plants ($P<0.001$; Fig. 9.7). Mean DW was greater in BCH12 than in sp5, sp12, G28 and G29 plants ($P<0.001$), while the values for sp12 were greater than for sp5, G28 and G29 ($P<0.001$). At 30 DAE, there was no detectable difference between WT, BCH12 and sp12 plants, and the values for all three lines were greater than for sp5, G28 and G29 plants ($P<0.001$). At final harvest (40 DAE), there was no significant difference between the WT, BCH12, sp12 and sp5 lines, indicating that the biomass of sp5 and sp12 plants had increased over the course of the four successive harvests

to become equivalent to that of WT plants. However, the DW values for all four lines were still greater than those for both G28 and G29 ($P<0.001$); biomass was greater for G28 than for G29 ($P<0.001$).

9.5 DISCUSSION

9.5.1 Transgenic 'high ABA' seeds have increased dormancy

ABA is essential for the induction of seed dormancy (King *et al*., 1976) and studies using ABA deficient tomato mutants have shown that low seed dormancy is correlated with low levels of ABA within the seed (e.g. Groot and Karassen, 1992). Seed from plants which over-express an ABA biosynthetic gene often display increased dormancy. For example, Frey *et al*. (1999) reported that *N. plumbaginifolia* plants over-expressing *NpZEP* (driven by the CaMV 35s promoter) showed a three day increase in dormancy; when the same species was transformed with construct containing the *PvNCED1* gene, plants exhibited a four day delay in germination (Qin and Zeevaart, 2002). In the 'high ABA' lines described here, the extent of the increase in seed dormancy was correlated with the level of ABA over-production.

Seed of BCH12, which over-expresses *LeBCH2* alone, showed germination characteristics which did not differ significantly from WT control seed, whereas the 'sp' lines, which over-express *LeNCED1*, showed increased dormancy relative to the WT control. The extent of the increase in dormancy differed greatly between the two 'sp' lines, with the milder sp12 showing only a five day increase in the mean time required for germination compared to sp5, which only achieved 25% germination 14 days after 100% germination had occurred in WT. This is consistent with Thompson *et al*. (2000), who reported that the transgenic sp lines exhibited increased dormancy relative to WT seed, with this phenotype being far more extreme in sp5. The observation that over-expression of *LeNCED1* has a greater impact on seed dormancy than over-expression of *LeBCH2* is to be expected on the basis that NCED is a major rate-limiting step in the ABA biosynthetic pathway (Qin and Zeevaart, 1999; Thompson *et al*., 2000).

The germination characteristics of the double gene 'high ABA' genotypes are of particular interest in the present study. These lines display simultaneous over-expression of both *LeBCH2* and *LeNCED1* transgenes and were created by crossing the homozygous *LeBCH2* over-expressing line (BCH12) with the two homozygous sp lines (sp12 and sp5) to eventually create the double homozygous lines G28 (BCH12/sp12) and G29 (BCH12/sp5). The combined over-expression of both ABA biosynthetic genes provided further increases in transpiration efficiency (TE_p) compared to the parental lines (BCH12, sp12 and sp5), and this effect appeared to be additive (*cf.* Chapter 7). However, the effect appeared to be more than additive for seed germination, as neither 'double transgene' line showed any germination under normal germination conditions i.e. when imbibed with water. This represents a dramatic increase in seed dormancy relative to the parental lines; for example, when considering G28 seed, parental line BCH12 shows no increase in dormancy and the increase for sp12 seed is minimal, yet the severity of dormancy in G28 seed means that it does not germinate in the absence of treatment with norflurazon. Analysis of sp12 and G28 seed has established that sp12 seed has greater ABA concentration than that of WT and BCH12, whilst G28 seed has a further, significant increase in seed ABA content in comparison with sp12. Both sp12 and G28 seed also contain greater levels of DPA (an ABA catabolite; *cf.* Section 1.2.3.5), than both WT and BCH12 seed (Sonneveld *et al*. in prep.) The effect of simultaneously over-expressing both *LeBCH2* and *LeNCED1* transgenes appears to have a synergistic effect on seed dormancy (*cf.* Chapter 10 for further discussion).

9.5.2 Development of Sowing Protocols

Within this study, all long-term growth and gravimetric experiments required synchronisation of plant size and leaf area at the beginning of the experiments to ensure that any differences in water use or growth rate could be attributed to true treatment/line effects rather than germination of establishment effects. As described above, seed from the 'high ABA' lines exhibit differing germination times and uniformity, as well as differing rates of growth during the early establishment phase. For this reason, the experiments described here were used to develop two sets of sowing protocols to allow synchronisation of: (1) seedling emergence; and (2) plant

size at the four-leaf-stage, for all five transgenic ABA over-producing lines relative to WT controls (*cf*. Chapter 3 for full list of protocols).

ABA concentration within cells is maintained dynamically in an equilibrium of constant synthesis and degradation (Cutler and Krochko, 1999), and *de novo* seed ABA synthesis is required for the maintenance of dormancy (Debeaujon *et al*., 2000). Gas chromatography-mass spectrometry revealed that ABA concentration in the seed of both sp lines (sp12 and sp5) was threefold greater than in WT seeds (Thompson *et al*., 2000). Therefore, if ABA synthesis in the 'high ABA' seed is blocked, the net ABA concentration will eventually fall to levels where germination is no longer inhibited.

During the trials described here, the influence of the concentration and duration of the norflurazon treatment was examined to determine optimal conditions for each 'high ABA' line which exhibited severely increased seed dormancy and therefore required norflurazon treatment to allow quick and uniform germination. However, as a herbicide, norflurazon also causes photobleaching of emerging cotyledons when seeds are treated at high concentrations or for prolonged periods. Carotenoids are important in dissipating excess energy during photosynthesis, and carotenoid deficiency resulting from norflurazon application leads to the degradation of chlorophyll by highly oxidising singlet oxygen molecules. This causes the emerging cotyledons to assume a bleached appearance due to lack of chlorophyll. As the cotyledons are the primary photosynthetic organs during early seedling growth (Hussey, 1962), seedlings may be unable to photosynthesise effectively when bleaching is severe and therefore die once the energy reserves within the seed are exhausted. It is therefore imperative that the norflurazon treatment is sufficient to break dormancy but not so severe that it causes the seedlings die.

The highest dose of norflurazon caused severe bleaching and some seedling death in both the WT and sp5 lines (Fig. 9.5), whereas application of the same treatment induced only mild bleaching of some cotyledons in the G28 genotype. In general, WT and sp5 seedlings showed greater sensitivity to norflurazon, whereas G28 seeds required the most extreme treatment to initiate any level of germination. This effect may have occurred because the timing of germination affected the duration of the

period when metabolically active seeds were exposed to the norflurazon. WT and sp5 seeds germinated more rapidly than those of G28 in the high norflurazon treatment, and so would have been exposed as metabolically active seeds for longer. Alternatively, activity of phytoene desaturase could have been greater in G28 seeds, meaning a higher dose of norflurazon is required to inhibit it.

The development of a new sowing method was also important in providing reliable seedling establishment for the 'high ABA' lines. Tung *et al*. (2008) reported that the 'ultra high' ABA rbcS plants exhibited a reduced ability for expanding cotyledons to escape from the testa, termed the 'lollipop' phenotype. This phenotype, whereby the radicle emerges unrestricted but the cotyledons do not emerge from the testa, has also been observed to a lesser extent when seeds of sp genotypes were germinated in petri dishes (Symonds, 2002; Tung *et al*., 2008). Reciprocal crosses involving both rbcS (Tung *et al*., 2008) and sp12 (Symonds, 2002) lines demonstrate that additional ABA production in the embryo is responsible for this phenotype, rather than the maternal parent, while the treatments involving applications of norflurazon demonstrate that increased seed-sourced ABA biosynthesis prior to imbibition is the cause of this phenotype (Tung *et al*., 2008). It has been proposed that transgenic seeds with increased ABA biosynthesis may experience increased resistance to germination from the testa (Frey *et al*., 1999). This theory is supported by the observation that the testa of the ABA deficient, tomato mutant, *sitiens*, is thinner than in WT seeds (Hilhorst and Downie, 1996).

The increased toughness of the testa in 'high ABA' seeds often makes it necessary to soften the testa with droplets of water to facilitate its loss when seeds are germinated in Petri dishes. For this reason, an alternative direct sowing method was included in the second norflurazon trial involving the WT, sp5 and G28 lines, in which seeds were sown directly into compost following norflurazon treatment; all pots were covered with 9 cm Petri dish lids to maintain high humidity in the air space above the compost, with the aim of softening of the testa to aid emergence of the expanding cotyledons. The lids were removed following emergence of the hypocotyl hook to avoid hindering development and allow the cotyledons to emerge in normally. This approach successfully eradicated the 'lollipop' phenotype in all 'high ABA' lines and was therefore included in the sowing protocol (*cf*. Chapter 3).

9.5.3 Seedling establishment and early growth of 'high ABA' plants

When germination of all 'high ABA' lines was synchronised, plants exhibited slower growth rates during the establishment phase in all 'high ABA' lines; however, as with the increased seed dormancy phenotype, the extent of this effect and time taken to overcome it depended on the level of ABA over-production. BCH12 plants had a smaller biomass than WT controls after 10 days of growth but then recovered to attain with WT values by 20 DAE (Fig. 9.7). A similar effect was found for sp12 (the milder sp line) plants, which were slower to establish during the first 20 days after germination but then exhibited increased growth rates between 20 and 30 DAE to achieve a similar biomass to WT plants by 30 DAE. Lines sp5 and G28 took slightly longer to recover, but again achieved biomass accumulation that was not significantly to WT plants by 40 DAE. G29 was the only line in which biomass was significantly reduced at the end of the experimental period. The results for leaf length confirm this effect of ABA over-production on early growth as all 'high ABA' lines had shorter leaves at the beginning of the experiment and the time required to recover to WT values depended on the degree of ABA over-production. The data reported here therefore suggest that, whilst 'high ABA' plants are initially slightly slower to establish, their relative growth rates later exceeded those of WT plants, thereby achieving parity of size by 40 DAE.

Of particular interest was the difference in cotyledon length between WT and 'high ABA' lines (Fig 9.6). Genotypes which over-produced ABA had significantly smaller cotyledons than WT controls and, whilst the cotyledons of WT seedlings continued to expand throughout the experimental period, those of 'high ABA' genotypes did not, with the exception of the mild BCH12 line. Cotyledons are the primary photosynthetic organ during the early stages of seedling growth (Hussey, 1962), and it is well documented that damage to the cotyledons may adversely affect seedling development. Reductions in growth resulting from damage to the cotyledons by herbivory have been shown to have negative implications for competitive ability during the establishment phase and reproductive success at maturity (Hanley and May, 2006). In species with leaf-like cotyledons, the cotyledons undergo rapid growth following emergence due to increases in cell

number and size. Rapid cotyledon expansion is associated with the production of chlorophyll, while the presence of functional stomata facilitates the uptake of CO_2 for photosynthesis (Lovell and Moore, 1970). Bisognin *et al*. (2005) demonstrated that cucumber seedlings only depend on seedling reserves for pre-emergence growth and further seedling development depends on the photosynthetic capacity of the cotyledons until the first true leaf is completely unfolded. Emergence of fully viable cotyledons is therefore crucial to the establishment of tomato seedlings.

It is possible that damage to the expanding cotyledons of 'high ABA' seedlings caused by increased testa toughness (*cf.* Section 9.5.2) may reduce photosynthetic capacity, so reducing their growth until the first true leaves expand. Alternatively, increased ABA biosynthesis may directly affect cotyledon expansion and early growth. Lopez-Molina *et al*. (2001) reported that ABA may reversibly arrest the development of embryos in *Arabidopsis* during a narrow developmental time interval between germination and the onset of vegetative growth. This has been proposed as a developmental checkpoint which enables plants to monitor water status within their immediate environment, thereby protecting them from drought (Lopez-Molina *et al*., 2001).

As mentioned previously, reliable and uniform seed germination and seedling establishment are important characteristics when considering the production of commercial tomato crops. The work described here has demonstrated that, whilst germination of 'high ABA' transgenic lines may require treatment with herbicide to break dormancy, reliable seed germination can be achieved once the optimal dosage has been determined. 'High ABA' seedlings were initially slower to establish but, when this first phase of growth was completed, growth rates then increased to match WT plants. Commercial production of 'high ABA' plants would therefore require adjustment to the sowing protocol and schedules normally used to produce tomato plants for transfer to glasshouse/field production systems.

10 GENERAL DISCUSSION

10.1 Summary of the key findings in this research programme

The phytohormone abscisic acid (ABA) is a fundamental component of plants response to water stress. ABA plays a major part in the control of transpiration *via* a reduction in stomatal aperture (Zeevaart and Creelman, 1988; Borel *et al*., 2001). It is possible to mimic the effect of mild water stress by long-term exposure to elevated internal ABA concentrations, thus increasing water use efficiency (WUE; Wong *et al*., 1979; Bradford *et al*., 1983). It was suggested that by using tomato lines that are pre-adapted to water stress *via* increased ABA biosynthesis, improvements in whole plant WUE could be achieved. ABA is synthesised via the oxidative cleavage of C_{40} epoxycarotenoid precursors, a reaction catalysed by a key rate limiting enzyme NCED (Thompson *et al*., 2000a). Over-expression of *LeNCED1* driven by the Gelvin 'super-promoter' led to the production of stable transgenic lines with increased bulk leaf ABA concentrations (Thompson *et al*., 2000a). This research project examined the implications of long-term elevation of ABA in these and other 'high ABA' lines.

One initial objective was to of identify suitable irrigation regimes under which any differences in WUE and productivity between 'high ABA' and WT plants could be clearly revealed. This was addressed using several techniques; including progressive drought, demand-led and gravimetric methods of determining irrigation levels. Initial trials, performed under sub-optimal and optimal irrigation levels, demonstrated that the mildest 'high ABA' line (sp12) exhibited more restrained use of water than corresponding WT controls. As these plants continuously generate a water stress signal, they are not as profligate in their water use as WT plants, thus conserving soil moisture. This meant that under progressive drought conditions, growth rates could be maintained for longer. The sp12 plants produced a significantly larger amount of biomass than WT plants when irrigation was sub-optimal and maintained similar growth rates to WT plants that received double the quantity of irrigation water (Fig. 4.4). It was concluded that due to the elevated level of bulk leaf ABA exhibited by the *LeNCED1* over-producing, 'high ABA' plants (Thompson *et al*., 2000a),

stomatal behaviour was affected, limiting stomatal conductance in conditions where water supply is plentiful, when WT plants appear to be exhibiting super-optimal stomatal opening. Despite maintaining growth and avoiding plant water deficits for longer, the 'high ABA' plants subjected to this irrigation regime did eventually experience plant water deficit and in these circumstances behaved as WT plants, accumulating similar levels of xylem sap ABA, albeit starting from different basal levels (Thompson *et al*., 2007a). Whilst 'high ABA' plants outperform WT controls when water is limited, the key finding of the initial trials was their restricted maximal stomatal opening in well-watered conditions, which allowed water to be 'saved up' for its later use (Fig. 4.7A and 4.10A).

As transpiration rates are positively correlated with crop yield (Boyer, 1982), it has often been assumed that long-term restriction of stomatal opening would excessively limit photosynthesis and therefore growth rates (Condon *et al*., 2004; Jones, 2004). In spite of this prediction, it was observed that under both optimal and suboptimal irrigation conditions during the initial growth trials, the sp12 plants maintained growth rates at least equal to those of WT plants (*cf*. Chapter 4). In these initial experiments, a simple trickle irrigation system was used and consequently there was no way of accurately recording the total amount of water transpired by individual plants. Later on in the research programme a gravimetric method of determining whole plant transpiration efficiency (TE_p) was utilised, to accurately asses any difference in growth rate and water use between WT and 'high ABA' plants.

The over-expression of *LeNCED1* alone was found to provide a significant increase in TE_p but when the over-expression of *LeNCED1* and *LeBCH2* were combined a further improvement was clearly observed. The two constructs simultaneously expressed in these plants did not lead to any negative impact on biomass accumulation, suggesting that a sufficiently high level of ABA had not yet been reached to reduce assimilation rate to an extent that long-term biomass production was limited (*cf*. Section 10.4.1.). When over-expression of *LeNCED1* was driven by an alternative tissue-specific/strongly light responsive promoter (rbcS), this resulted in lines with 'ultra-high' ABA over-production (Tung *et al*., 2008). These plants exhibited severe phenotypes presumably as a result of their 'ultra high' level of ABA over-production (*cf.* Section 10.2).

The potential use of 'high ABA' plants as rootstocks grafted onto WT scions, in order to potentially improve whole plant WUE was evaluated. It was found that the 'double transgene' line (G29), over-expressing *LeNCED1* and *LeBCH2*, did not provide a strong enough root-sourced ABA signal to affect scion stomatal behaviour and therefore whole plant WUE. It has been pointed out that in comparison with leaf ABA biosynthesis, alongside NCED, enzymes catalysing other steps in the biosynthetic pathway are more limiting to root-sourced ABA production (Thompson *et al*., 2007b). Roots have drastically reduced carotenoid levels in comparison with shoots (Parry and Horgan, 1992; Fraser *et al*., 1994) and C_{40} carotenoids are required as precursors for the synthesis of ABA (Taylor *et al*., 2005). The colourless compound phytoene is the first C_{40} carotenoid in the pathway and its formation is catalysed by phytoene synthase (PSY) (Bartley *et al*., 1992). To increase the flux of carotenoid/xanthophyll precursors through the pathway the upregulation of *PSY* was combined with the previously upregulated *NCED* and *BCH* genes, with the objective of producing a rootstock that would produce sufficient root-synthesised ABA to have an effect on stomatal behaviour of WT scions. Unfortunately, at the time of writing, the success or otherwise of this 'three transgene root-sourced approach is still uncertain, as this part of the research programme is still ongoing.

10.2 Implications of ABA over-production on plant growth and development

Alongside the possibility that the growth of 'high ABA' plants would be impaired due to a limited assimilation rate as a consequence of reduced stomatal apertures, the possibility that long-term increases in basal ABA content i.e. resting (non-stress levels) would have a direct negative affect on plant growth and development must also be considered. As discussed in Sections 1.2.2.2 and 4.1.1.1, the effect of ABA on plant growth has been a topic of much controversy. Many earlier experiments reported ABA to be an inhibitor of growth (Quarrie and Jones, 1977; Creelman *et al.*, 1990). However analysis of ABA interactions led to the suggestion that ABA appears to have a role in promoting leaf growth. This could occur either through its antagonistic interaction with ethylene which restricts leaf

growth (Sharp *et al*., 2000; Sharpe, and LeNoble, 2002) or *via* an alternative, ethylene-independent mechanism (LeNoble *et al*., 2004; Dodd *et al*., 2009).

Analysis of the growth of 'high ABA' and 'ultra-high ABA' plants in this research programme did not give a totally clear cut or simple picture. Despite suggestions that biomass production might have been expected to be inhibited in 'high ABA' plants, the data obtained from every experiment in which long-term biomass production of mature plants was measured, demonstrated that the transgenic plants consistently exhibited growth that equalled or even exceeded that of WT controls. However, this was clearly not the case with the 'ultra high' ABA lines (Tung *et al*., 2008). Nevertheless, growth of the 'high ABA' plants during the early establishment phase i.e. the first month was shown to be significantly reduced. It should be emphasised that this is only a temporary effect as the data reported in Chapter 9 (Fig. 9.7) demonstrated that whilst 'high ABA' plants were initially slightly slower to establish, their relative growth rates later typically exceed those of WT plants, in order to catch up and be of equal size after about 40 days of growth. In this context it should be pointed out that 'high ABA' plants had reduced cotyledon lengths, with reduced expansion rates. This is important because cotyledons are the main photosynthetic organs during the period immediately after seedling emergence (Hussey, 1962). It is possible that the reduction in photosynthetic area associated with the cotyledons could be mainly responsible for the slower initial growth rates of 'high ABA' plants (Hanley and May, 2006). It is possible that increased ABA levels could have a direct inhibitory effect on cotyledon expansion and early seedling growth (Lopez-Molina *et al*., 2001).

It has been reported that plants from the 'high ABA' sp5 line, exhibit longer petioles and reduced leaf epinasty (Thompson *et al*., 2007a). Data from the progressive drought trials (Chapter 4) support this, with all 'high ABA' lines having significantly longer leaves than WT controls (Table 4.1). When grown under well watered conditions sp12, sp5 and G28 plants also had significantly greater leaf lengths than WT controls, however this effect was not seen in G29 plants, despite the fact that they over-produce ABA to a greater extent than sp5 and are also derived from the sp5 line (sp5 x BCH12). As G29 plants over-produce ABA to a greater extent than lines sp12, sp5 and G28, this could conceivably be a threshold effect by which the

level of ABA over-production in G29 plants has exceeded that in which leaf growth is promoted and that the level of ABA is nearer optimal for promotion of leaf growth in sp5 plants.

Experiments using ABA-deficient mutants have demonstrated that ABA has an important role in restricting ethylene evolution, which is believed to have an inhibitory effect on growth (Sharp and LeNoble, 2002). When tomato plants were grown under a PRD system, leaf growth inhibition was correlated with increased ethylene evolution, consistent with the idea that ethylene was limiting leaf growth (Sobeih *et al*., 2004). Stronger evidence provided by the same study, came from transgenic plants that have low stress induced ethylene production which exhibited no increase in ethylene evolution in response to PRD treatment and also did not exhibit leaf growth inhibition. ABA and ethylene have an antagonistic relationship; with ABA-deficient mutants producing more ethylene (Tal *et al*., 1979) and therefore exhibit some morphological symptoms characteristic of excess ethylene (Tal, 1966). The stunted growth of ABA-deficient maize and tomato plants is believed to be partly due to excess ethylene production, which is normally inhibited by endogenous ABA production, rather than being caused only by abnormal water relations (Sharp *et al*., 2000). It is possible that in sub-optimal irrigation conditions (e.g. during the progressive drought experiment), the increased ABA content in 'high ABA' plants acts to further reduce stress-induced ethylene production, in comparison with WT plants, thus maintaining leaf growth. However, it has also been reported that by using grafting techniques involving *flc* scions and WT rootstocks, it can be shown that ABA can have an ethylene-independent effect, promoting leaf growth directly (Dodd *et al*., 2009).

Increased ABA accumulation in 'high ABA' lines may also help maintain plant water status, in conditions where WT plants may exhibit deficits, thus increasing turgor driven growth. As mentioned previously, 'high ABA' plants have been shown to be more sensitive to VPD and may show greater restriction in the extent of stomatal opening during the midday period when VPD is greatest and WT plants could exhibit reductions in plant water status. When under stress, plants can control plant water status by increasing root water availability/uptake, through root growth (Sharp *et al*., 2002) or by increased root hydraulic conductivity (Lp_r; Kaldenhoff *et*

al., 2008) and this is often correlated with increased root ABA concentration (Vysotskaya *et al*., 2004). The application of exogenous ABA to root systems increased hydraulic conductivity (Hose, 2000; Sauter *et al*., 2002) and the hydraulic conductance of roots is partly regulated by ABA-induced changes in the activity and abundance of aquaporins (Hose *et al*., 2000; Seimens and Zwiazek, 2004). Transgenic 'high ABA' lines exhibit increased Lp_r and it has been suggested that the increased conductance of the root system could help maintain water supplies to the shoot, in conditions of high evaporative demand, thus having a positive effect of shoot water status and also facilitating turgor driven growth (Thompson *et al*., 2007a).

Plants that constitutively over-produce ABA exhibit increased seed dormancy. This could have been predicted, as ABA is known to play a key role in controlling dormancy (King *et al*., 1976; Groot *et al*., 1991; Finkestein *et al*., 2002; Kermode, 2005). The severity of ABA induced dormancy was shown to increase with the extent of ABA over-production in 'high ABA' lines, with the mildest line (sp12) exhibiting germination that was delayed by 5 days and the most extreme 'double transgene' (G29) exhibiting no germination without the application of norflurazon, a herbicide that blocks carotenoid and therefore ABA biosynthesis (Smith, 1997). This represents a drastic alteration of phenotype from the non-dormant WT seed to highly dormant G29 line. Whereas in an already dormant species, e.g. *N.plumbaginifolia*, over-expression of ABA biosynthetic genes has often been shown to delay germination for only a few days (Frey *et al*., 1999; Qin and Zeevaart, 2002).

Whilst the problem of prolonged seed dormancy in 'high ABA' plants can be overcome *via* the use of norflurazon (Thompson *et al*., 2000a; Chapter 9), prolonged application of norflurazon can result in photobleaching of the cotyledons, which in severe cases caused seedling death (Figs. 9.4 and 9.5). The 'double transgene' lines, which provided the greatest gains in TE_p, required the greatest dose of norflurazon, however, they appeared to be less sensitivity to the norflurazon than 'WT and 'single transgene' lines. This is possibly because WT and 'single transgene' lines became metabolically active earlier and so were exposed to norflurazon in a metabolically active state for longer. Through detailed development of sowing protocols specifically tailored for each line (*cf*. Table 3.1), reliable germination and seedling

establishment can be achieved and due to the effect described above, the greater dosage requirement of 'double transgene' lines does not necessarily result in any greater occurrence of cotyledon bleaching. If 'high ABA' plants were to be grown commercially or on a large scale, it would require adjustment of current techniques to propagate young plants ready to transplant into the crop production system, which would be feasible but also more time consuming and costly. It is therefore desirable to develop 'high ABA' lines that exhibit ABA over-production only in green photosynthetic tissue, therefore retaining the agronomic benefit of more restrained water use, whilst decoupling this from the negative impacts of increased dormancy and the slightly prolonged seedling establishment phase (*cf.* Section 10.4.1.2).

High level expression of *LeNCED1*, driven by the *rbcS3C* promoter, achieved an 'ultra high' level ABA accumulation. This provided an opportunity to evaluate the effects of long-term elevation of ABA at higher levels than previously seen in the constitutive 'high ABA' lines. These plants exhibited several undesirable phenotypes as a direct or indirect effect of the 'ultra high' levels of ABA, including significantly reduced leaf chlorophyll contents, severely reduced growth rates and considerably altered reproductive development (*cf.* Chapter 6 for detail and more extensive discussion). This was a demonstration that it is possible to over-produce ABA at a super-optimal level. The negative impacts on reproductive development observed in the 'ultra high' plants were not exhibited in the constitutive 'high ABA' lines, with similar time taken to develop inflorescences between these and WT plants (*cf.* Chapter 7). It was reassuring to find that the preliminary analysis of fruit production of the mildest 'high ABA' line showed no difference total fruit production. However when the 'ultimate' water saving 'high ABA' line is finally created, commercial production style trials would be required to definitively determine whether long-term elevation of ABA accumulation would have any adverse impact on fruit yield.

Extreme levels of ABA over-production are clearly of no benefit in an agricultural, crop production context. However, it should be noted that when these plants are grown over a long period, the extreme symptoms described previously become slightly less severe and they are able to grow and maintain plant water status in conditions of very low water availability, where WT and the milder 'high ABA' plants exhibited visual signs of drought stress. Such a level of ABA accumulation

could conceivably allow growth in arid areas, in which the hot dry conditions would otherwise prevent growth of non-adapted crops. It must be considered however that such a drastic reduction in stomatal aperture, as exhibited by these plants, may have significant impacts on other physiological processes.

Loveys *et al.* (2004) suggested that reducing transpiration rate by manipulating the ABA biosynthetic pathway might reduce evaporative cooling and significantly increase leaf temperature. A problem of temperature stress has not been observed during this research programme; however plants have been consistently grown in conditions of optimal light and temperature and as transpiration is important for evaporative cooling in hot environments, significantly reducing transpiration rates may result in temperature stress in extreme conditions.

10.3 'High ABA' technology and its application in agriculture

10.3.1 The GM approach

The most obvious and advantageous consequence of increased ABA accumulation is the improved TE_p observed in all 'high ABA' lines (excluding the 'ultra high' rbcS lines) (*cf.* Chapters 5 and 7; Thompson *et al.*, 2007a). The greatest increase in TE_p was achieved by combining the over-expression of two ABA biosynthetic genes (*NCED* and *BCH*), which resulted in a 51% increase in TE_p when compared with WT controls (*cf.* Section 10.4.1 for discussion of 'optimal' levels of ABA over-production).

Water scarcity imposes huge restrictions on crop yield (Boyer, 1982) and the situation is likely to worsen as increased population and global climate change increase the pressure on finite water resources (Mannion, 1995; Somerville and Briscoe, 2001; Richter and Semenov, 2005; Kalra *et al.*, 2007; Ansink and Ruijis, 2008). The worlds population is expected to increase by a further 35% in the next twenty years (Ali and Talukder, 2008), therefore the development of methods to improve WUE for both rain-fed and irrigated crop production is currently of major importance (Anderson *et al.*, 1999; Hamdy *et al.*, 2003; Parry *et al.*, 2005). The research described here has demonstrated that *via* genetic manipulation of the ABA

biosynthetic pathway, large improvements in TE_p can be achieved. This novel method of providing plants which can sustain current levels of productivity, whilst requiring less water, would undoubtedly be of huge advantage in a climate of reduced water availability. This technique does however require the crop to be genetically manipulated.

There is still much controversy over the pro's and con's of GM technology, despite reviews concluding that there is no evidence that GM crops are more harmful to the consumer and the environment than other current agricultural practices (e.g. Dale *et al*., 2002) and due to negative public opinion GM crops are currently not grown in the UK (Horlick-Jones *et al*., 2006). If public opinion were to change about the use of GM food, this 'high ABA' technology could be applied to other crops including potato, another *Solanum* species, for which lines over-expressing *NCED* and *BCH* genes are already available. The potato crop dominates irrigated agriculture in the UK, consuming 51% of the total irrigation water utilised in England and Wales (Stansfield and Hutchings, 1997).

10.3.2 'High ABA' rootstocks

Grafted rootstock are commonly used in many crop species to protect against soil-borne pests and diseases, to reduce plant stature or to provide resistance to abiotic stresses (Bhatt *et al*., 2002; Fernandez-Garcia *et al*., 2002; Csiznszky *et al*., 2005). The use of non-GM scions grafted onto 'high ABA' GM rootstocks may be a way of alleviating some of the objections to this technology. This 'semi-GM' approach would use a transgenic rootstock to convey a long-distance root-sourced stress signal to a non-GM, commercial cultivar scion. Therefore the fruit produced and consumed by the public would not be GM. If it was possible to develop a 'high ABA' transgenic rootstock line capable of fulfilling this role, it would still be interesting to find out whether this approach would be seen by the public as acceptable. Such strong views are often held about the whole concept of GM, rather than the specific risks of growing and consuming particular GM plants/products. It must also be considered that this approach would be too expensive for use with low value crops, such as potato, relative to glasshouse tomato plants grown in Northern Europe.

It remains to be seen whether or not this grafting technique can be used to demonstrate that the over expression of *LeNCED1*, *LeBCH2* and *LePSY1*, generates sufficient root-synthesised ABA to affect scion stomatal behaviour. If it does, the use of a root-specific promoter could also be considered to overcome undesirable effects of constitutive over expression (e.g. seed dormancy) and to provide high ABA roots for crops where the use of rootstocks is not economic. There are lots of reports of root-specific promoters in various other species; it should be noted that promoters do not necessarily behave in the same manner when transformed into a heterologous host (Vaughn *et al*., 2006). For example, Jones (2007) demonstrated that when a root-specific promoter from the *Arabidopsis* geranylgeranyl pyrophosphate synthase3 (*AtGGPS3)* gene (Okada *et al*., 2000) was transformed into tomato, it was observed to be expressed weakly in all organs of the plant and did not function in the same root specific manner. A suitable tomato root-specific promoter has since been identified (Jones *et al*., 2008). The novel *SlREO* promoter is highly expressed in roots but had a very low level of expression in aerial plant organs and is ideal for providing strong and specific gene expression in the bulk of tomato root tissue growing in soil (Jones *et al*., 2008).

10.3.3 The non-GM approach to creating tomato lines with elevated ABA accumulation

Water-stress induced ABA accumulation in leaves has been shown to closely correlate with increases in *NCED* mRNA in many species including bean (Qin and Zeevaart, 1999), tomato (Thompson *et al*., 2000) and Arabidopsis (Iuchi *et al*., 2001) and the enzyme encoded is known to catalyse a key regulatory step in the ABA biosynthetic pathway. The cultivated tomato species *Solanum lycopersicum* L. has many wild relatives which originate from dry habitats in South America (Taylor, 1986) and several are known to have improved salt and drought tolerance (Tal, 1971; Rick, 1974; Denan and Tal, 1979). It is possible that due to natural selection in their native environments, the *NCED1* alleles from such *Solanum* wild species may have evolved promoters driving higher expression levels which could confer greater WUE when introgressed into a commercial *S.lycopersicum* genetic background. These wild tomato relatives are currently undergoing a classical backcrossing as part of a

DEFRA-funded programme with the objective of introgressing divergent wild species *NCED1* alleles into the common genetic background of a common tomato cultivar, to assess their effect on WUE. It has been suggested that this could be an alternative non-GM approach to increasing ABA accumulation exploiting a combination of natural resources and knowledge gained from genetic manipulation. Preliminary gravimetric WUE experiments have shown that plants possessing the *S. pennellii NCED1* allele have improved WUE when compared with those possessing the Ailsa Craig allele (H. Hilton/A. Thompson, pers. comm.)

10.4 Achieving the optimal level of ABA over-production

10.4.1 Currently available 'high ABA' tomato lines

At a whole plant level, the basic definition of WUE is the ratio of the quantity of biomass produced to the quantity of water transpired (Jones, 2004). In well-watered conditions with a good level of light, WT plants can increase their stomatal apertures past the point that provides any useful gain in CO_2 absorption and therefore assimilation rate. In 'high ABA' plants, maximal stomatal apertures appear to be limited and are also more sensitive to VPD than WT plants (Thompson *et al*., 2007a). This strategy of restrained stomatal aperture consistently resulted in improved TE_p. It is possible that this effect could be achieved in other species that over-express one or more ABA biosynthetic genes, with plants over-expressing an *NCED* gene showing reduced transpiration and improved drought tolerances (based on visual analysis of wilting symptoms; Iuchi *et al*., 2001; Qin and Zeevaart, 2002).

It has been established that 'high ABA' technology has potential to provide great agronomic benefit. The constitutive 'high ABA' lines (sp12 and sp5) characterised in this research program, appear to have less than optimal levels of ABA over-production. The optimum could be described as the level of ABA that achieves the maximum possible reduction in transpiration obtainable before growth rates would become adversely affected. When considered at the individual leaf level, the leaf ABA concentration which restricts maximal stomatal aperture to the point where any further stomatal opening would not result in any usable increase in CO_2 absorption

and therefore no further net gain in carbon assimilation (i.e. photosynthesis is limited by other factors).

The first *LeNCED1* over-expressing lines to be created (sp12 and sp5) display relatively modest increases in ABA accumulation, in comparison with those exhibited by water stressed WT plants (Thompson *et al*., 2007b; Tung *et al*., 2008). Plants from the line sp5 have fairly consistently shown greater levels of transgene expression than sp12 (Thompson *et al*., 2007b) and often appear to have more extreme physiological responses e.g. seed dormancy. However in the experiments reported here there was no significant difference between the two sp lines, when analysing TE_p. It is clear from characterisation of the rbcS 'ultra high' ABA plants that it is possible to obtain levels of ABA over-production which appear to be above the optimal level (Section 10.2). In order to attempt to increase ABA accumulation beyond that of the sp12 and sp5 level, the 'double transgene' lines were created and these provided a further increase in TE_p (*cf.* Chapter 7). The extent of over-production in these lines (G28 and G29) has not exceeded the 'optimal' level defined above, as they saved more water without any loss of biomass production. Whilst the effect of combining the over-expression of the two ABA biosynthetic transgenes (*LeNCED1* and *LeBCH2*) appears to simply have an additive effect on TE_p, this was not the case for all parameters measured (e.g. seed dormancy; *cf.* Chapter 9). There is evidence that the combination of the two transgenes has a synergistic, rather than additive effect on concentration of ABA in the leaves, suggesting that there could be a non-linear relationship between leaf ABA concentration and TE_p (Sonneveld *et al*. in prep.).

Despite the potential agronomic benefits which could be provided by the constitutive 'double transgene' over-expressing lines, as with the sp lines, these plants also exhibit some negative impacts associated with constitutive over-expression. As mentioned above, sp lines over-expressing *LeNCED1* alone have increased seed dormancy and the simultaneous over-expression of *LeNCED1* and *LeBCH2* in the 'double transgene' lines has a synergistic effect on seed dormancy. This is likely to be due to a threshold effect. The levels of ABA accumulation in the seed of lines G28 and G29 appear to have exceeded an agronomically acceptable level in the seeds, resulting in an extreme increase in dormancy. There is also a more severe

effect on early growth, with G29 plants taking significantly longer to pass through the 'seedling establishment' phase than the more mild 'high ABA' lines. The above factors combine to mean that despite the constitutive 'double transgene' approach possibly achieving close to optimal control over stomatal behaviour, the accompanying consequences of the constitutive over-production of ABA can not always be predicted and mean that these plants clearly do not represent the ideal 'high ABA' line. To generate transgenic 'high ABA' lines with optimal levels of ABA over-production for improved TE_p, without the negative impact of constitutive expression on seed germination and growth rate during seedling establishment, research is currently focussing on designing an *LeNCED1* over-expression construct, using a promoter with more selective patterns and appropriate levels of expression.

10.4.2 Generating 'high ABA' lines using alternative promoters

Transformation to create the original *sp*::*LeNCED1* lines (sp12 and sp5) involved the use of constructs containing a chimaeric "super-promoter" (sp) to potentially provide strong constitutive expression (Ni *et al*., 1995). Surprisingly this resulted in only a few over-expressing primary transformants, all with relatively mild levels of ABA over-production (Thompson *et al*., 2000a; Thompson et al., 2007a). During the tissue culture stage, high-expressing *sp*::*LeNCED1* shoots could have shown signs of chlorosis possibly as a direct or indirect effect of very high levels of ABA synthesis. It is therefore possible that they were discarded as non-transformed, kanamycin-sensitive shoots (which would have been chlorotic), meaning that only lines in which the integration site limited the over-expression level were inadvertently selected.

The native tomato RuBisCO small subunit is encoded by a small family of genes which are only transcribed in photosynthetically active organs, including the leaves of mature plants and cotyledons of seedlings grown in light (Wanner and Gruissem, 1991). The promoter for one of these, the *rbcS3C* gene, was selected to drive *LeNCED1* expression, with the primary objective of decoupling the positive effect of *LeNCED1* over-expression on stomatal behaviour, from the negative impacts on germination and early growth. Transformation of tomato plants with the *rbcS3C::LeNCED1* construct allowed the recovery of many transgenic plants with

very high levels of ABA (Tung *et al*., 2008). The rbcS3C promoter is switched off in the dark (Wanner and Gruissem, 1991) and is also suppressed in tomato leaves containing a high sucrose content (Vanoosten and Besford, 1994), therefore the relatively low light and high sugar environment in the tissue culture may have prevented full expression of the rbcS3C::*LeNCED1* construct until the transformants were transferred to the higher light conditions provided by the glasshouse. The use of this tissue specific promoter was successful, in the sense that the rbcS lines showed only mild increases in seed dormancy in comparison with what might be expected from plants with very high constitutive ABA accumulation. The tissue specificity and 'ultra high' levels of ABA over-production in photosynthetically active leaves did however result in a severe inhibition of growth (Fig. 6.3A).

The use of the identical tomato rbcS3C::*LeNCED1* constructs to transform *Arabidopsis* resulted in 'high ABA' transgenic plants with similar effects on growth and stomatal behaviour to those of the *sp*::*LeNCED1* lines of tomato. These plants exhibited a significant increase in WUE for both gravimetric (WUE_p) and instantaneous gas exchange (WUE_{iGE}) measurements (Deswarte *et al*. in prep.) and this is thought to be because the tomato promoter resulted in weaker expression in a heterologous host. A reciprocal experiment is currently in progress, whereby tomato plants are being transformed with a construct that utilises the *Arabidopsis rbcS1A* promoter to drive *LeNCED1* expression (*At-rbcS1A::LeNCED*). Initial data indicates that the *At-rbcS1A::LeNCED1* construct has allowed the production of at least one line that has a good germination rate, low stomatal conductance, and that grows well under glasshouse conditions (Dr A. Thompson, pers.comm.).

Whilst previous approaches focussed on designing constructs to produce transgenic 'high ABA' plants with optimal spatial and temporal transgene expression, an alternative approach is to use a transposon-based system (Taylor, Thompson and Awan, unpublished), in which hundreds of independent transgenic lines can rapidly be generated, with a large variation in transgene expression. This system could hold several advantages over the traditional construct-based system and avoids the previously discussed issues with generating and identifying 'high ABA' lines by selection for kanamycin-resistance during tissue culture. These plants can then be screened for desirable phenotypes associated with ABA over-production, hopefully

identifying lines with improved WUE but with normal seed germination, establishment and growth. One possible method of rapidly screening for the desirable stomatal characteristics in plants with optimal levels of ABA-overproduction would be through a thermal imaging method, in which the different levels of thermal cooling *via* transpiration would be identified between WT and 'high ABA' plants (Merlot *et al*., 2002).

The alternative methods of generating 'high ABA' lines described above, provide various advantages, however they all rely on the upregulation of one ABA biosynthetic gene (*NCED*). As well as providing substrate for NCED, the various xanthophyll pools play important roles in photosynthetic and photoprotective mechanisms (Demmig-Adams and Adams, 1992; Kuhlbrandt *et al*., 1994; Demmig-Adams *et al.*, 1996; Howitt and Pogson, 2006; Johnson *et al*., 2008). In plants over-expressing *LeNCED1*, there is increased demand for xanthophyll precursors and endogenous levels of enzymes catalysing previous steps in the pathway, including BCH, may be insufficient to maintain precursor pool sizes. It has been shown that the over-expression of *LeNCED1* results in depletion of xanthophyll pools (Balasubramanian, 2007; Tung *et al*., 2008), therefore engineering plants that over-express *LeNCED1* to a greater extent than in the current 'sp lines' may have physiological consequences similar to that observed in the 'ultra high' ABA over-producing lines (*cf*. Chapter 6). The over-expression of two or more ABA biosynthetic genes may therefore be the most effective method of optimising ABA over-production, without affecting other key physiological mechanisms.

10.4.3 Potential advantages and disadvantages of using 'high ABA' technology in a variety of crop production situations

It has been shown during this project that, at least under the controlled glasshouse conditions utilised (*cf.* Chapter 3), it is possible to modify stomatal behaviour through the over-expression of ABA biosynthetic genes, resulting in tomato plants with improved TE_p (*cf*. Chapters 5 and 6). The optimal level of over-expression (for the environmental conditions used during this project) can be described as that which limits the amount of water transpired without causing a penalty in biomass production. The benefits of 'high-ABA' technology may be most

easily obtained in glasshouse tomato crop production and in other irrigated crops grown in protected environments, in which plants are ideally spaced, temperature fluctuations are controlled, atmospheric turbulence is minimised and any reduction in total irrigation requirement would be of some financial advantage. However, it is necessary to consider several additional factors which might potentially have an impact on the effectiveness of this technology in a variety of crop production situations (e.g. open field cropping systems).

There is a wide range of opinions on the most effective strategies for increasing food production in a future climate of decreased water availability (e.g. Condon *et al*., 2004; *cf*. Section 1.1). Blum (2009) argues that a key determinant of plant productivity under conditions of limited water supply is the effective use of water (EUW), rather than simply high WUE. This author suggests that maximal plant production requires higher stomatal conductance over time, in order to allow increased carbon fixation per unit land area. Therefore, higher yielding genotypes of several crop species would be likely to have increased stomatal conductance and transpiration rate under drought stress and well-watered conditions (Fisher *et al*., 1998; Sanguineti *et al*., 1999; Araus *et al*., 2002; Horie *et al*., 2006; Blum, 2009).

In open field cropping systems, a large proportion of soil water that could potentially be available to the plants is evaporated directly from the soil. For example in Australian wheat production, it has been estimated that up to 40% of all available soil water is lost via this process (Siddique *et al*., 1990). It has therefore been suggested that plant breeders should direct breeding programmes towards generating vigorous seedling and root system growth, in order to increase the percentage of soil water taken up by the crop (Rebetzke and Richards, 1999). Several researchers have formed the view that plants with improved water use efficiency, through alteration of stomatal behaviour, will not allow the most efficient capture of maximal soil water. In an open field context, restriction of stomatal aperture may result in water remaining unused in the soil for longer periods, which could result in it being lost via soil evaporation. Optimal stomatal aperture may be somewhere between fully open and that giving maximal gain in WUE (Jones, 1992).

Although it may represent a 'special case', the research reported here does not appear to be consistent with the theory that WUE and EUW cannot be achieved simultaneously (Blum, 2009). The 'high ABA' tomato lines shown to have increased TE_p (Section 10.4.1), have consistently exhibited growth rates that equal, or even exceed those of WT plants in mildly sub-optimal conditions and when well-watered. In this case, there was no reduction in leaf area and therefore ground cover production appears likely to be unimpaired, even if these 'high ABA' plants had been grown in open field crop systems. These transgenic tomato plants also exhibited several phenotypes related to their increased basal level of ABA which might be considered to be advantageous, when considering the aim for breeders of rapid leaf area production in order to achieve maximal capture of soil water. Petiole length is consistently greater in sp5 plants than WT controls, with epinasty also being reduced (Thompson *et al*., 2007a). This together with increased leaf area potentially facilitates quicker ground coverage, preventing water loss directly from the soil surface. The 'high ABA' tomato plants have also been reported to exhibit increased root hydraulic conductivity (Thompson *et al*., 2007a), possibly due to ABA induced changes in aquaporin abundance and activity (Hose *et al*., 2000). This might be expected to increase potential water uptake per unit surface area and time (Glinka and Reinhold, 1971).

At any one point in time there is an optimal stomatal conductance that would result in maximal instantaneous WUE; however the optimal use of water over a longer period of time (such as a growing season) requires variation in stomatal opening as the environment changes (Jones, 1992). One way of optimising stomatal behaviour to improve WUE is to ensure that the periods when stomata are relatively widely open and photosynthesis is rapid coincide with times of the day when evaporative demand is fairly low (e.g.in the morning). Stomatal closure around midday/early afternoon, when VPD is often greatest, is a well known mechanism often leading to improvements in WUE (Chaves *et al*., 2002). Reducing transpiration when atmospheric demand was greatest, by establishing a maximum transpiration rate, was successful in improving WUE during simulations involving sorghum (Sinclair *et al*., 2005). In this context it should be noted that the mildest 'high ABA' transgenic tomato plants from line sp12 were shown to exhibit reduced apertures in comparison with WT at high VPD, but apertures were similar to WT at low VPD; allowing

maximum CO_2 assimilation when atmospheric demand for water is relatively low (Thompson *et al.*, 2007a). When considering optimising levels of ABA production within a 'whole crop', a level at which stomatal behaviour is modified enough to reduce water loss and therefore potentially limit plant water-stress during times of high evaporative demand, whilst maintaining carbon assimilation over an entire growing season, is clearly the optimal strategy. It would be inappropriate to genetically engineer plants with a permanently reduced stomatal aperture, which although likely to have maximal instantaneous WUE, would not be able to respond dynamically to changes in environmental conditions.

Another important factor to keep in mind when considering the potential effectiveness of 'high-ABA' technology on a field scale, is whether or not a crop is effectively 'coupled' with its atmosphere. A plant is considered well coupled when mass and energy exchange between the plant and the bulk atmosphere is efficient, so that leaf temperature follows air temperature (Jones, 2004). In large areas of aerodynamically smooth, short crops (such as cereals) coupling is often poor. In these conditions transpiration rate is not necessarily proportional to stomatal conductance and is often driven at an equilibrium rate determined by the radiation load on the leaf (Jones, 1985). It has been pointed out that because a reduction in stomatal conductance initially reduces water loss, it thereby slightly increases the leaf surface temperature. This in itself provides a somewhat greater driving force for leaf transpiration which could, in theory, cancel out the effect of reducing stomatal aperture in the first place (Jarvis and McNaughton, 1985). The present research work, carried out on the 'high ABA' tomato lines in order to determine individual plant water loss gravimetrically, is not consistent with this idea of 'cancelling out' the effect of reducing stomatal aperture.

However, it should be noted that Jones (2004) suggests that caution is required when extrapolating from data showing improvements in WUE on single plants in controlled environments to crop production in the field, as 'scale effects' have not been taken into account. This problem has been previously encountered during the testing of antitranspirants which were effective on single plants in controlled environments, but utilised on a field scale, success was relative to the extent of coupling between the vegetation boundary layer and the turbulence of the bulk

atmosphere (Loveys *et al*., 2004). Improvements in WUE observed during small scale trials may therefore fail to be reflected at crop scale (Jones, 2004).

The long-term growth trials conducted during this project (*cf*. Chapter 4) simulated tomato crop growing conditions as closely as possible, with the same plant spacing as seen in commercial glasshouses. In these experiments the water saving capacity of 'high-ABA' plants was maintained even during summer months, with the reduced transpiration rates not causing the plants to exhibit any visual signs of heat stress. It is, however, reasonable to assume that the tomato plants investigated in these trials were relatively well coupled with the atmosphere within the glasshouse. Without large scale field trials of the existing GM tomato lines (difficult to organise with public hostility in the UK) and in the absence of an ideal non-GM, 'high ABA' cereal crop species, it is very difficult to be sure whether or not plant-atmosphere de-coupling would alter the effectiveness of this 'high ABA' technology. What is certain is that improvements in WUE have been regularly and consistently observed in the glasshouse grown 'high ABA' tomato lines investigated during the research programme reported in this thesis.

10.5 Alternative approaches to manipulate plant responses to stress

The research described here has focussed on the manipulation of the ABA biosynthetic pathway to alter the basal ABA concentration in the absence of any water-stress stimuli. Research involving the manipulation of other candidate genes is also providing potential improvements in WUE. For example the over-expression of genes involved with ion transporters and transcription factors have both provided improved tolerance of abiotic stresses in many species (*cf*. Zhang *et al*., 2004 for a review). In wheat for example, stress-induced expression of the drought-related *Arabidopsis* transcription factor *DREB1A* has been shown to convey improved tolerance of drought conditions (Pelligrineschi *et al*., 2004). Over-expression of another drought-related transcription factor from *Arabidopsis*, (the *ABF3*gene) in transgenic rice had similar effects, with enhanced drought tolerance coupled with normal growth rates and no visible phenotypic alterations (Oh *et al*., 2005).

Aquaporins (AQPs) are membrane proteins which increase permeability to water (Kaldenhoff and Fisher, 2006). It has now been established that AQPs play a key role in plant water balance and WUE (Kaldenhoff and Maurel, 2007). It has been reported that when the tobacco gene *NtAQP1* is silenced, root hydraulic conductivity was reduced (Siefritz *et al*., 2002). When *NtAQP1* was constitutively over-expressed in transgenic tomato plants, TE_p was increased when plants were exposed to salt stress (Sade *et al*., 2010).

There have been a number of recent advances in the identification of ABA receptors (Sheard and Zheng, 2009) and this could provide future opportunities to manipulate plant responsiveness to ABA. In the most recent advances, members of the START protein family, known as PYR/PYL/RCAR have been shown to bind ABA and inhibit the activity of specific protein phosphatase enzymes, which were previously implicated in the ABA response (Ma *et al*., 2009; Park *et al*., 2009). A G-protein-coupled receptor (GCR2, Liu *et al*., 2007) has been shown to be specifically implicated in the stomatal response to ABA and its over-expression leads to increased ABA sensitivity and reduced stomatal opening (Liu *et al*., 2007). Whilst the role of ABA in the control of plants responses to abiotic stress is well-documented, the identification of ABA receptors has been unusually challenging (Sheard and Zheng, 2009), with current understanding of ABA perception being still at an early stage.

Finally, an alternative method to that utilised during this research programme, of producing plants with increased levels of basal ABA, is through manipulation of ABA catabolism. The catabolic degradation of ABA occurs in most plant cell types (Cutler and Krochko, 1999) and ABA concentration in plant tissues depends both on its rate of synthesis and catabolism (Verslues and Zhu, 2007). The main pathway of catabolism involves the hydroxylation of ABA to form PA and DPA (*cf*. Section 1.2.4.5; Nambara and Marion-Poll, 2005). Since ABA accumulation triggers its own degradation, the manipulation of ABA catabolism is an important target for genetic engineering of ABA levels in plants (Finkelstein and Rock, 2002). As the regulation of ABA catabolism is suggested to be key in determining the level of stress-induced ABA accumulation (Priest *et al*., 2006), sustained elevation of ABA accumulation in

transgenic plants could potentially be prevented by a coincident increase in the rate of ABA catabolism (Cutler *et al*., 1997). The inducible over-expression of *PuNCED1* in tobacco not only resulted in the increase in ABA concentration, but also resulted in increased accumulation of its catabolite PA (Qin and Zeevaart, 2002). Transgenic tomato seeds over-expressing two ABA biosynthetic genes (*LeNCED1* and *LeBCH2*) had significantly increased DPA, as well as increased seed ABA content (Sonneveld *et al*. in prep.), an effect that may be widespread throughout the tissues of 'high ABA' plants. These findings both suggest that in 'high ABA' plants over-expressing one or more ABA biosynthetic genes, the level of ABA accumulation is at least in part reduced by its catabolism. The ABA 8'-hydroxylase-encoding *Arabidopsis* gene, *cyp707a2*, is reported to play a major role in the control of seed ABA concentration, with null mutants of this gene accumulating six-fold more ABA than WT (Kushiro *et al*., 2004). It is possible that upregulation of ABA biosynthetic genes could be combined with the downregulation of ABA catabolic genes to produce plants with elevated ABA concentrations.

ABA conjugates, including ABA-glucose ester (ABA-GE), are biologically inactive (Cutler and Krochko, 1999). The activity of β-glucosidases, which hydrolyse ABA glucose esters to release biologically active ABA, also has an influence over ABA accumulation. For example, *Arabidopsis* plants that are deficient in β-glucosidase exhibit lower ABA concentration in leaves and increased sensitivity to stress (Lee *et al*., 2006). It is likely that plants with high ABA-GE specific β-glucosidase activity would have strong ABA-signals, derived from ABA-GE in the xylem (Wilkinson and Hartung, 2009). One advantage of the 'high ABA' lines reported in this research programme is that whilst they do exhibit more restrained maximal stomatal conductances, their stomata still respond dynamically to changes in environmental conditions (Table 5.1, Fig. 5.1). The danger with altering too many of a plant's intrinsic mechanisms of adjusting the ABA content of a specific tissue would be that plants may exhibit a permanent and severe restriction of stomatal opening, even in optimal conditions, thus resulting in a situation similar to that of the 'ultra high' rbcS lines, in which growth rates were severely affected.

10.6 Conclusions

With an increasing world population and decreasing water resources available for crop production, the need to improve water productivity or produce 'more crop per drop' is becoming ever more vital. Whilst the word 'drought' evokes images of arid environments, devoid of any rainfall, many forms of plant water deficit can have an impact on the diverse crop production systems throughout the world. For example in the UK, insufficient soil moisture frequently limits crop productivity because rainfall often does not occur when and where crops need it most. The UK is one of the world's most efficient producers of arable crops, yet approximately 30% of the current wheat area is grown on drought-prone land and loss of yield due to drought costs UK agriculture in excess of £60 million per year (Foulkes *et al*., 2007).

It has been suggested that different strategies must be combined in order to increase agricultural water productivity (Passioura, 2006). Plant breeders have improved crop yields in dry conditions with the aid of tools such as marker assisted selection (MAS), in which improvements in drought tolerance of upland rice (Steele *et al*., 2006) and pearl millet (Serraj *et al*., 2005) have been observed. Breeding programmes are currently selecting for key features, such as improved intrinsic transpiration efficiency (Rebetzke *et al*., 2002) and optimal timing of flowering (Richards, 1991). Agricultural scientists have improved management techniques for saving water, from the improved management of soil and stubble that increases available soil water and minimises soil evaporation (Silburn and Glanville, 2002), to the modification of crop rotations and sowing schedules (Passioura, 2002). Plant scientists have furthered understanding of plant responses to water deficit and are now manipulating these processes to improve WUE. For example, novel irrigation techniques such as RDI and PRD (*cf.* Section 1.1.1) are now utilised to improve water productivity (Dorji *et al*., 2005; Liu *et al*., 2006; Ali *et al*., 2007).

This research programme has demonstrated that by using transgenic lines that are pre-adapted to water stress *via* increased ABA biosynthesis, improvements in whole plant WUE can be achieved. The simultaneous over-expression of two ABA biosynthetic genes (*LeNCED1* and *LeBCH2*) was shown to provide the greatest improvement in WUE. This 'high ABA' technology could certainly be of

agricultural benefit and could possibly be integrated with other strategies discussed above, in order to sustain crop production under conditions of reduced rainfall or irrigation availability. Future work will investigate alternative promoters to achieve the ‘optimal’ level of long-term ABA over-production, with the ultimate goal of achieving transgenic lines with maximal gain in WUE, without impacting on seed germination, plant growth or crop productivity. It is also possible that the ‘high ABA’ approach could be utilised to provide non-GM tomato fruit by the development of ‘high ABA’ rootstocks to be grafted onto commercial tomato cultivars, or via introgression of wild species versions of key ABA biosynthetic genes, into conventional varieties.

11 **BIBLIOGRAPHY**

Ackerson, R. (1984) Regulation of soyabean embryogenesis by Abscisic acid. *Journal of Experimental. Botany*, **35**: 403-413.

Ackerson, R.C. and Radin, J.W. (1983) Abscisic acid accumulation in cotton leaves in response to dehydration at high pressure. *Plant Physiol*ogy **71**: 432-433

Addicott, F.T. (1983). *Abscisic acid*. Edited by Addicott, F.T. Praeger: New York.

Addicott, F.T. and Lyon, J.L. (1969). Physiology of Abscisic acid and related substances. *Annual review of Plant Physiology* **20:** 139-164

Addicott, F.T., Lyon, J.L., Ohkuma, K., Thiessen, W.E., Carns, H.R., Smith, O.E., Cornforth, J.W., Milborrow, B.V., Ryback, G., Wareing, P.F. (1968) Abscisic acid: a new name for abscisin II *(*Dormin). *Science* **159**: 1493.

Algre, S., Girona, J., Marsal, J., Mata, M., Montagut, D., Texido, F., Motilva, M.J. and Romero, M.P. (1999) Regulated deficit irrigation of olive trees. *Acta Horticulturae* **474**: 373-376

Ali, M.H. and Talukder, M.S.U. (2008) Increasing water productivity in crop production—a synthesis *Agricultural Water Management* **95**: 1201–1213

Ali, M.H., Hoque, M.R., Hassan, A.A. and Khair, A. (2007) Effects of deficit irrigation on yield, water productivity, and economic returns of wheat. *Agricultural Water Management* **92**: 151–161

Ali-Rachedi, S., Bouinot, D., Wagner, M.H., Bonnet, M., Sotta, B., Grappin, P., and Jullien, M. (2004). Changes in abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta* **219:** 479-488

Allen, G., Chu, S., Schumacker, K., Shimazaki, C., Vafeados, D., Kemper, A., Hawke, S., Tallman, G., Tsien, R and Harper, J. (2000) Alterations of stimulus specific guard cell calcium oscillations and stomatal closing in *Arabidopsis det3* mutant. *Science* **289**: 2338-2342

Anderson, R.L., Bowman, R.A., Nielsen, D.C., Vigil, M.F., Aiken, R.M. and Benjamin, J.G. (1999) Alternative crop rotations for the central Great Plains. *Journal of Production in Agriculture* **12**: 95–99

Ansink, E., and A. Ruijs (2008) Climate change and the stability of water allocation agreements. *Environmental and Resource Economics* **41**: 133-287

Araus, J.L., Slafer, G.A., Reynolds, M.P. and Roy, O.C. (2008) Plant breeding and water-stress in C3 cereals: What to breed for? *Annals of Botany* **89**: 925-940

Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A., and Zenk, M.H. (1997). Terpenoid biosynthesis from 1-deoxy-D-xylulose in higher plants by intramolecular skeletal rearrangement. *Proceedings of the National Academy of Sciences of the USA* **94:** 10600-10605

Aroca, R., Ferrante, A., Vernieri, P. and Chrispeels, M.J. (2006) Drought, abscisic acid and transpiration rate effects on the regulation of PIP aquaporin gene expression and abundance in *Phaseolus vulgaris* plants. *Annals of Botany* **98:** 1301-1310

Assmann, S.M. (1993) Signal transduction in guard cells. *Annual Review of Cell Biology* **9:** 345–375

Aswath, C.R., Kim, S.H., Mo, S.Y. and Kim, D.H. (2005) Transgenic plants of creeping bent grass harboring the stress inducible gene, 9-*cis*-epoxycarotenoid dioxygenase, are highly tolerant to drought and NaCl stress. *Plant Growth Regulation* **47:** 129-139

Atherton J.G. and Harris, G.P. (1986) Flowering. In: *The Tomato Crop* 167–200. Edited by, Atherton J.G. and Harris, G.P. Chapman, Hall, London.

Audran, C., Borel, C., Fre,y A., Sotta, B., Meye,r C., Simonneau, T. and Marion-Poll, A. (1998) Expression studies of the zeaxanthin epoxidase gene in *Nicotiana plumbaginifoli*a. *Plant Physiology* **118:** 1021-1028

Bacon, M.A. (2004). Water use efficiency in plant biology. In *Water use efficiency in plant biology*. Edited by M. A. Bacon: Blackwell Pub. ; CRC Press.

Balasubramanian, G. (2007) Manipulation of multiple ABA-biosynthesis genes in tomato, PhD Thesis, The University of Nottingham.

Bano, A., Hansen, H., Dorffling, K. and Hahn, H. (1994) Changes in the content of free and conjugated abscisic acid, phaesic acid and cytokinins in the xylem sap of drought stressed sunflower plants. *Phytochemsitry* **37**: 345-347

Bartholomew, D.M., Bartley, G.E. and Scolnik, P.A. (1991) Abscisic acid control of *rbcS* and *cab* transcription in tomato leaves. *Plant Physiol*ogy **96**, 291-296

Bartley, G.E. and Scolnik, P. (1993) cDNA cloning, expression during development, and genome mapping of *PSY2*, a second tomato gene encoding phytoene synthase. *Journal of Biological Chemistry* **268:** 25718-25721

Bartley, G.E., Viitanen, V.P., Bacot, K.O. and Scohik, P.A. (1992) A tomato gene expressed during fruit ripening encodes an enzyme of the caroteniod biosynthesis pathway. *The Journal of Biological Chemistry* **267**: 5036-5039

Bassi, R., Pineau, B., Dainese, P. and Marquardt, J. (1993) Carotenoid-binding proteins of photosystem II. *European Journal of Biochemistry* **212:** 297-303

Beardsell, M.F. and Cohen, D. (1975) Relationships between leaf water status, abscisic acid levels, and stomatal resistance in maize and sorghum. *Plant Physiol*ogy **56**: 207-212

Benfey, P.N. and Ren, L., Chua, N. (1989) The CaMV 35S enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. *EMBO Journal* **8:** 2195-2202

Bennet, R.D., Norman, S.M. and Maier, V.P. (1981) Biosynthesis of abscisic acid from [1,2-$^{13}C^{2}$] acetate in *Cercospora rosicola*. *Phytochemistry* **20**: 2343-2344

Bewley, J.D. (1997). Seed germination and dormancy. *Plant Cell* **9**: 1055-1066

Bewley, J.D. and Black, M. (1994). *Seeds: Physiology of Germination and Development* (Second Edition*)*. 382-385. Plenum Press: New York.

Bhatt, R.M., Srinivasa, N.K. and Sadashive, A.T. (2002) Rootstock as a source of drought tolerance in tomato. *Indian Journal of Plant Physiology* **7**: 338-342

Bird, C.R., Ray, J.A., Fletcher, J.D., Boniwell, J.M., Bird, A.S., Blain, I., Bramley, B.M. and Schuch, W. (1991) Using antisense RNA to study gene function: Inhibition of carotenoid biosynthesis in transgenic tomatoes. *Biotechnology*, **9**: 635-639

Bird, C.R., Smith, C.J.S., Ray, J.A., Moureau, P., Bevan, M.W., Bird, A.S., Hughes, S., Morris, P.C., Grierson, D. and Schuch, W. (1988) The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. *Plant Molecular Biol*ogy **11**: 651-662

Blackman, P.G. and Davies, W.J. (1985) Root to shoot communication in maize plants of the effects of soil drying. *Journal of Experimental Bot*any **36**: 39-48.

Blatt, M.R. (2000) Ca^{2+} signalling and control of guard cell volume in stomatal movements. *Current Opinion in Plant Biology* **3**: 196-204

Blum, A. (2009) Effective use of water (EUW) and not water-use efficiency (WUE) is the target of crop yield improvement under drought-stress. *Field Crops Research* **112**: 119-123

Bisognin, D.A., Velasques, L. and Widders, I. (2005) Cucumber seedling dependence on cotyledonary leaves for early growth. *Pesq. agropec. bras. Brasília* **40**: 531-539

Boland, A.M., Mitchell, P.D., Jerie,P.H. and Goodwin, I. (1993) The effect of regulated deficit irrigation on tree water use and growth of peach. *Journal of Horticultural Science* **68**: 261-274

Borel, C., Fre,y A., Marion-Pol,l A., Tardieu, F., Simonneau, T. (2001) Does engineering abscisic acid biosynthesis in *Nicotiana plumbaginifolia* modify stomatal response to drought? *Plant Cell and Environment* **24:** 477-489

Boulard, T. and Baille, A. (1993) A simple greenhouse climate control model incorporating the effects of aeration and evaporative cooling. *Agric. For. Meteorology* **65**: 145-57

Bouvier, F., Keller, Y., d'Harlingue, A., and Camara, B. (1998). Xanthophyll biosynthesis: molecular and functional characteri–zation of carotenoid hydroxylases from pepper fruits *(Capsicum annuum L.*). *Biochimica et Biophysica Acta* **1391:** 320-328.

Bouvier, F., O'Harlingue, A., Backhaus, R.A., Kumagai, M.H. and Camara, B. (2000) Identification of neoxanthin synthase as a carotenoid cyclase paralog. *European Journal of Biochemistry* **267:** 6346-6352

Boyer, J.S. (1982) Plant productivity and environment (crop genetic improvement). *Science* **218:** 443-448

Boyer, D.G., Wright, R.J., Winant, W.M. and Perry, H.D. (1990). Soil water relations on a hilltop cornfield in central Appalachia. *Soil Science* **149**:383–392

Boyer JS **and Westgate** ME. **(**2004**)** Grain yields with limited water. *Journal of Experimental Botany* **55**: 2385–2394

Boyle, M.G., Boyer, J.S. and Morgan, P.W. (1991) Stem infusion of liquid culture medium prevents reproductive failure of maize at low water potentials. *Crop Science* **31**: 1246-1252

Bradford, K.J., Sharkey, T.D. and Farquar, G.D. (1983) Gas exchange, stomatal behaviour and $\delta^{13}C$ values of the *Flacca* tomato mutant in relation to abscisic acid. *Plant Physiology* **72**: 245-250

Bramley, P., Teulieres, C., Blain, I., Bird, C. and Schuch, W. (1992) Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5. *The Plant Journal* **2:** 343-349

Britton, G. (1995). Structure and properties of carotenoids in relation to function. *Federation of American Societies for Experimental Biology* **9:** 1551-1558

Burbidge, A., Grieve, T., Terry, C., Corlett, J., Thompson, A. and Taylor, I.B. (1997a) Structure and expression of a cDNA encoding zeaxanthin epoxidase, isolated from a wilt-related tomato (*Lycopersicon esculentum* Mill.) library. *Journal of Experimental Botany* **48:** 1749-1750

Burbidge, A., Grieve, T., Jackson, A., Thompson, A. and Taylor, I.B. (1997b) Structure and expression of a cDNA encoding a putative neoxanthin cleavage enzyme (NCE), isolated from a wilt-related tomato (*Lycopersicon esculentum* Mill.) library. *Journal of Experimental Botany* **48:** 2111-2112

Burbidge, A., Grieve, TM., Jackson, A., Thompson, A., McCarty, D.R., Taylor, I.B. (1999) Characterization of the ABA-deficient tomato mutant *notabilis* and its relationship with maize*Vp14*. *The Plant Journal* **17:** 427-431

Busch, M., Seuter, A., Hain, R. (2002) functional analysis of the early steps of carotenoid biosynthesis in tobacco. *Plant Physiology* **128:** 439-453

Cadman, C.S.C., Toorop, P.E., Hilhorst, H.W.M. and Finch-Savage, W.E. (2006) Gene expression profiles of *Arabidopsis* Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant Journal* **46**: 805-822

Cantliffe, D. J. and Vansickle, J. J. (2001) Competitiveness of the Spanish andDutch greenhouse industries with the Florida fresh vegetable industry. *Proc. Fla. State Hort. Soc.* **114**: 283-287

Carrasco, P., Manzara, T. and Gruissem, W. (1993) Developmental and organ-specific changes in DNA-protein interactions in the tomato rbcS3B and rbcS3C promoter regions. *Plant Molecular Biology* **21**: 1-15

Carretero-Paulet, L., Ahumada, I., Cunillera, N., Rodriguez-Concepcion, M., Ferrer, A., Boronat, A. and Campos, N. (2002) Expression and molecular analysis of the *Arabidopsis* DXR gene encoding 1-deoxy-D-xylulose 5-phosphate reductoisomerase, the first committed enzyme of the 2-C-methyl-D-erythritol 4-phosphate pathway. *Plant Physiology* **129:** 1581-1591

Carretero-Paulet, L., Cairo, A., Botella-Pavia, P., Besumbes, O., Campos, N., Boronat, A. and Rodriguez-Concepcion, M. (2006) Enhanced flux through the methylerythritol 4-phosphate pathway in *Arabidopsis* plants overexpressing deoxyxylulose 5-phosphate reductoisomerase. *Plant Molecular Biology* **62:** 683-695

Chalmers, D.J., Burge, G., Serie, P.H. and Mitchell, P.D. (1986) The mechanisms of regulation of Bartlett pear fruit and vegetative growth by irrigation withholding and regulation deficit irrigation. *Journal of American Society for Horticultural Science* **111**: 904-907

Chapagain, A.K., and Orr, S. (2009) An improved water footprint methodology linking global consumption to local water resources: A case of Spanish tomatoes, *Journal of Environmental Management*, **90**: 1219-1228

Chaves, M.M. and Oliveira, M.M. (2004) Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. *Journal of Experimental Botany* **55:** 2365-2384

Chen, G., Lips, S.H. and Sagi, M. (2002) Biomass production, transpiration rate and endogenous abscisic acid levels in grafts of flacca and wildtype tomato (Lycopersicon esculentum). *Functional Plant Biology* **29**: 1329–1335

Chen, W., Provart, N. J., Glazebrook, J., Katagiri, F., Chang, H.-S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Chen, X., Lam. S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J.L., Wang, X. and Zhu, T. (2002) Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. *Plant Cell* **14:** 559-574

Cheng, W., Endo, A., Zhou, L., Penney, J., Chen, H., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T., Sheen, J. (2002) A unique short-chain dehydrogenase/reductase inA*arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell* **14:** 2723-2743

Chernys, J.T. and Zeevaart, J.A.D. (2000) Characterization of the 9-*cis*-epoxycarotenoid dioxygenase gene family and the regulation of abscisic acid biosynthesis in Avocado. *Plant Physiology* **124:** 343-353

Christmann, A., Hoffmann, T., Telpova, L. and Grill, E. (2005) Generation of active pools of abscisic acid revealed by *in vivo* imaging of water-stressed *Arabidopsis* plants. *Plant Physiology* **137**: 209-219

Christmann, A., Weiler, E.W., Steudle, E., Grill, E. (2007) A hydraulic signal in root-to-shoot signalling of water shortage. *The Plant Journal* **52**:167–174

Condon, A.G., Richards R.A., Rebetzke G.J., Farquhar G.D. (2004). Breeding for high water use efficiency. *Journal of Experimental Botany* **55**: 2447-2460

Comstock, J. P. (2002). Hydraulic and chemical signaling in the control of stomatal conductance and transpiration. *Journal of Experimental Botany* **53**:195-200

Cornforth, J.W., Milborrow, B.V., Ryback, P.F., Wareing, P.F. (1965) Identity of sycamore 'dormin' with abscisin II. *Nature* **205:**1269

Cornish, K. and Zeevaart, J.A.D. (1985) Movement of Abscisic acid into the apoplast in response to water stress in *Xanthuim strumarium* L. *Plant Physiol*ogy **78**: 623-626

Cornish, K. and Zeevaart, J.A.D. (1988) phenotypic expression of wild-type tomato and three wilty mutants in relation to abscisic acid accumulation in roots and leaflets of reciprocal grafts. *Plant Physiology* **87:** 190-194

Costa, J.M. and Heuvelink, E. (2005). The tomato crop and industry. In *Tomatoes*. 1-19 Edited by Heuvelink. E.CAB Publishing, Oxford.

Creelman, R.A., Mason, H.S., Bensen, R.J., Boyer, J.S., Mullet, J.E. (1990) Water deficit and abscisic acid cause differential inhibition of shoot versus root growth in soybean seedlings. *Plant Physiology* **92**: 205-14

Creelman, R.A. and Zeevaart, J.A.D. (1984) Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. *Plant Physiology* **75:** 166-169

Csizinsky, A.A. (2005). Production in the open field. In *Tomatoes*. 1-19. Edited by Heuvelink. E. CAB Publishing, Oxford.

Cunningham, Jr F.X. and Gantt, E. (1998) Genes and enzymes of carotenoid biosynthesis in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **49:** 557-583

Cunningham, Jr F.X., Pogson, B., Sun, Z., McDonald, K.A., DellaPenna, D. and Gantt, E. (1996) Functional analysis of the and lycopene cyclase enzymes of A*rabidopsis* reveals a mechanism for control of cyclic carotenoid formation. *Plant Cell* **8:** 1613-1626

Cunningham, Jr F.X., Sun, Z.R., Chamovitz, D., Hirschberg, J., and Gantt, E. (1994). Molecular structure and enzymatic function of lycopene cyclase from the cyanobacterium *Synechococcus* sp. strain PCC7942. *Plant Cell* **6:** 1107-1121

Cutler, A.J. and Krochko, J.E. (1999) Formation and breakdown of ABA. *Trends in Plant Science* **4:** 472-477

Cutler, A., Squires, T., Loewen, M., and Balsevich, J. (1997). Induction of (+)-abscisic acid 8' hydroxylase activity by (+)-abscisic acid in cultured maize cells. *Journal of Experimental Botany* **48:** 1787-1795

Cuttriss, A.J. and Pogson, B.J. (2004) Carotenoids. In: *Plant Pigments and their Manipulation. Annual Plant Reviews* **14**: pp 57-91 Blackwell Publishing, Oxford, UK.

Davies, W.J. (1995) Plant hormones and ecophysiology of conifers. *In* Physiological Ecology: Ecophysiology of Coniferous Forests. 63–78. Edited by Smith, W.K. and Hinckley, T.M. Academic Press, London.

Davies, W.J., Bacon, M.A., Thompson, D.S., Sobeih, W. and Rodríguez, L.G. (2000) Regulation of leaf and fruit growth in plants growing in drying soil: exploitation of the plants' chemical signalling system and hydraulic architecture to increase the efficiency of water use in agriculture. *Journal of Experimental Botany* **51**, 1617-162

Davies, W.J., Kudoyarova, G., and Hartung, W. (2005). Long-distance ABA Signaling and Its Relation to Other Signaling Pathways in the Detection of Soil Drying and the Mediation of the Plant's Response to Drought. *Journal of Plant Growth Regulation* **24:** 285-295

Davies, W.J. and Hartung, W. (2004) Has the extrapolation from biochemistry to crop functioning worked to sustain plant production under water scarcity? In; New Directions for a Diverse Planet, *Proceedings of the 4th International Crop Science Congress, Australia.*

Davies, W.J., Mansfield, T.A. and Orton P.J. (1978) *Stratergies employed by plants to conserve water: can we improve on them?* Monograph, British Crop Protection Council, 45-54

Davies, W.J., Tardieu, F. and Trejo, C.L. (1994) How do chemical signals work in plants that grow in drying soil? *Plant Physiol*ogy **104**: 309-314

Davies, W.J., Thompson, D.S. and Taylor, J.E. (1998) Manipulation of growth of horticultural crops under environmental stress. In; *Genetic and Environmental Manipulation of Horticultural Crops.* Edited by Cockshull, K., CAB, Wallingford.

Davies, W.J., Wilkinson, S. and Loveys, B. (2002) Stomatal control by chemical signalling and the exploitation of this mechanism to increase water use efficiency in agriculture. *New Phytologist* **153:** 449-460

Davies, W.J., Zhang, J. (1991) Root signals and the regulation of growth and development of plants in drying soil. *Annual Review of Plant Physiology Plant Molecular Biology* **42:** 55-76

Davison, .PA., Hunter, C.N. and Horton, P. (2002) Overexpression of β-carotene hydroxylase enhances stress tolerance in *Arabidopsis*. *Nature* **418:** 203-206

Debeaujon, I. Leon-Kloosterziel K.M. and Koornneef, **M.** (2000) Influence of the testa on seed dormancy, germination and longevity in *Arabidopsis thaliana*. *Plant Physiology* **122**: 403–414

Dehan, K. and Ta,l M. (1978) Salt tolerance in the wild relatives of the cultivated tomato: Responses of *Solanum pennellii* to high salinity. *Irrigation in Science* **1**: 71–76

DeKoning, A.M.N. (2000) Development and dry matter distribution in glasshouse tomato. PhD Thesis, Wageningen Agricultural University, The Netherlands.

Delseny, M., Bies-Etheve, N., Carles, C.H., Hull, G., Vicient, C., Raynal, M., Grellet, F. and Aspart, L. (2001) Late embryogenesis abundant (LEA) protein gene regulation during *Arabidopsis* seed maturation. *Plant Physiol*ogy **158**: 419-427

Demmig-Adams, B. (1990) Carotenoids and photoprotection in plants: A role for the xanthophyll zeaxanthin. *Biochim. biophys. Acta* **1020**: 1-24, 1990

Demmig-Adams, B., Adams, III W.W.I. (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends in Plant Science* **1:** 21-26

Demmig-Adams, B. and Adams, III W.W. (1992). The xanthophyll cycle. In: *Carotenoids in Photosynthesis*. Edited by Young, A. and Britton, G., Springer: Berlin/ Heidelberg

Demmig-Adams, B. and Adams III W.W. (2002) Antioxidants in photosynthesis and human nutrition. *Science* **298:** 2149-2153

Desikan, R., Cheung, M.K., Bright, J., Henson, D., Hancock, J.T. and Neill, S.J. (2004) ABA, hydrogen peroxide and nitric oxide signaling in stomatal guard cells. *Journal of Experimental Botany* **55:** 205–212

Dielman, J.A. and Heuvelink, E. (1992) Factors affecting number of leaves proceeding the first inflorescence in the tomato. *Journal of Horticultural Science* **67**: 1-10

Dietz, K.J., Saute, A., Wichert, K., Messdaghi, D. and Hartung, W. (2000) Extracellular B-glucosidase activity in barley involved in the hydrolysis of ABA glucose conjugate in leaves. *Journal of Experimental Biology* **51**: 937-944

Dodd, I.C. (2005) Root-to-shoot signalling: assessing the roles of 'up' in the up and down world of long-distance signalling *in planta*. *Plant and Soil* **274**:251–270

Dodd, I.C., Theobald, J. C., Richer, S. K. and Davies W. J. (2009) Partial phenotypic reversion of ABA-deficient flacca tomato (Solanum lycopersicum) scions by a wild-type rootstock: normalizing shoot ethylene relations promotes leaf area but does not diminish whole plant transpiration rate. *Journal of Experimental Biology* **60**: 4029 – 4039

Dorji, K., Behboudian, M.H., Zegbe-Domínguez, J.A. (2005) Water relations, growth, yield, and fruit quality of hot pepper under deficit irrigation and partial rootzone drying. *Sci. Hort.* **104**: 137-149

Dry, P.R. and Loveys, B.R. (1998) Factors influencing grapevine vigour and the potential for control with partial root-zone drying. *Australian Journal for Grapevine Research* **4**: 140-148

Dry, P.R. and Loveys, B.R. (1999) Grapevine shoot growth and stomatal conductance are reduced when part of the root system is dried. *Vitis*, **38**: 151-154

Dry, P.R., Loveys, B.R., Botting, D and During, H. (1996) Effects of partial root-zone drying on grapevine vigour, yield, composition of fruit and water use. *Proceedings of the 9th Australian Wine Industry Technical Conference*: 126-131

Duckham, S.C., Linforth, R.S.T. and Taylor I.B. (1991) Abscisic-acid-deficient mutants at the ABA gene locus of *Arabidopsis thaliana* are impaired in the epoxidation of zeaxanthin. *Plant, Cell and Environment* **14:** 601-606

Ellis, R.J .(1979) The most abundant protein in the world. *Trends inBiochemical Science* **4**: 241–244

Enfissi, E.M.A., Fraser, P.D., Lois, L., Boronat, A., Schuch, W. and Bramley, P.M. (2005) Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato. *Plant Biotechnology Journal* **3:** 17-27

Estevez, J.M., Cantero, A., Reindl, A., Reichler, S. and Leon, P. (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *Journal of Biological Chemistry* **276:** 22901-22909

Fambrini, M., Vernieri, P., Toncelli, M.L., Rossi, V.D. and Pugliesi, C. (1995) Characterization of a wilty sunflower (Helianthus annuus L) mutant. III. Phenotypic interaction in reciprocal grafts from wilty mutant and wildtype plants. *Journal of Experimental Botany* **46**: 525–530

Farquhar, G.D., Ehleringer, J.R. and Aubick, K.T. (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Molecular Biology* **40**: 503-537

Farquhar, G.D., O'Leary, M.H. and Berry, J.A. (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Australian Journal of Plant Physiology* **9:** 121-137

Farquhar, G.D. and Richards, R.A. (1984) Isotopic composition of plant carbon correlates with water-use efficiency of wheat genotypes. *Australian Journal of Plant Physiology* **11:** 539-552

Fernandez-Garcia, N., Martinez, V., Cerda, A. and Carvajal, M. (2002) Water and nutrient uptake of grafted tomato plants grown under saline conditions. *Journal of Plant Physiology* **159**: 899-905

Field, C. B., Berry, J.A. and Mooney, H.A. (1982) A portable system for measuring carbon 15 dioxide and water vapour exchange of leaves. *Plant Cell Environment* **5**: 179-186.

Finch-Savage, W.E., Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytologist* **171:** 501-23

Finkelstein, R.R., Gampala, S.S.L. and Rock, C.D. (2002) Abscisic acid signaling in seeds and seedlings. *Plant Cell* **14:** 515-545

Fisher, R.A., Rees, D., Sayre, K.D., Lu, Z.M., Condon, A.G. and Saavedra, A.L. (1998) Wheat yield progress associated with higher stomatal conductance and photosynthetic rate and cooler canopies. *Crop Science* **38:** 1467-1475

Fischer, R.A. (1981) Optimising the use of water and nitrogen through breeding of crops. *Plant and Soil* **58**: 249–279

Foulkes, M.J., Sylvester-Bradley, R., Weightman, R. and Snape, J.W. (2007) Identifying physiological traits associated with improved drought resistance in winter wheat. *Field Crops Research* **10**3: 11-24

Fraser, P.D., Enfissi, E.M.A., Halket, J,M,, Truesdale, M.R., Yu, D., Gerrish, C. and Bramley, P.M. (2007). Manipulation of phytoene Levels in Tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell* **19**: 3194 – 3211

Fraser, P.D., Truesdale, M.R., Bird, C.R., Schuch, W. and Bramley, P.M. (1994) carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). *Plant Physiology* **105:** 405-413

Fray, R.G. and Grierson, D. (1993) Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. *Plant Molecular Biology* **22:** 589-602

Fray, .RG., Wallace, A., Fraser, P.D., Valero, D., Hedden, P., Bramley, P.M. and Grierson, D. (1995) Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant Journal* **8:** 693-701

French, R.J. and Schultz, J.E. (1984) Water use efficiency in wheat in a mediterranean type environment: II. Some limitations to efficiency. *Australian Journal of Agricultural Research* **35**: 765–775

Frey, A., Audran, C., Marin, E., Sotta, B. and Marion-Poll, A. (1999) Engineering seed dormancy by the modification of zeaxanthin epoxidase gene expression. *Plant Molecular Biology* **39:** 1267-1274

Frey, A., Boutin, J.P., Sotta, B., Mercier, R. and Marion-Poll, A. (2006) Regulation of carotenoid and ABA accumulation during the development and germination of *Nicotiana plumbaginifolia* seeds. *Planta* **224**: 622–632

Frey, A., Godin, B., Bonnet, M., Sotta, B., Marion-Poll, A. (2004) Maternal synthesis of abscisic acid controls seed development and yield in *Nicotiana plumbaginifolia*. *Planta* **218:** 958-964

Fusada, N., Masuda, T., Kuroda, H., Shimada, H., Ohta, H., and Takamiya, T. (2005) Identification of a novel **cis**-element exhibiting cytokinin-dependent protein binding in vitro in the 5'-region of NADPH-protochlorophyllide oxidoreductase gene in cucumber. *Plant Molecular Biology* **59:** 631-45

Galpaz, N., Ronen, G., Khalfa, Z., Zamir, D. and Hirschberg, J. (2006) A chromoplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato *white-flower* locus. *Plant Cell* **18:** 1947-1960

Gamble, P.E. and Mullet, J.E. (1986). Inhibition of carotenoid accumulation and abscisic acid biosynthesis in fluridone treated dark grown barley. *European Journal of Biochemistry* **160**: 117-121

Garcia-Mata, C., Gay, R., Sokolovski, S., Hills, A., Lamattina, L. and Blatt MR (2003**)** Nitric oxide regulates K^+ and Cl^- channels in guard cells through a subs**et** of abscisic acid-evoked signaling pathways. *Proceedings of the National Academy of Sciences of the USA* **100:** 11116–11121

Gaskell, G., Allum, N., Wagner, W., Kronberger, N., Torgersen, H., Hampel, J. and Bardes, J. (2004) GM foods and the misperception of risk perception. *Risk Analysis*, **24**: 185-94.

German, M.A, Kandel-Kfir, M., Swarzberg, D., Matsevitz, T. and Granot, D. (2003) A rapid method for the analysis of zygosity in transgenic plants. *Plant Science* **164:** 183-187

Gittins, J.R., **Hiles, E.R.**, **Pellny, T.K.**, **Biricolti, S. and James, D.J**. (2001) The *Brassica napus extA* promoter: a novel alternative promoter to CaMV 35S for directing transgene expression to young stem tissues and load bearing regions of transgenic apple trees (*Malus pumila* Mill). *Molecular Breeding* **7:**51–62

Gittins, J.R., Pellny, T.K., Hiles, E.R., Rosa, C., Biricolti, S. and James, D.J. (2000) Transgene expression driven by heterologous ribulose-1,5-biphosphate carboxylase/oxygenase small-subunit gene promoters in the vegetative tissues of apple (*Malus pumila* Mill.). *Planta* **210**: 232-240

Giuliano, G., Bartley, G.E., Scolnik, P.A. (1993) Regulation of carotenoid biosynthesis during tomato development. *Plant Cell* **5:** 379-387

Giuliano, **G., Al-Babili, S., von Lintig, J.** (2003) Carotenoid oxygenases: cleave it or leave it. *Trends in Plant Science* **8**:145–149

Glinka, Z. (1980) Abscisic acid promotes both volume flow and ion release to the xylem in sunflower roots. *Plant Physiology* **65:** 537-540

Glinka, Z., and Reinhold, L. (1971) Abscisic acid raises the permeability of plant cells to water. *Plant Physiology* **48**: 103-105

Gollan, T., Passioura, J.B. and Munns, R. (1986) Soil water status affects the stomatal conductance of fully turgid wheat and sunflower leaves. *Australian Journal of Plant Physiology* **13**: 459-464

Gollan, T., Schurr, U. and Schulze, E.D. (1992) Stomatal responses to drying soil in relation to changes in xylem sap composition of *Helianthus annus*, the concentration of cations, anions, amino acids and pH of the xylem sap. *Plant, Cell and Environment* **15**: 551-559

González-Guzmán, M., Apostolova, N., Bellés, J.M., Barrero, J.M., Piqueras, P., Ponce, M.R., Micol, J.L., Serrano, R. and Rodriguez P.L. (2002) The short-chain alcohol dehydrogenase ABA2 catalyzes the conversion of xanthoxin to abscisic aldehyde. *Plant Cell* **14:** 1833-1846

Goodwin, I. and Jerie, P.H. (1992) Regulated deficit irrigation from concept to practice. *Australia and New Zealand Wine Industry Journal* **5**: 131-133

Goodwin, T.W. (1980). The Biochemistry of the Carotenoids. Chapman & Hall, London

Götz, T., Sandmann, G., and Römer, S. (2002). Expression of a bacterial carotene hydroxylase gene (*crtZ*) enhances UV tolerance in tobacco. *Plant Molecular Biology* **50:** 129-142

Gowing, D.J., Davies, W.J., and Jones, H.G. (1990). A positive root-sourced signal as an indicator of soil drying in apple. *Journal of Experimental Botany* **41:** 1535-1540

Grant, O.M. Stoll, M. and Jones, H.G. (2004) Partial rootzone drying does not affect fruit yield of raspberries. *Journal of Horticultural. Science and Biotechnology* **79**: 125-130

Grappin, P., Bouinot, D., Sotta, B., Miginiac, E., Jullien, M. (2000) Control of seed dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta* **210:** 279-285

Griffiths, A. and Bray, E.A. (1996) Shoot induction of ABA-requiring genes in response to soil drying. *Journal of Experimental Botany* **47**: 1525-1531

Groot, S.P.C. and Karssen, C. (1992) Dormancy and germination of abscisic acid-deficient tomato seeds. *Plant Physiol*ogy **99**: 952-958

Groot, S.P.C., Kieliszmksa-Rokicka, B., Vermeer, E. and Karssen, C. (1988) Gibberellin induced hydrolysis of endosperm cell walls in gibberellin-deficient tomato seeds prior to radicle protrusion. *Planta* **174**: 500-504

Groot, S.P.C., Van Yperen, I., and Karssen, C.M. (1991). Strongly reduced levels of endogenous abscisic acid in developing seed of the tomato mutant *sitiens* do not influence *in vivo* accumulation of dry matter and storage proteins. *Plant Physiology* **81:** 7

Guichard, S., Bertin, N., Leonard, C. and Gary, C. (2001) Tomato fruit quality in relation to water and carbon fluxes. *Agronomie* **21**: 385–392

Hall, A.E., Richards, R.A., Condon, A.G., Wright, G.C. and Farquar, G.D. (1994) Carbon isotope discrimination and plant breeding. *Plant Breeding Review* **4**: 81-113

Hamblin, A.P. and Hamblin, J. (1985) Root characteristics of some temperate legume species and varieties on deep, free-draining entisols. *Australian Journal of Agricultural Research* **36:** 63–72

Hamdy, A., Ragab, R. and Scarascia-Mugnozza, E. 2003. Coping with water scarcity: water saving and increasing water productivity. *Irrigation and Drainage* **52**: 3–20

Hanley, M. E. and May, O. C. (2006) Cotyledon damage at the seedling stage affects growth and flowering potential in mature plants. *New Phytologist* **169**:243–250

Hartung, W. and Davies W.J. (1991) Drought induced changes in physiology and ABA. In: *Abscisic Acid, Physiology and Biochemistry*. Edited by Davies, W.J. and Jones H.G., BIOS Scientific, Oxford, UK.

Hartung, W. and Jeschke, W.D. (1999) Abscisic acid: a long-distance stress signal in salt-stressed plants. In: *Plant Responses to Environmental Stress*. *From Phytohormones to Genome reorganization.* Edited by Lerner, H.R., New York, NY: Marcel Dekker.

Hartung, W., Kaiser, W.M., and Burschka, C. (1983). Release of abscisic acid from leaf strips under osmotic stress. *Zeitschrift für Pflanzenphysiologie* **122:** 131-138

Hartung, W. and Radin, J.W. (1989) Abscisic acid in the mesophyll apoplast and in the root system of sap of water stressed plants: The significance of pH gradients. *Current Topics in Plant Biochemistry and Physiology* **8**: 110-124

Hartung, W., Sauter, A. and Hose, E. (2002) Abscisic acid in the xylem: where does it come from, where does it go to? *Journal of Experimental Botany* **53:** 27-32

Harvey, M., Quilley, S., and Beynon, H. (2002). Exploring the tomato. In: *Transformations of nature, society and economy*. Edgar Publishing, Cheltenham, UK.

Heuvelink, E. (2005). Developmental Processes. In *Tomatoes*. 53-85 Edited by Heuvelink. E, CAB Publishing, Oxford.

Hoeffler, J.-F., Grosdemange-Billiard, C. and Rohmer, M. (2001) Elucidation of the 2-*C*-methyl-d-erythritol 4-phosphate pathway for isoprenoid biosynthesis: straightforward syntheses of enantiopure 1-deoxy-d-xylulose from pentose derivatives. *Tetrahedron Letters* **42**: 3065–3067

Horlick-Jones, T. (2006) On evaluating the GM Nation? Public debate about the commercialization of transgenic crops in Britain. *New Genetics and Society* **25**: 265-88

Hilhorst, H.W.M. (1995) A critical update on seed dormancy. *Seed Science Research* **5**: 61-73

Hilhorst, H.W.M. and Downie, B. (1996) Primary dormancy in tomato *(Lycopersicon esculentum* cv. Moneymaker): studies with the *sitzens* mutant. *Journal of Experimental Botany* **47:** 89-97

Hilhorst, H.W.M., Groot, S.P.C and Bino, R.J. (1998) The tomato seed as a model system to study seed development and germination. *Acta Bot Neerl* **47**: 169–183

Hiron, R.W.P. and Wright, S.T.C. (1973) The role of endogenous abscisic acid in the response of plants to stress. *Journal of Experimental Botany* **24:** 769-781

Hirschberg, J. (1998). Molecular biology of carotenoid biosynthesis. In: *Carotenoids - Biosynthesis and Metabolism.* 149-194 Edited by Britton, G., Linaen-Jensen, S., and Pfander, H., Birkhauser Verlag, Basel.

Hirschberg, J. (2001) Carotenoid biosynthesis in flowering plants. *Current Opinion in Plant Biology* **4:** 210-218

Hoad, G.V. (1995). Transport of hormones in the phloem of higher plants. *Plant Growth regulation* **16:** 173-182

Holbrook, N.M., Shashidhar, V.R., James, R.A., Munns, R. (2002) Stomatal control in tomato with ABA-deficient roots: Response of grafted plants to soil drying. *Journal of Experimental Botany* **53:** 1503-1514

Hopkins, W.G. (1999) *Introduction to Plant Physiology* (Second Edition) John Wiley and Sons Inc, New York.

Horie, T., Matsuura, S., Takai, T., Kuwasaki, K., Ohsumi, A. and Shiraiwa, T. (2006) Genotypic differences in canopy diffusive conductance measured by a new remote sensing method and its association with the differences in rice yield potential. *Plant, Cell and Environment* **29**: 653-660

Horton, P., Ruben, A.V., and Walters, R.G. (1996). Regulation of light harvesting in green plants. *Annual review of Plant Physiology and Plant Molecular Biology* **47:** 655-684

Hose, E., Steudle, E. and Hartung, W. (2000). Abscisic acid and hydraulic conductivity of maize roots: a study using cell- and root pressure probes. *Planta* **211:** 874-882

Howitt, C.A. and Pogson, B.J. (2006) Carotenoid accumulation and function in seeds and non-green tissues. *Plant Cell Environment* **29**: 435-445

Huang, D., Jaradat, M.R., Wu, W., Ambrose, S.J., Ross, A.R., Abrams, S.R. and Cutler, A.J. (2007) Structural analogues of ABA reveal novel features of ABA perception and signalling in *Arabadopsis. The Plant Journal* **50**: 414-428

Hussain, A., Black, C.R., Taylor, I.B. and Roberts, J.R. (1999) Soil compaction. A role for ethylene in regulating leaf expansion and shoot growth in tomato? *Plant Physiology* **121**:1227-1237

Hussey, G. (1963). Growth and development in the young tomato. I. The effect of temperature and light intensity on growth of the shoot apex and leaf primordia. *Journal of Experimental Botany* **14:** 316-25

Hwang, J.U. and Lee, Y. (2001) Abscisic acid-induced actin reorganization in guard cells of dayflower is mediated by cytosolic calcium levels and by protein kinase and protein phosphatase activities. *Plant Physiology* **125**: 2120–21

Ingram, J. and Bartels, D. (1996) The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47:** 377-403

Inskeep, W. P. and Bloom, P. R. (1985) Extinction coefficients of chlorophyll a and b in N,N-Dimethylformamide and 80% Acetone. *Plant Physiology*, **77**: 483-485

Irving, H., Gehring, C. and Parish, R. (1992) Changes in cystolic pH and calcium of guard cells precede stomatal movements. *Proceedings of the National Academy of Sciences of the USA* **89**: 1790-1794

Iuchi, S., Kobayashi, M., Yamaguchi-Shinozaki, K., Shinozaki, K. (2000) A stress-inducible gene for 9-*cis*-epoxycarotenoid dioxygenase involved in abscisic acid biosynthesis under water stress in drought-tolerant cowpea. *Plant Physiology* **123:** 553-562

Iuchi, S., Kobayashi, M., Taji, T., Naramoto, M., Seki, M., Kato, T., Tabata, S., Kakubari, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2001) Regulation of drought tolerance by gene manipulation of 9-*cis*-epoxycarotenoid dioxygenase, a key enzyme in abscisic acid biosynthesis in *Arabidopsis*. *The Plant Journal* **27:** 325-333

Jacobsen, J.V., Pearce, D.W., Poole, A.T., Pharis, R.P., and Mander, L.N. (2002). Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. *Plant Physiology* **115:** 428-441

Jeschke, W.D., Peuke A.D., Pate J.S. and Hartung, W. (1997) Transport, synthesis and catabolism of abscisic acid (ABA) in intact plants of castor bean (*Ricinus communis* L.) under phosphate deficiency and moderate salinity. *Journal of Experimental Botany* **48**: 1737–1747

Jia, W.S. and Davies, W.J. (2007) Modification of leaf apoplastic pH in relation to stomatal sensitivity to root-sourced ABA signals. *Plant Physiology* **143**:68–77

Jia, W.S. and Zhang, J.H. (1999) Stomatal closure is induced rather by prevailing xylem abscisic acid than by accumulation amount of xylem derived abscisic acid. *Physiologia Plantarum* **106**: 268-275

Jiang, F. and Hartung, W. (2008) Long-distance signalling of abscisic acid (ABA): the factors regulating the intensity of the ABA signal. *Journal of Experimental Botany* **59**: 37–43

Jiang, F., Jeschke, W.D. and Hartung, W. (2004) Abscisic acid (ABA) flows from *Hordeum vulgare* to the hemiparasite *Rhinanthus minor* and the influence of infection on host and parasite abscisic acid relations. *Journal of Experimental Botany* **55**: 2323–2329

Johnson, M.P., Havaux, M., Triantaphylidès, C., Ksas, B., Pascal, A.A., Robert, B., Davison, P.A., Ruban, A.V. and Horton, P. (2007) Elevated zeaxanthin bound to oligomeric LHCII enhances the resistance of Arabidopsis to photooxidative stress by a lipid-protective, antioxidant mechanism. *Journal of Biological Chemistry* **282**: 22605-22618

Jones, H.G. (1976) Crop characteristics and the ratio between assimilation and transpiration. *Journal of Applied Ecology* **13**: 605–622

Jones, H.G. (1985) Partitioning stomatal and non-stomatal limitations to photosynthesis. *Plant, Cell and Environment* **8**: 95-104

Jones, H.G. (1992) Further analysis of WUE. In: *Plants and microclimate* Edited by Jones, H.G. Press Syndicate of the University of Cambridge.

Jones, H. G. (2004) What is water use efficiency. In: *Water use efficiency in plant biology*. 27–41 Edited by Bacon, M. A., Blackwell Publishing, Oxford.

Jones, R.J. and Brenner, M.L. (1987). Distribution of abscisic acid in maize kernels during grain filling. *Plant Physiology* **83:** 905-909

Jones, H.G., Luton, M.T., Higgs, K.H. and Hamer, P.J. (1983) Experimental control of water status in an apple orchard. *Journal of Horticultural Science* **58**: 301-316

Jones, M.O., Manning, K., Andrews, J., Wright, C., Taylor, I.B. and Thompson, A.J. (2008) The promoter from SlREO, a highly-expressed, root-specific *Solanum lycopersicum* gene, directs expression to cortex of mature roots. *Functional Plant Biology* **35:** 1224 – 1233

Jones, R.J. and Mansfield, R. (1970) Suppression of stomatal opening in leaves treated with abscisic acid. *Journal of Experimental Botany* **21**: 714-719

Kaiser, W.M. and Hartung, W. (1981) Uptake and release of abscisic acid by isolated photoautotrophic mesophyll cells, depending on pH gradients. *Plant Physiology* **68:** 202-206

Kaldenhoff, R., Ribas-Carbo, M., Flexas, J., Lovisolo, C., Heckwolf, M. and Uehlein, U. (2008) Aquaporins and plant water balance. *Plant Cell Environment* **31**: 658–666

Kalituho, L., Rech, J. and Jahns, P. (2007) The roles of specific xanthophylls in light utilization. *Planta*, **225**: 423-439

Kaldenhoff, R. and Fischer, M. (**2006**) Aquaporins in plants. *Acta Physiologia* **187**: 169–176

Kalra, N. (2007) Impacts of climate change on agriculture. *Outlook on Agriculture* **36**: 109–118

Kang, S. and Zhang, J. (2004) Controlled alternate partial root-zone irrigation: its physiological consequences and impact on water use efficiency. *Journal of Experimental Botany* **55**: 2437-2446

Kang, S., Zhang, L., Hu, X. and Jerie, P. (2000) An improved water use efficiency for hot pepper grown under controlled alternate drip irrigation on partial roots. *Scientia Horticulturae* **89**: 257-267

Karssen, C.M., Brinkhorst-van der Swan, D.L.C., Breekland, A.E. and Koornneef, M. (1983) Induction of dormancy during seed development by endogenous abscisic acid: studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.). *Planta* **157:** 158-165

Kermode, A.R. (2005) Role of abscisic acid in seed dormancy. *Journal of Plant Growth Regulation* **24:** 319-344

Kerr, E.A. (1963) Germination of tomato seed as affected by fermentation time, variety, fruit maturity and harvest date. Report of the horticultural experiment station and products laboratory, Ontario. In: *Horticulture Abstracts* **34**: 1090

King, R.W. (1976) Abscisic acid in developing wheat grains and its relationship to grain growth and maturation. *Planta* **132**: 43-51

Kirda, C., Topcu, S., Kaman, H., Ulger, A.C., Yazici, A., Centin, M. and Derici, M.R. (2005) Grain yield response and N-fertiliser recovery of Maize under deficit irrigation. *Field Crop Research* **93**: 132-141

Knight, C.D., Seghal, A., Atwal, K., Wallace, J.C., Cove, D.J., Coates, D., Quantrano, R.S., Bahadur, S., Stockley, P.G. and Cuming, A.C. (1995) Molecular responses to abscisic acid and stress are conserved between moss and cereals. *Plant Cell*, **7**: 499-506.

Koorneef, M., Reuling, G. and Karassen, C.M. (1984) The isolation and characterisation of ABA insensitive mutants of *Arabidopsis*. *Physiologia Plantarum* **61:** 377-383

Kramer, P.J. (1980) Drought stress and origins of adaption. In: *Adaption of Plants to Water and High Temperature Stress.* Edited by Turner, N.C. and Kramer, P.J. John Wiley and Sons, New York.

Krochko, J.E., Abrams, G.D., Loewen, M.K., Abrams, S.R. and Cutler, A.J. (1998) (+)-abscisic acid 8′-hydroxylase is a cytochrome P450 monooxygenase. *Plant Physiology* **118:** 849-860

Kühlbrandt, W., Wang, D.N. and Fujiyoshi, Y. (1994) Atomic model of plant light-harvesting complex by electron crystallography. *Nature* **367**: 614-621

Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T, Hirai N, Koshiba T, Kamiya Y, Nambara E (2004) The *Arabidopsis* cytochrome P450 CYP707A encodes ABA 8′-hydroxylases: key enzymes in ABA catabolism. *EMBO Journal* **23:** 1647-1656

Lawlor, A. (2000) *Photosynthesis*, (Third Edition) BIOS: Scientific Publishers.

Lee, K.H., Piao, H.L., Kim, H.Y., Choi, S.M., Jiang, **F., Hartung, W., Hwang, I., Kwak, J.M., Lee, I.J., Hwang, I.** (2006) Activation of glucosidase via stress-induced polymerization rapidly increases active pools of abscisic acid. *Cell* **126**:1109–1120

Lefebvre, V., North, H., Frey, A., Sotta, B., Seo, M., Okamoto, M., Nambara, E. and Marion-Poll, A. (2006) Functional analysis of *Arabidopsis NCED6* and *NCED9* genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. *Plant Journal* **45:** 309-319

Leishman, M. R. and. Westoby, M. (1994) The role of large seed size in shaded conditions: experimental evidence. *Functional Ecology* **8**: 205–214

Leon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A.D., Koornneef, M. (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *The Plant Journal* **10:** 655-661

LeNoble, M.E., Spollen, W.G. and Sharp, R.E. (2004) Maintenance of shoot growth by endogenous ABA: genetic assessment of the involvement of ethylene suppression. *Journal of Experimental Botany* **55**: 237–245

Leung, J. and Giraudat, J. (1998) Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology* **49:** 199-222

Leydecker, M.T., Moureaux, T., Kraepiel, Y., Schnorr, K. and Caboche, M. (1995) Molybdenum cofactor mutants, specifically impaired in xanthine dehydrogenase activity and abscisic acid biosynthesis, simultaneously over-express nitrate reductase. *Plant Physiology* **107:** 1427-1431

Liang, J., Zhang, J. and Wong, M.H. (1997) How do roots control xylem sap ABA concentration in response to soil drying. *Plant and Cell Physiology* **38**: 10-16

Li, X-P., Bjorkman, O., Shih, C., Grossman, A.R., Rosenquist, M., Jansson, S., and Niyogi, K. A. (2000). Pigment-binding protein essential for regulation of photosynthetic light harvesting. *Nature* **403:** 391-395

Lichtenthaler, H.K., Rohmer, M. and Schwender, J. (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. *Physiolia Plantanum* **101:** 643-652

Lindgren, L.O., Stålberg, K.G. and Höglund, A.S. (2003) Seed-specific overexpression of an endogenous *Arabidopsis* phytoene synthase gene results in delayed germination and increased levels of carotenoids, chlorophyll, and abscisic acid. *Plant Physiol*ogy **132**: 779-785

Linforth, R.S.T., Bowman, W.R., Griffin, D.A., Marples, B.A. and Taylor, I.B. (1987) 2-trans-ABA-alcohol accumulation in the wilty tomato mutants *flacca* and *sitiem. Plant Cell Environment* **10**: 599-606

Liotenberg, S., North, H. and Marion-Poll, A. (1999) Molecular biology of Abscisic acid biosynthesis in plants. *Plant Physiological Biochem*istry **37**: 341-350

Liu, F., Shahnazari, A., Andersen, M.N., Jacobsen, S-E. and Jensen, C.R. (2006) Physiological responses of potato (*Solanum tuberosum* L.) to partial root-zone drying: ABA signalling, leaf gas exchange, and water use efficiency. *Journal of Experimental Botany* **57**: 3727-3735

Liu, X., Yue, Y., Li, B., Nie, Y., Li, W., Wu, W. and Ma, L. (2007) A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. *Science* **315:** 1712-1716

Lois, L.M., Campos, N., Putra, S.R., Danielsen, K., Rohmer, M. and Boronat, A. (1998) Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamine, and pyridoxol biosynthesis. *Proceedings of the National Academy of Science USA* **95:** 2105-2110

Lopez-Molina, L., Mongrand, S. and Chua, N.-H. (**2001**) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in *Arabidopsis*. *Proceedings of the National Academy of Science USA* **98:** 4782-4787

Lovell, P.H. and Moore, K.G. (1970) A comparative study of cotyledons as assimilatory organs. *Journal of Experimental Botany* **21**: 1017-1030

Loveys, B.R. (1991) What use is knowledge of ABA physiology for crop improvement? In; *Environmental Plant Biology. Physiology and Biochemistry of ABA.* 245-259.Oxford, Bios Scientific Publishers.

Loveys, B.R., Dry, P.R., Stoll, M. and McCarty, M.G. (2000) Using plant physiology to improve the water use efficiency of horticultural crops. *Acta Horticulturae* **537**: 187-199

Loveys, B.R., Stoll, M. and Davies, W.J. (2004) Physiological approaches to enhance water use efficiency in agriculture: exploiting plant signalling in novel irrigation practice. In: *Water Use Efficiency in Plant Biology*. 113–141. Edited by Bacon M.A., Blackwell Publishing: Oxford.

Ma, Y., Szostkiewicz, I., Korte, A., Moes, D., Yang, Y., Christman, A. and Grill, E. (2009) Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science* **324:** 1064-1068

MacRobbie, E.A.C. (1988) Stomatal guard cells. In: *Solute Transport in Plant Cells and Tissues.* 453-491, Edited by Barker D.A. and Hall J.L., John Wiley and Sons, Inc, New York.

Mandel, M., Feldmann, K.A., Herrera-Estrella, L., Rocha-Sosa, M. and León, P. (1996) *CLA1*, a novel gene required for chloroplast development, is highly conserved in evolution. *Plant J*ournal **9**: 649-658

Mannion, A. 1995. *Agriculture and Environmental Change.* John Wiley and Sons, Toronto, Ontario, Canada.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey, A. and Marion-Poll, A. (1996) Molecular identification of zeaxanthin epoxidase of Nicotiana plumbaginifolia, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. *EMBO Journal* **15:** 2331-2342

Marin, E. and Marion-Poll, A. (1997) Tomato *flacca* mutant is impaired in ABA aldehyde oxidase and xanthine dehydrogenase activities. *Plant Physiology Biochemistry* **35**: 369-372

Martínez-Fernández, J. and Selma, M. A. (2004). Assessing the sustainability of Mediterranean intensive agricultural systems through the combined use of dynamic system models, environmental modelling and geographical information systems. *Handbook of sustainable development planning: studies in modelling and decision support.* Cheltenham: Edward Elgar Publishing.

Matthews, M.A. and Anderson, M.M. (1988) Fruit ripening in *Vitis vinifore* L. responses to seasonal water deficit. *Viti*c **39**: 313-320

Matthews, M.A., Ishii, R., Anderson, M.M. and O'Mahony, M. (1990) Dependence of wine sensory attributes on vine water status. *Journal of the Science of Food and Agriculture* **51**: 321-335

Maunders, M.A., Holdsworth, M.J., Slater, A., Knapp, J.E., Bird, C.R., Schuch, W. and Grierson, D. (1987) Ethylene stimulates the accumulation of ripening-related mRNAs in tomatoes. *Plant, Cell and Environment* **10**: 177-184

Maximov, N.A. (1929) *Plants in Relation to Water*. Allen and Unwin Ltd., London.

McAinsh, M.R., Brownlee, C.and Hetherington, A.M. (1990) Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. *Nature* **343:** 186-188

McCarthy, M.G. (1997) The effects of transient water deficit on berry development of *cv.* Shiraz (*Vitis vinifera* L.). *Australian Journal of Grape and Wine Research* **3**: 102-108

Merlot, S., Mustilli, A.C., Genty, B., North, H., Lefebvre, V., Sotta, B., Vavasseur, A. and Giraudat, J. (2002) Use of infrared thermal imaging to isolate *Arabidopsis* mutants defective in stomatal regulation. *Plant J*ournal **30**: 601-609

Milborrow, B.V. (1967) The identification of (+)-abscisicin II ((+)-dormin) in plants and measurements of its concentration. *Planta*, **76**: 93-113

Millar, A.A., Jacobsen, J.V., Ross, J.J., Helliwell, C.A., Poole, A.T., Scofield, G., Reid, J.B. and Gubler, F. (2006) Seed dormancy and ABA metabolism in *Arabidopsis* and barley: the role of ABA 8'-hydroxylase. *Plant Journal* **45**: 942-954.

Mingo, D.M., Bacon, M.A. and Davies W.J. (2003) Non-hydraulic regulation of fruit growth in tomato plants (*Lycopersicon esculentum* cv. Solairo) growing in drying soil. *Journal of Experimental Botany* **54**:1205-1212

Mingo, D.M., Theobald, J.C., Bacon, M.A., Davies, W.J. and Dodd, I.C. (2004) Biomass allocation in tomato (*Lycopersicon esculentum*) plants grown under partial rootzone drying: enhancement of root growth. *Functional Plant Biol*ogy **31**: 971-978

Monteith, J.L. and Unsworth, M. (1990) *Principles of Environmental Physics*. Edward Arnold: London.

Mousseron-Canet, M. J. C., Mani, J. C., Dalle, J. P., and Olive, J. L. (1968). Photooxydation sensibilisee de quelques composes apparentes a la dehydro-â-ionone. Synthese de l'ester methylique de la (±)-abscisine. *Bulletin de la Societe Chimique de France* **12:** 3864-3878

Munns, R. (1992) A leaf elongation assay detects an unknown growth inhibitor in xylem sap from wheat and barley. *Australian Journal of Plant Physiology* **19**: 127-35

Munns, R. and Sharp, R.E. (1993) Involvement of abscisic acid in controlling plant growth in soils of low water potential. *Australian Journal of Plant Physiology* **20**: 425-437

Murata, Y., Pie, Z.-M., Mori, I.C. and Schroeder, J. (2001) Abscisic acid activation of plasma membrane Ca2+ channels in guard cells requires cystolic NAD(P)H and is differentially distributed upstream and downstream of reactive oxygen species production in aba1 and *aba 2* protein2 mutants. *Plant Cell* **13**: 2513-2523

Mustilli, A.C., Merlot, S., Vavasseur, A., Fenzi, F. and Giraudat, J. (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. *Plant Cell* **14**: 3089-3099

Nagel, O.W., Konings, H. and Lambers, H. (1994) Growth rate, plant development and water relations of the ABA-deficient tomato mutant *sitiens*. *Physiologia Plantarum* **92**: 102–108

Nambara, E. and Marion-Poll, A. (2003). ABA action and interactions in seeds. *Trends in Plant Science* **8**: 213-217

Nambara, E. and Marion-Poll, A. (2005) Abscisic acid biosynthesis and catabolism. *Annual Review of Plant Biology* **56:** 165-185

Neill, S.J., Desikan, R., Clarke, A. and Hancock, J. (2002) Nitric oxide is a novel component of abscisic acid signalling in stomatal guard cells. *Plant Physiology* **128**: 13-16

Neill, S.J., Horgan, R. and Rees, A.F. (1987) Seed development and vivipary in *Zea mays* L. *Planta* **171**: 358-364

Ng, C.K.-Y., Carr, K., McAnish, M.R., Powell, B. and Hetherington, A.M. (2001) Drought induced guard cell signal transduction involves sphigosive1-phosphate. *Nature* **410**: 596-599

Ni, M., Cui, D., Einstein, J., Narasimhulu, S., Vergara, C.E. and Gelvin, S.B. (1995) Strength and tissue-specificity of chimeric promoters derived from the octopine and mannopine synthase genes. *Plant* Journal **7**: 661-676.

Niyogi, K.K. (2000) Safety valves for photosynthesis. *Current Opinion Plant Biology* **3**: 455–560.

Niyogi, K.K., Grossman, A.R. and Bjorkman, O. (1998) *Arabidopsis* mutants define a central role for the xanthophyll cycle in the regulation of photosynthetic energy conversion. *Plant Cell* **10:** 1121-1134

North, H.M., De Almeid, A., Boutin, J.P., Frey, A., To, A., Botran, L., Sotta, B. and Marion-Poll, A. (2007) The Arabidopsis ABA-deficient mutant aba4 demonstrates that the major route for stress-induced ABA accumulation is *via* neoxanthin isomers. *The Plant Journal* **50**: 810-24

Ogren, W.L. and Bowes, G. (1971) Ribulose diphosphate carboxylase regulates soybean photorespiration. *Nat. New Biol*ogy **230**: 159-160

Oh, S.-J., Song, S.I., Shic, K.Y., Jang, H.-J., Kim, S.Y., Kim, M., Kim, Y.-K., Nahm, B.H., and Kim, J.K. (2005) *Arabidopsis* CBF3/DREB1A and ABF3 in transgenic rice increased tolerance to abiotic stress without stunting growth. *Plant Physiology* **138**: 341–351

Ohkuma, K., Addicott, F.T., Smith, O.E., and Thiessen, W.E. (1965). The structure of Abscisin II. *Tetrahedron Letters* **29:** 321-332

Ohkuma, K., Lyon, J.L., Addicott, F.T. and Smith, O.E. (1963) Abscisin II, an abscission-accelerating substance from young cotton fruit. *Science* **142**: 1592-1593

Okada, K., Saito, T., Nakagawa, T., Kawamukai, M. and Kamiya, Y. (2000) Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in *Arabidopsis*. *Plant Physiology* **122:** 1045-1056

Okamoto, M., Kuwahara, A., Seo, M., Kushiro, T., Asami, T., Hirai, N., Kamiya, Y., Koshiba, T. and Nambara, E. (2006) CYP707A1 and CYP707A2, which encode abscisic acid 8′-hydroxylases, are indispensable for proper control of seed dormancy and germination in *Arabidopsis.* *Plant Physiology* **141:** 97-107

OMFRA (2003) Growing greenhouse vegetables 2003 supplement. *Publications 371S*. Ontario Ministry for Food Agriculture and Rural Affairs, Canada

Ooms, J.J.J., Leon-Kloosterziel, K.M., Bartels, D., Koornneef, M., Karssen, C.M. (1993) Acquisition of desiccation tolerance and longevity in **seed**s of *Arabidopsis thaliana* (a comparative study using abscisic acid-insensitive abi3 mutants). *Plant Physiol*ogy **102**: 1185–1191

Outlaw, WH. (2003) Integration of cellular and physiological functions of guard cells *Critical Reviews in Plant Sciences* **22**: 503–529

Oweis, T., Zhang, H., and Pala, M. (2000) Water use efficiency of rain-fed and irrigated bread wheat in a Mediterranean environment. *Agronomy Journal* **92**:231-238

Page, J.E., Hause, G., Raschke, M., Gao, W.Y., Schmidt, J., Zenk, M.H. and Kutchan, T.M. (2004) Functional analysis of the final steps of the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway to isoprenoids in plants using virus-induced gene silencing. *Plant Physiol*ogy **134**: 1401-1413

Park, S-Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y., Lumba, S., Santiago, J., Rodrigues, A., Chow, T-S., Alfred, S.E., Bonetta, D., Finkelstein, R., Provart, N.J., Desveaux, D., Rodriguez, P.L., McCourt, P., Zhu, J-K., Schroeder, J.I., Volkman, B.F. and Cutler, S.R. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of ABA-binding START proteins. *Science* **324**: 1068-1071

Parry, M.A.J., Flexas, J. and Medrano, H. (2005) Prospects for crop production under drought: research priorities and future directions. *Annuals of Applied Biol*ogy **147**: 211-226

Parsons, R., Weyers, J.D.B., Lawson, T. and Godber, I.M (1997) Rapid and straightforward estimates of photosynthetic characteristics using a portable gas exchange system. *Photosynthetica* **34**: 265-279

Parry, M.A.J., Neill, J.J. and Horgan, R. (1988) Xanthoxin levels and metabolism in wildtype and wilty mutants of tomato. Planta, **173**: 392-404

Passioura, J.B. (1977) Grain yield, harvest index and water useof wheat. *Journal of Australian Institute of Agricultural Science* **43**: 117–120

Passioura, J.B. (2002) Environmental biology and crop improvement. *Functional Plant Biol*ogy **29**: 537-546

Pecker, I., Gabbay, R., Cunningham, F.X. and Hirschberg, J. (1996) Cloning and characterization of the cDNA for lycopene-cyclase from tomato reveals decrease in its expression during fruit ripening. *Plant Molecular Biology* **30:** 807-819

Peet, M.M. (2005). Irrigation and fertilisation. In *Tomatoes*. 171-199 Edited by Heuvelink. E.CAB Publishing: Oxford.

Pei, Z.M. and Kuchitsu, K. (2005) Early ABA signalling events in the guard cells. *Journal of Plant Growth Regulation* **24**: 296-307

Pei, Z.M., Murata, Y., Bennig, G., Thomine, S., Kluster, B., Allen, G., Grill, E. and Schroeder, J. (2000) Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* **406**: 731-734

Pelligrineschi, A., Reynolds, M., Pacheco, M., Brito, R.M., Almeraya, R., Yamaguchi- Shinozaki, K. and Hoisington, D. (2004) Stress-induced expression in wheat of *Arabidopsis thaliana* DREB1A gene delays water stress symptoms under greenhouse conditions. *Genome* **47**: 493-500

Pierce, M.L. and Raschke, K. (1980) Correlation between loss of turgor and accumulation of abscisic acid in detached leaves. *Planta* **148**: 174-182

Picken, A.J.F. (1984) A review of pollination and fruit set in tomato. *Journal of Horticultural Science* **59**: 1-13

Picken, A.J.F., K. Stewart and K. Klapwicjk. 1986. Germination And Vegetative Development. In: *The Tomato Crop*. 167-200. Edited by Chapman and Hall, London.

Pogson, B., McDonald, K.A., Truong, M., Britton, G. and DellaPenna, D. (1996) *Arabidopsis* carotenoid mutants demonstrate that lutein is not essential for photosynthesis in higher plants. *Plant Cell* **8:** 1627-1639

Pogson, B.J. and Rissler, H.M. (2000). Genetic manipulation of carotenoid biosynthesis and photoprotection. *Philosophical Transactions of the Royal Societyof London* **355:** 1395-1403

Portree, J. (1996) Greenhouse vegetable production guide. British Columbia Ministry of Agriculture Fisheries and Food, British Columbia.

Priest, D.M., Ambrose, S.J., Vaistij, F.E., Elias, L., Higgins, G.S., Ross, A.R.S., Abrams, S.R. and Bowles, D.J. (2006) Use of the glucosyltransferase UGT71B6 to disturb abscisic acid homeostasis in *Arabidopsis thaliana*. *Plant Journal* **46**: 492-502.

Qin, X. and Zeevaart, J.A.D. (1999) The 9-cis-epoxycarotenoid cleavage reaction is the key regulatory step of abscisic acid biosynthesis in water-stressed bean. *Proceedings of the National Academy of Sciences USA* **96:** 15354-15361

Qin, X. and Zeevaart, J.A.D. (2002) Overexpression of a 9-*cis*-epoxycarotenoid dioxygenase gene in *Nicotiana plumbaginifolia* increases abscisic acid and phaseic acid levels and enhances drought tolerance. *Plant Physiology* **128:** 544-551

Quarrie, S.A. (1987) Use of genotypes differing in endogenous abscisic acid levels in studies of physiology and development. In: *Hormone Action in Plant Development: A Critical Appraisal.* 89–105 Edited by Hoad, G.V., Lenton, J.R., Jackson, M.B., Atkin, R.K. London: Butterworths.

Quarrie, S.A. and Jones, H.G. (1977) Effects of abscisic acid and water stress on development and morphology of wheat. *Journal of Experimental Botany* **28:** 192-203

Quarrie, S.A., Whitford, P.N., Appleford, N.E.J., Wang, T.L., Cook, S.K., Henson, I.E. and Loveys, B.R. (1988) A monoclonal antibody to (S)-abscisic acid: its characterisation and use in a radioimmunoassay for measuring abscisic acid in crude extracts of cereal and lupin leaves. *Planta* **173:** 330-339

Quayle, J.R., Fuller, R.C., Benson, A.A. and Calvin, M. (1954) Enzymatic carboxylation of ribulose diphosphate. *Journal of American Chemical Society* **76**: 3610-3612

Raschke, K., Shabahang, M. and Wolf, R. (2003) The slow and the quick anion conductance in whole guard cells: their voltage-dependent alternation, and the modulation of their activities by abscisic acid and CO2. *Planta* **217:** 639-650

Raz, V., Bergervoet, J. H. W., and Koornneef, M. (2001). Sequential steps for developmental arrest in Arabidopsis seeds. *Development* **128:** 243-255

Razem, F.A., El-Kereamy, A., Abrams, S.R. and Hill, R.D. (2006) The RNA-binding protein FCA is an abscisic acid receptor. *Nature* **439:** 290-294

Rebetzke, G.J., Condon, A.G. Richards, R.A. and Farquhar, G.J. (2002) Selection for reduced carbon-isotope discrimination increases aerial biomass and grain yield of rainfed bread wheat. *Crop Science* **42**: 739-745

Richter, G.M. and Semenov, M.A. (2005) Modelling impacts of climate change on wheat yields in England and Wales - assessing drought risks. *Agricultural Systems* **84**: 77-97

Robertson, D.S. (1955) The genetics of vivipary in maize. *Genetics* **40:** 745-760

Rock, C. and Zeevaart, J. (1991) The *aba* mutant of *Arabidopsis thaliana* is impaired in epoxy-carotenoid biosynthesis. *Proceedings of the National Academy of Sciences USA* **88:** 7496-7499

Rock, C. and Quatrano, R. (1995) The Role of Hormones During Seed Development. In: *Plant Hormones. Physiology, Biochemistry and Molecular Biology*, (Second Edition) 671-697. Edited by P Davies, Klewer Academic Publishers: Dordrecht, The Netherlands.

Rodermel, S.R., Abbott, M.S. and Bogorad, L. (1988) Nuclear-organelle interactions: nuclear antisense gene inhibits ribulose biphosphate carboxylase enzyme levels in transformed tobacco plants. *Cell* **55**: 673-681

Rodrigo, M-, Alquezar, B. and Zacarias, L. (2006) Cloning and characterization of two 9-*cis*-epoxycarotenoid dioxygenase genes, differentially regulated during fruit maturation and under stress conditions, from orange (*Citrus sinensis* L. Osbeck). *Journal of Experimental Botany* **57:** 633-643

Rodriguez-Concepcion, M., Ahumada, I., Diez-Juez, E., Sauret-Gueto, S., Loist, L.M., Gallego, F., Carretero-Paulet, L., Campos, N., and Boronat, A. (2001) 1-Deoxy-D-xylulose 5-phosphate reductoisomerase and plastid isoprenoid biosynthesis during tomato fruit ripening. *Plant Journal* **27:** 213-222

Rodriguez-Concepcion, M. and Boronat, A. (2002) Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids. A metabolic milestone achieved through genomics. *Plant Physiology* **130:** 1079-1089

Ronen, G., Carmel-Goren, L., Zamir, D. and Hirschberg, J. (2000) An alternative pathway to beta –carotene formation in plant chromoplasts discovered by map-based cloning of Beta and old-gold color mutations in tomato. *Proceedings of the National Academy of Sciences USA* **97:** 11102-11107

Ronen, G., Cohen, M., Zamir, D. and Hirschberg, J. (1999) Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant *Delta*. *The Plant Journal* **17:** 341-351

Rossel, J.B., Wilson, I.W. and Pogson, B.J. (2002) Global changes in gene expression in response to high light in *Arabidopsis*. *Plant Physiol*ogy **130**: 1109-1120

Royle, P. (2004) Regulation of the ABA biosynthetic pathway. PhD Thesis, University of Nottingham

Rudich, J. and Luchinsky, U. (1986) Water economy. In: *The tomato crop: a scientific basis for improvement.* Edited by Atherton, J.G. and Rudich, J., London, New York: Chapman and Hall.

Saab, I.N., Sharp, R.E., Pritchard, J. and Voetberg, G.S. (1990) Increased endogenous abscisic acid maintains primary root growth and inhibits shoot growth of maize seedlings at low water potentials. *Plant Physiology* **93:** 1329-1336

Sade, N., Gebretsadik, M., Seligmann, R., Schwartz, A., Wallach, R and Moshelion, M (2010) The Role of Tobacco Aquaporin1 in Improving Water Use Efficiency, Hydraulic Conductivity, and Yield Production Under Salt Stress. *Plant Physiology* **152**: 245-254

Sagi, M., Scazzocchio, C. and Fluhr, R. (2002) The absence of molybdenum cofactor sulfuration is the primary cause of the *flacca* phenotype in tomato plants. *Plant J*ournal **31**: 305-317

Saliendra, N.Z., Sperry, J.S. and Comstock, J.P. (1995) Influence of leaf water status on stomatal response to humidity, hydraulic conductance, and soil drought in *Betula occidentalis. Planta* **196:** 357-66

Saltvielt, M.E. (2005). Fruit ripening and fruit qualtiy. In *Tomatoes*. 145-171. Edited by Heuvelink. E. CAB Publishing: Oxford.

Sanguineti, M.C., Tuberosa, R., Landi, P., Maccaferri, M., Casarini, E. and Conti, S. (1999) QTL analysis of drought related traits and grain yield in relation to genetic variation for leaf abscisic acid concentration in field grown maize. *Journal of Experimental Botany* **50**: 1289-1297

Santos, H.P. and Buckeridge, M.S. (2004) The role of the storage carbon of cotyledons in the establishment of seedlings of *Hymenaea courbaril* under different light conditions. *Annals of Botany* **94**: 819-830

Sauter, A., Davies, W. J., and Hartung, W. (2001). The long-distance abscisic acid signal in the droughted plant: the fate of the hormone on its way from root to shoot. *Journal of Experimental Botany* **52:** 1991-1997

Sauter, A., Dietz, K. and Hartung, W. (2002) A possible stress physiological role of abscisic acid conjugates in root-to-shoot signalling. *Plant, Cell and Environment* **25:** 223-228

Sauter, A. and Hartung, W. (2000) Abscisic acid conjugates – Do they play a role as long-distance stress signal in the xylem. *Journal of Experimental Botany* **51**: 929-936

Sauter, A., Pietz, K.J. and Hartung, W. (2002) A possible stress physiological role of Abscisic acid conjugates in root-to-shoot signalling. *Plant, Cell and Environment* **25**: 223-228

Schachtman, D.P. and Goodger, J.Q. (**2008**) Chemical root to shoot signaling under drought. *Trends Plant Science* **13:** 281–287

Schroeder, J.I., Allen, G.J., Hugouvieux, V., Kwak, J.M. and Waner, D. (2001) Guard cell signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biol*gy **56**: 627-658

Schulze, E.D. (1986) Carbon dioxide and water vapour exchange in response to drought in the atmosphere and in the soil. *Annual Review of Plant Physiology* **37**: 247–274

Schurr, U., Gollan, T. and Schulze, E.D. (1992) Stomatal response to drying soil in relation to changes in xylem sap composition of *Helius annus*; Stomatal sensitivity to abscisic acid imported from the xylem sap. *Plant, Cell and Environment* **15**: 561-567

Schwartz, S.H., Leon-Kloosterziel, K.M., Koornneef, M. and Zeevaart, J. (1997) Biochemical characterization of the *aba2* and *aba3* mutants in *Arabidopsis thaliana*. *Plant Physiology* **114:** 161-166

Schwartz, S.H., Qin, X. and Zeevaart, J.A.D. (2003) Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. *Plant Physiology* **131:** 1591-1601

Sekimoto, H., Seo, M., Kawakami, N., Komano, T., Desloire, S., Liotenberg, S., Marion-Poll, A., Caboche, M., Kamiy,a Y. and Koshiba T (1998) Molecular cloning and characterization of aldehyde oxidases in *Arabidopsis thaliana*. *Plant and Cell Physiology* **39:** 433-442

Seo, M., Peters, A.J.M., Koiwai, H., Oritani, T., Marion-Poll, A., Zeevaart, J.A.D., Koornneef, M., Kamiya, Y. and Koshiba, T. (2000) The *Arabidopsis* aldehyde oxidase 3 (AAO3) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. *Proceedings of the National Academy of Sciences USA* **97:** 12908-12913

Seo, M. and Koshiba, T. (2002) Complex regulation of ABA biosynthesis in plants. *Trends in Plant Science* **7:** 41-48

Serraj, R., Hash, C.T., Rizvi, S.M.H., Sharma, A., Yadav, R.S. and Bidinger, F.R. (2005) Recent advances in marker-assisted selection for drought tolerance in pearl millet. *Plant Production Science* **8**: 334-337

Sharp, R.E. (2002) Interaction with ethylene: Changing views on the role of abscisic acid in root and shoot growth responses to water stress. *Plant, Cell and Environment* **25:** 211-222

Sharp, R.E. and LeNoble, M.E. (2002). ABA, ethylene and the control of shoot and root growth under water stress. *Journal of Experimental Botany* **53**: 33-37

Sharp, R.E., Poroyko, V., Hejlek, L.G., Spollen, W.G., Springer, G.K., Bonhert, H. J., and Nguyen, H. T. (2004). Root growth maintenance during water deficits: physiology to functional genomics. *Journal of Experimental Botany* **55**: 2343-2351

Sheard, L. and Zheng, N. (2009) Plant biology: Signal advance for abscisic acid. *Nature* **462**: 575-576

Siddique, K.H.M., Tennant, D., Perry, M.W. and Belford, R.K. (1990) Water use and water use efficiency of old and modern wheat cultivars in a medditeranean-type environment. *Australian Journal of Agricultural Research* **41**: 431-447

Siefritz, F., Tyree, M.T., Lovisolo, C., Schubert, A. and Kaldenhoff, R. (2002) PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. *Plant Cell* **14:** 869–876

Siemens, J.A. and Zwiazeck, J.J. (2004) Changes in root water flow properties of solution culture-grown trembling aspen (*Populus tremuloides*) seedlings under different intensities of water-deficit stress. *Physiologia Plantarum* **121:** 44-49

Silburn, D.M. and Glanville, S.F. (2002) Management practices for control of runoff losses from cotton furrows under storm rainfall. I. Runoff and sedimentation. *Australian Journal of Soil Research* **40**: 1–20

Silva, R.F., Koch, R.B., and Moore, E.L. (1982). The effect of extraction procedures on tomato (*Lycopersicon lycopersicum*) seed germination and vigour. *Seed Science and Technology* **10:** 187-191

Slovik, S., Daeter, W. and Hartung, W. (1995) Compartmental redistribution of long-distance transport of Abscisic acid (ABA) in plants as influenced by environmental changes in the rhizosphere – a biomathmatical model. *Journal of Experimental Botany* **46**: 881-894

Slovik, S. and Hartung, W. (1992) Compartmental distribution and redistribution of abscisic acid in intact leaves. III. Analysis of the stress-signal chain. *Planta*, **187**: 37-47

Smith, C.D.S. (1997) In *The Pesticide Manual (11th Edition)*. Edited by Smith, C.D.S. The British Crop Protection Council Publishing.

Sobeih, W.Y., Dodd, I.C., Bacon, M.A., Grierson, D. and Davies, W.J. (2004) Long-distance signals regulating stomatal conductance and leaf growth in tomato (*Lycopersicon esculentum*) plants subjected to partial root-zone drying. *Journal of Experimental Botany* **55:** 2353-2363

Sokolovski, S., Hills, A., Gay, R., Garcia-Mata, C., Lamattina, L. and Blatt, M.R. (2005) Protein phosphorylation is a prerequisite for intracellular Ca2+ release and ion channel control by nitric oxide and abscisic acid in guard cells. *Plant Journal* **43:** 520-529

Somerville, C. and Briscoe, J. (2001) Genetic Engineering and Water. *Science* **292:** 2217

Sprenger, G.A., Schorken, U., Wiegert, T., Grolle, S., de Graaf, A.A,. Taylor, S.V., Begley, T.P., Bringer-Meyer, S. and Sahm, H. (1997) Identification of a thiamine-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isoprenoids, thiamine and pyridoxol. *Proceedings of The National Academy of Sciences USA* **94:** 12857-12862

Stansfield, C.B. and Hutchings, S.J. (1997). Irrigation in England: A review of the 1995 MAFF Survey. *Landwards* **52**: 2–6

Steele, K.A., Price, A.H., Shashidhar, H.E. and Witcombe, J.R. (2006). Marker-assisted selection to introgress rice QTLs controlling root traits into and Indian upland rice variety. *Theoretical and Applied Genetics* **112**: 208-221

Stoll, M., Loveys, B.R. and Dry, P.R. (2000) Hormonal changes induced by partial rootzone drying of irrigated grapevine. *Journal of Experimental Botany* **51**: 1627-1634

Strand, A., Kvernberg, K., Karlsen, A. and Liaaen-Jensen, S. (2000) Geometrical E/Z isomers of (6R)- and (6S)-neoxanthin and biological implications. *Biochemical Systematics and Ecology* **28:** 443-455

Sugita, M. and Gruissem, W. (1987) Developmental, organ-specific, and light-dependent expression of the tomato ribulose-1,5-biphosphate carboxylase small subunit gene family. *Proceedings of The National Academy of Sciences USA* **84**: 7104-7108

Sugita, M., Manzara, T., Pichersky, E., Cashmore, A. and Gruissem, W. (1987) Genomic organization, sequence analysis and expression of all five genes encoding the small subunit of ribulose-1,5-biphosphate carboxylase/oxygenase from tomato. *Molecular and General Genetics* **209:** 247-256

Sun, Z., Gantt, E., and Cunningham, F.X. (1996). Cloning and the functional analysis of the α-carotene hydroxylase of *Arabidopsis thaliana. Journal of Biological Chemistry* **271:** 24349-24352

Symonds, R.C. (2002). The manipulation of the ABA biosynthetic pathway. PhD Thesis, The University of Nottingham.

Taiz, L. and Zeiger, E. (2006) *Plant physiology.* (Fourth Edition). Sunderland, MA: Sinauer Associates Inc.

Tal, M. (1966) Abnormal stomatal behaviour in wilty mutants of tomato. *Plant Physiology* **41:**1387–1391

Tal, M. (1971) Salt tolerance in the wild relatives of the cultivated tomato: Responses of *Lycopersicon esculentum*, *L. peruvianum*, and *L. esculentum minor* to sodium chloride solution. *Australian Journal of Agricultural Research* **22**: 631–638

Tal, M., **Imber, D.**, **Erez, A. and Epstein, E**. (1979) Abnormal stomatal behavior and hormonal imbalance in *flacca*, a wilty mutant of tomato. II. Effect of abscisic acid on indoleacetic acid metabolism and ethylene evolution. *Plant Physiology* **63:**1044–1048

Tal, M., Imber, D. and Itai, C. (1970) Abnormal stomatal behaviour and hormonal imbalance in *flacca*, a wilty mutant of tomato. I. Root effect and kinetin-like activity. *Plant Physiology* **46**: 367-372

Tal, M. and Nevo, Y. (1973) Abnormal stomatal behaviour and root resistance, and hormonal imbalance in three wilty mutants of tomato. *Biochemical Genetics* **8**: 291–300

Tan, B.C., Schwartz, S.H., Zeevaart, J.A. and McCarty, D.R. (1997) Genetic control of abscisic acid biosynthesis in maize. *Proceedings of the National Academy of Sciences USA* **94:** 12235-12240

Tan, B., Joseph, L.M., Deng, W., Liu, L., Li, Q., Cline, K., McCarty, D.R. (2003) Molecular characterization of the *Arabidopsis* 9-*cis* epoxycarotenoid dioxygenase gene family. *The Plant Journal* **35:** 44-56

Tang, L-S., Li, Y. and Zhang, J. (2005) Physiological and yield responses of cotton under partial rootzone irrigation. *Field Crops Res*earch **94**: 214-223

Tardieu, F. and Davies, W.J. (1992) Stomatal response to abscisic acid is a function of current plant water status. *Plant Physiology* **98**: 540-545

Tardieu, F., Zhang, J., Katerji, N., Bethenod, O., Palmers, S. and Davies, W.J. (1992) Xylem ABA controls the stomatal conductance of filed grown maize subjected to soil compaction or soil drying. *Plant Cell and Environment* **15**: 193-197

Taylor, I.B. (1986) Biosystematics of the tomato. In: *The tomato crop: a scientific basis for improvement*. 1–34. Edited by Atherton JG, Rudich J, Chapman and Hall, London.

Taylor, I.B., Linforth, R.S.T., Al-Naieb, R.J., Bowman, W.R. and Marples, B.A. (1988) The wilty tomato mutants *flacca* and *sitiens* are impaired in the oxidation of ABA-aldehyde to ABA. *Plant, Cell and Environment* **11:** 739-745

Taylor, I.B., Burbidge, A. and Thompson, A.J. (2000) Control of abscisic acid synthesis. *Journal of Experimental Botany* **51:** 1563-1574

Taylor, I.B., Sonneveld, T., Bugg, T.D.H. and Thompson, A.J. (2005) Regulation and manipulation of the biosynthesis of abscisic acid, including the supply of xanthophyll precursors. *Journal of Plant Growth Regulation* **24:** 253-273

Thompson, P.A. (1974) Characterisation of the germination responses to temperature of vegetable seeds. I. Tomatoes. *Scientia Horticulturae* **2**: 35–54

Thompson, A.J., Jackson, A.C., Symonds, R.C., Mulholland, B.J., Dadswell, A.R., Blake, P.S., Burbidge, A. and Taylor, I.B. (2000a) Ectopic expression of a tomato 9-*cis*-epoxycarotenoid dioxygenase gene causes over-production of abscisic acid. Plant Journal **23:** 363-374

Thompson, A.J., Jackson, A.C., Parker, R.A., Morpeth, D.R., Burbidge, A. and Taylor, I.B. (2000b) Abscisic acid biosynthesis in tomato: Regulation of zeaxanthin epoxidase and 9-*cis*-epoxycarotenoid dioxygenase mRNAs by light/dark cycles, water stress and abscisic acid. *Plant Molecular Biology* **42:** 833-845

Thompson, A.J., Thorne, E.T., Burbidge, A., Jackson, A.C., Sharp, R.E.and Taylor, I.B. (2004) Complementation of *notabilis*, an abscisic acid-deficient mutant of tomato: importance of sequence context and utility of partial complementation. *Plant, Cell and Environment* **27:** 459-471

Thompson, A.J., Andrews, J., Mulholland, B.J., McKee, J.M.T., Hilton, H.W., Horridge, J.S., Farquhar, G.D., Smeeton, R.C., Smillie, I.R.A., Black, C.R. and Taylor, I.B. (2007a) Overproduction of abscisic acid in tomato increases transpiration efficiency and root hydraulic conductivity and influences leaf expansion. *Plant Physiology* **143:** 1905-1917

Thompson, A.J., Mulholland, B.J., Jackson, A.C., McKee, J.M., Hilton, H.W., Symonds, R.C., Sonneveld, T., Burbidge, A., Stevenson, P., and Taylor, I.B. (2007b) Regulation and manipulation of ABA biosynthesis in roots. Plant, Cell and Environment *30:* 67-78

Tian, L. and DellaPenna, D. (2001) Characterization of a second carotenoid β-hydroxylase gene from *Arabidopsis* and its relationship to the *LUT1* locus. *Plant Molecular Biology* **47:** 379-388

Tung, S.A., Smeeton, R.C., White, C.A., Black, C.R., Taylor, I.B., Hilton, H.W. and Thompson, A.J. (2008) Over-expression of LeNCED1 in tomato (Solanum lycopersicum L.) with the rbcS3C promoter allows recovery of lines that accumulate very high levels of abscisic acid and exhibit severe phenotypes. *Plant Cell and Environment* **31:** 968 – 981

Turner, N.C. (1997) Further progress in crop water relations. *Advances in Agronomy* **58**: 293-338

Turner, N.C. (2004) Agronomic options for improving rainfall-use efficiency of crops in dryland farming systems. *Journal of Experimental Botany* **55**: 2413–2425

Vanoosten, J.J. and Besford, R.T. (1994) Sugar feeding mimics effect of acclimation to high CO_2 - rapid down-regulation of RuBisCO small-subunit transcripts but not of the large subunit transcripts. *Plant Physiology* **143**: 306-31

Vaughan, S.P., James, D.J., Lindsey, K. and Massiah, A.J. (2006) Characterization of *FaRB7*, a near root-specific gene from strawberry (*Fragaria* x *ananassa* Duch.) and promoter activity analysis in homologous and heterologous hosts. *Journal of Experimental Botany* **57**: 3901-3910.

Verslues, P.E., Agarwal, M., Katiyar-Agarwal, S. and Zhu, J. (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. *The Plant Journal* **45:** 523-539

Verslues, P.E. and Zhu, J.K. (2005) Before and beyond ABA: Upstream sensing and internal signals that determine ABA accumulation and response under abiotic stress. *Biochemical Society Transactions* **33:** 375-379

Verslues, P.E. and Zhu, J.K. (2007) New developments in abscisic acid perception and metabolism. *Current Opinion in Plant Biology* **10**: 447-452

von Caemmerer, S. and Farquhar, G.D. (1981) Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. *Planta* **153:** 376-387

Vysotskaya, L.B., Arkhipova, T.N., Timergalina, L.N., Dedov, A.V., Veselov, S.Y. and Kudoyarova, G. (2004) Effect of partial root excision on transpiration, root hydraulic conductance and leaf growth in wheat seedlings. *Plant Physiological Biochemistry* **42**: 251-255

Wallace, J.S. (2000) Increasing agricultural water use efficiency to meet future food production. *Agriculture, Ecosystems and Environment* **82:** 105-119

Wanner, L.A. and Gruissem, W. (1991) Expression dynamics of the tomato *rbcS* gene family during development. *Plant Cell* **3**: 1289-1303.

Wan, X- and Li, L. (2006) Regulation of ABA level and water-stress tolerance of *Arabidopsis* by ectopic expression of a peanut 9-*cis*-epoxycarotenoid dioxygenase gene. *Biochemical and Biophysics Research Communications* **347:** 1030-1038

Wareing, P.F., Eagles, C.F., and Robinson, P.M. (1964). Natural inhibitors as dormancy agents. In. *Regulateurs Naturels de la Croissance Vegetale*. Edited by Nitsh, J.P.Centre National de Recherche Scientifique, Paris.

Wilkinson, S. (1999) pH as a stress signal. *Plant Growth Regulation* **29:** 87-99

Wilkinson, S. (2004) Water use efficiency and chemical signalling. In *Water use efficiency in plant biology*. Edited by M. A. Bacon. Blackwell Pub, CRC Press.

Wilkinson, S., Corlett, J.E., Oger, L. and Davies, W.J. (1998) Effects of xylem pH on transpiration from Wild-Type and *flacca* tomato leaves: a vital role for abscisic acid in preventing excessive water loss even from well-watered plants. *Plant Physiol*. **117**: 703-709

Wilkinson, S. and Davies, W.J. (1997) Xylem sap pH increase: A drought signal received at the apoplastic face of the guard cell that involves the suppression of saturable abscisic acid uptake by the epidermal symplast. *Plant Physiology* **113:** 559-573

Wilkinson, S. and Davies, W. J. (2002). ABA-based chemical signalling: the co-ordination of responses to stress in plants. *Plant, Cell and Environment* **25:** 195-210

Wilkinson, S. and Hartung, W. (2009) Food production: reducing water consumption by manipulating long-distance chemical signalling in plants *Journal of Experimental Botany* **60**: 1885 – 1891

Windsor, M.L. and Zeevaart, J.A.D. (1997) Induction of ABA 8'-hydroxylase by (+)-S-, (–)-R- and 8',8'8'-trifluoro-S-abscisic acid in suspension cultures of potato and Arabidopsis. *Phytochemistry* **45**: 931–934

Wong, S.C., Cowan, I.R. and Farquhar, G.D. (1979) Stomatal conductance correlates with photosynthetic capacity. *Nature* **282**: 424–426

Wright, S.T.C. (1977) The relationship between leaf water potential (Ψleaf) and the levels of abscisic acid and ethylene in excised wheat leaves. *Planta* **134:** 183-189

Wright, S.T.C. and Hiron, R.W.P. (1969) (+)-Abscisic acid, the growth inhibitor induced in detached wheat leaves by a period of wilting. *Nature* **224:** 719–720

Xiong, L., Ishitani, M., Lee, H. and Zhu, J.K. (2001) The *Arabidopsis LOS5/ABA3* locus encodes a molybdenum cofactor sulfurase and modulates cold and osmotic stress-responsive gene expression. *Plant Cell* **13**: 2063-2083

Xu, B., Li, F., Shan, L., Ma, Y., Ichizen, N. and Huang, J. (2006) Gas exchange, biomass potential and water relations of three grass seedlings under water-stress. *Weed biology Management* **6**: 79-88

Yamakawa, K. (1982) Use of rootstocks in Solanaceous fruit-vegetable production in Japan. *Japanese Agricultural Research Quarterly*. **15**: 175–179

Ye, X. and Beyer, P. (2000) Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm. *Science* **287:** 303-305

Zacarias, L. and Reid, M.S. (1990) Role of growth regulators in the senescence of Arabidopsis thaliana leaves. *Physiologia Plantarum* **80:** 549-554

Zeevaart, J.A.D. and Creelman, R.A. (1988) Metabolism and physiology of abscisic acid. *Annual Review of Plant Physiology and Plant Molecular Biology* **39:** 439-473

Zegbe-Domínguez, J.A., Behboudian, M.H. and Clothier, B.E. (2006) Responses of 'Peptoride' processing tomato to partial rootzone drying at different phonological stages. *Irrigation Sci*ence **24**: 203-210

Zhang, J. and Davies, W.J. (1987) Increased synthesis of ABA in partially dehydrated root tips and ABA transport from roots to leaves. *Journal of Experimental Botany* **38:** 2015-2023

Zhang, J. and Davies, W.J. (1989) Abscisic acid produced in dehydrating roots may enable the plant to measure the water status of the soil. *Plant, Cell and Environment* **12:** 73-81

Zhang, J. and Davies, W.J. (1991) Antitranspirant activity in xylem sap of maize. *Journal of Experimental Botany* **42**: 317-321

Zhang, J. and Davies, W.J. (1990) Does ABA in the xylem control the rate of leaf growth in soil dried maize and sunflower plants? *Journal of Experimental Botany* **41**: 1125-1132

Zhang, J. and Tardieu, F. (1996) Relative contribution of apices and mature tissues to ABA synthesis in droughted maize root systems. *Plant and Cell Physiology* **37**: 598-605

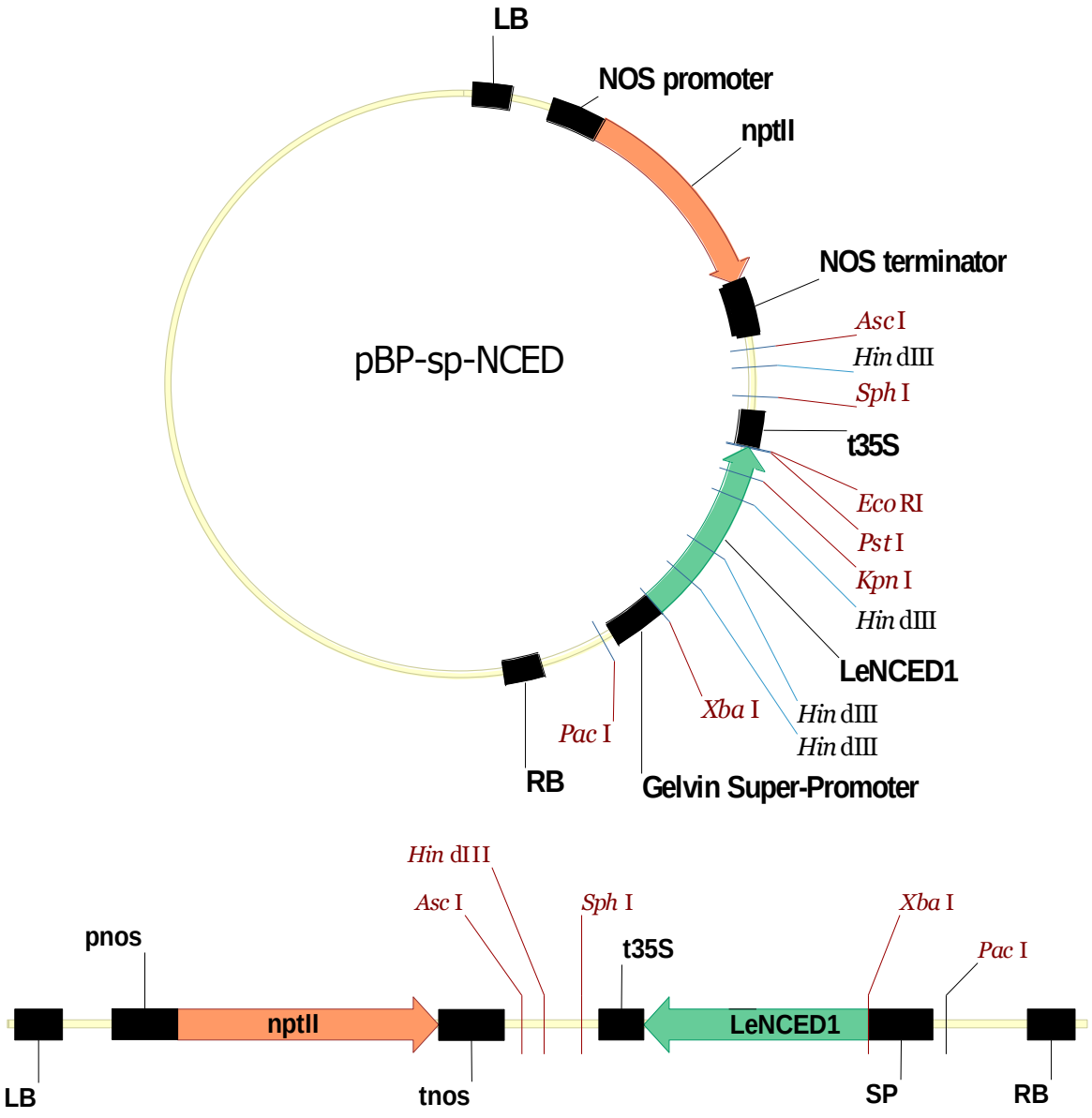
Zhang, J.Z., Creelman, R.A. and Zhu, J.K. (2004) From laboratory to field. Using information from *Arabidopsis* to engineer salt, cold, and drought tolerance in crops. *Plant Physiology* **135**: 615–621

Zhang, S.Q. and Outlaw, W.H. (2001) Abscisic acid introduced into the transpiration stream accumulates in the guard-cell apoplast and causes stomatal closure. *Plant, Cell and Environment* **24**: 1045-1054

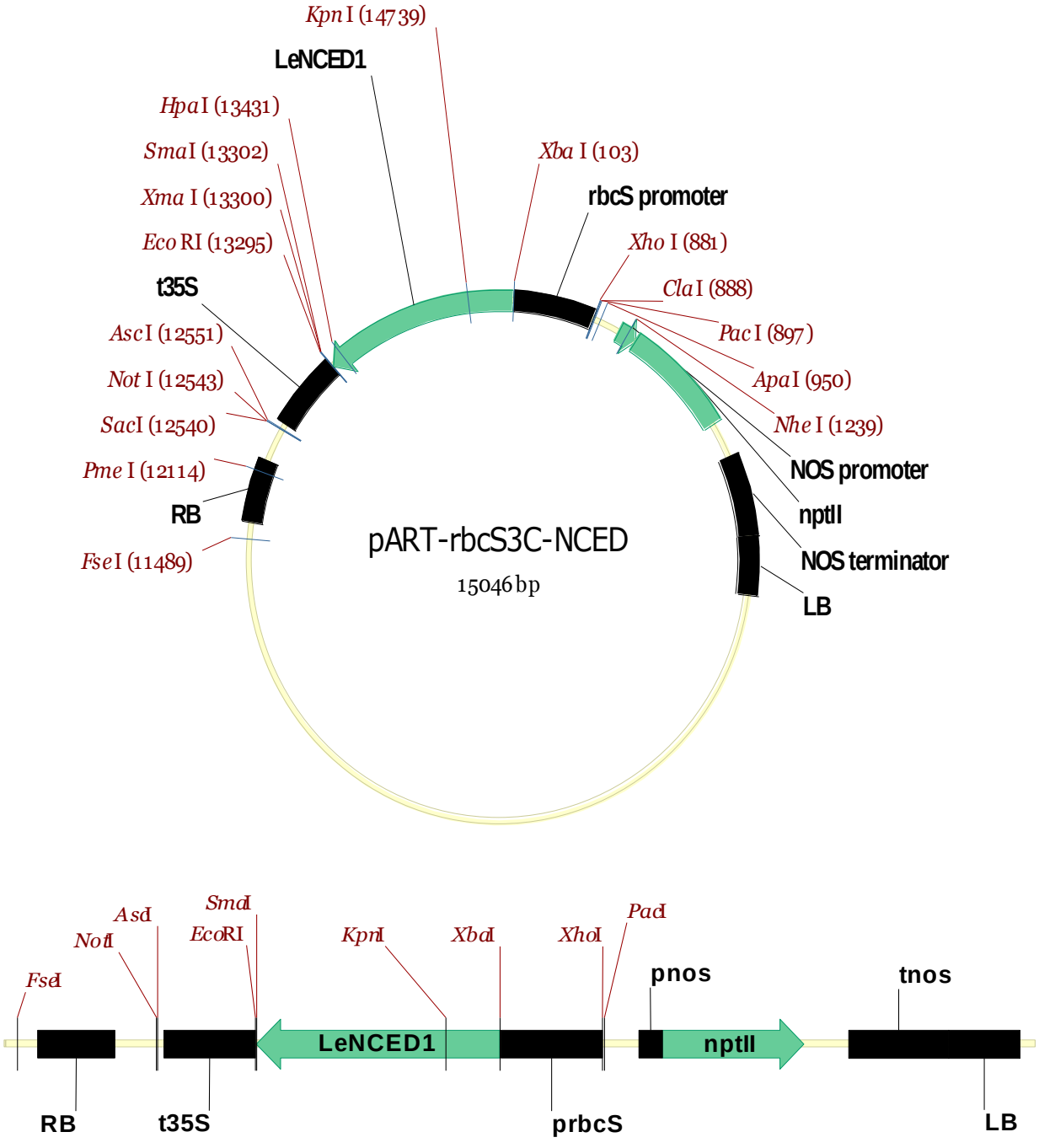
Zhang, X., Zhang, L., Dong, F., Gao, J., Galbraith, D.W. and Song, C.-P. (2001) Hydrogen peroxide is involved in abscisic acid-induced stomat**al** closure in *Vicia faba. Plant Physiology* **126:** 1438–1448

12 **APPENDICES**

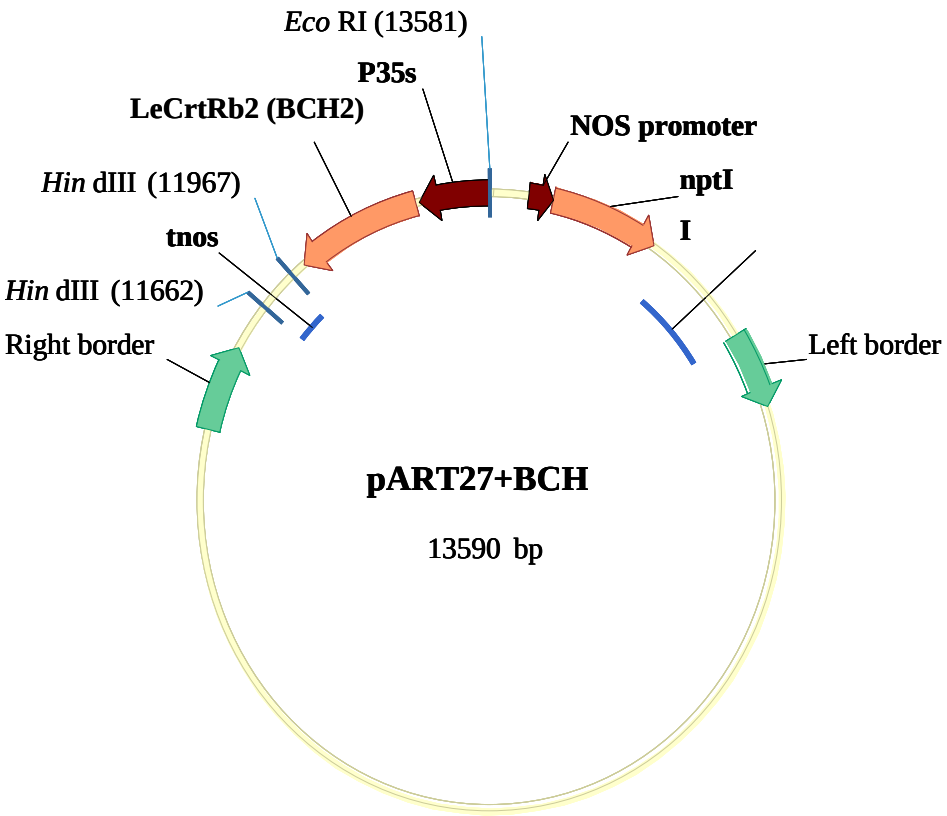
Appendix 1. **DNA region of construct pBP-sp-NCED.** *LeNCED1*, *LeNCED1* open reading frame; SP, Gelvin Super-Promoter; t35S, CaMV 35S polyadenylation signal; *npt*II, neomycin phosphotransferase II; p*nos* and t*nos*, promoter and terminator sequences from the nopaline synthase gene; LB and RB are the left and right T-DNA borders (from Tung, 2007).



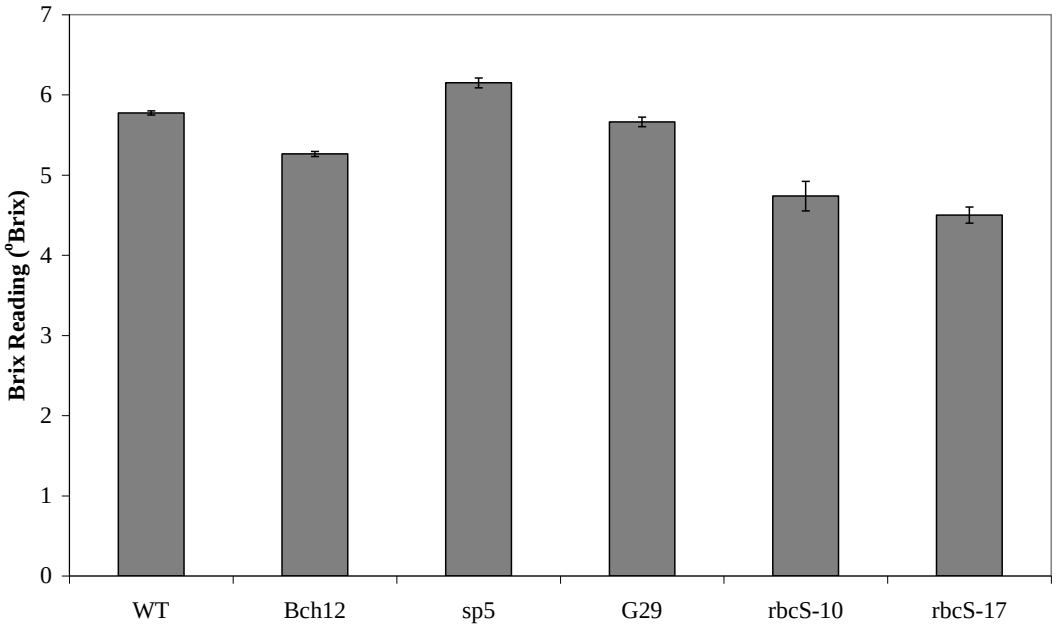
Appendix 2. **T-DNA region of construct pART-rbcS3C-NCED.** t35S, CaMV 35S polyadenylation signal; LeNCED1, *LeNCED1* open reading frame; p*rbcS*, tomato rbcS3C promoter; *npt*II, neomycin phosphotransferase II; p*nos* and t*nos*, promoter and terminator sequences from the nopaline synthase gene; RB and LB are the right and left T-DNA borders. Some restriction enzymes that are single cutters in the T-DNA are indicated (from Tung, 2007).



Appendix 3. **T-DNA region of construct pART27+BCH**. RB, right T-DNA border; LeCrtRb2, tomato BCH gene inserted in the sense direction; p35S, polyadenylation signals from CaMV 35S; nptII, neomycin phosphotransferase II gene; Pnos & Tnos, promoter and terminator sequences from the nopaline synthase gene; LB, left T-DNA border (from Balasubramanian, 2007).



Appendix 4**. Brix analysis of fruit quality** Brix analyses were carried out on fruit from the WT, sp5, BCH12, G29, 587-10 and 587-17 genotypes. Brix analysis provides a measure of the percent total soluble solid (TSS) within a given mass of plant juice. TSS was measured using a refractometer calibrated in ^{0}Brix, with each ^{0}Brix being equivalent to 1 g of TSS per 100 g of juice (Saltveit, 2004). Four plants of each genotype were grown under well-watered glasshouse conditions and fruit were sampled from trusses 2 and 3 except in the case of 587-10 and 587-17, for which fruit was sampled from the first two trusses to produce fruit. Two fruit were sampled from each truss, and three replicate samples of tissue were taken from the pericarp of each fruit. Samples were homogenised and centrifuged before placing 1 ml of the extracted juice on the optical prism of the refractometer and taking the reading.



Appendix 5. **Initial Biomass Data from Gravimetric TE_p Experiments.** Mean initial biomass of each line when experiment commenced with accompanying ANOVA analysis.

Line	Section 5.4 (WT, sp12 and sp5)	Section 7.4.1 (WT, BCH12, sp12 and G28)	Section 7.4.2 (WT, BCH12, sp5 and G29)	Section 7.4.3 (WT, BCH12, sp12, sp5, G28 and G29)
WT	3.31	3.633	3.97	4.255
BCH12	-	3.6	3.77	4.397
sp12	3.50	3.967	-	4.302
sp5	3.29	-	4.27	4.372
G28	-	3.867	-	4.273
G29	-	-	4.28	4.408
Fpr.	*0.751*	*0.418*	*0.407*	*0.878*
s.e.d.	0.292	2.45	0.348	0.158
l.s.d.	0.810	0.565	0.741	0.323

Appendix 6. **Biomass partitioning of WT BCH12, sp12 and G28.** Dry weight (DW) of individual components at time of destructive harvest during gravimetric TE_p experiment (Chapter 7; Section 7.41), with accompanying ANOVA analysis.

Line	Lamina DW (g)	Petiole DW (g)	Stem DW (g)	Truss DW (g)
WT	25.1	12.5	24.85	4.37
BCH12	27.8	11.7	25.13	4.72
sp12	26.2	15.5	23.43	15.07
G28	24.0	15.0	23.9	9.44
F pr.	*0.314*	*0.311*	*0.405*	*<0.001*
s.e.d.	3.255	12.88	1.215	2.168
l.s.d.	6.244	27.63	2.605	4.649

Appendix 7. **Accumulation of leaf ABA in 'single transgene' (*LeNCED1*) over-expression lines.** Fold and absolute differences are relative to WT non-dehydrated control within each experiment. Where appropriate, data from leaf dehydration experiments were adjusted to take account of water loss such that values are expressed as ABA per kilogram of the FW prior to dehydration. C: non-dehydrated control leaves; D 4h: leaves dehydrated and incubated for four hours. Adapted from Tung *et al.* (2008).

Reference	Genotype	Treatment	Leaf ABA (nmol kg-1 FW)	Fold Difference	Absolute difference (nmol kg-1 FW)
Thompson *et al*. (2007b)	WT	C	694		
	sp12	C	1174	1.7	480
	sp5	C	1130	1.6	436
Thompson *et al*. (2007a)	WT	C	1574		
	sp12	C	2001	1.3	427
Tung *et al*. (2008)	WT	C	760		
	WT	D 4h	3136	4.1	2376
	sp5	C	1090	1.4	330
	sp12	C	1347	1.8	587
	rbcS-4	C	1797	2.4	1037
	rbcS-10	C	1460	1.9	700
	rbcS-17	C	1736	2.3	976
	rbcS-18	C	1892	2.5	1132

Appendix 8. **Summary of the effect of 'single transgene' (*LeNCED1* or *LeBCH2*) and 'double transgene' (*LeNCED1*and *LeBCH2*) over-expression on mean leaf, root and seed ABA content.** Fold differences are relative to WT control within each experiment. For leaf ABA content, plants were grown under standard glasshouse conditions and a young leaf of each 6-8 week old plant was sampled (n = 12, P<0.001, SED: 9.86, LSD (5%): 19.83). For isolated root ABA content, roots were grown in liquid culture media for 6-8 weeks until sufficient root material (approximately 100mg DWt) had grown (n = 3, P<0.001, SED: 45.37, LSD (5%): 92.84). ABA was assayed using a radioimmunoassay, as previously described (Mulholland *et al.*, 2003). Seed ABA content was analysed after 48 h of imbibition, but prior to germination, using a UPLC/ESI-MS/MS method, (n = 6, P<0.001, SED: 7.92, LSD (5%): 16.88).

Reference and plant tissue	Genotype	ABA concentration	Fold difference
Sonneveld *et al.* (in prep.) **Leaf ABA content (ng.g^{-1} FWt)**	WT	105.5	
	BCH12 (*LeBCH2*)	145.3	1.38
	sp12 (*LeNCED1*)	182.1	1.72
	G28 (*LeNCED1*/ *LeBCH2*)	357.3	3.39
Balasubramanian (2007) **Isolated root ABA content (ng.g^{-1} DWt)**	WT	221.1	
	BCH12 (*LeBCH2*)	207.7	n/a
	sp12 (*LeNCED1*)	667.9	3.02
	G28 (*LeNCED1*/ *LeBCH2*)	2388.0	10.80
Sonneveld *et al.* (in prep.) **Seed ABA content (pmol g^{-1} DWT)**	WT	22.5	
	BCH12 (*LeBCH2*)	13.8	n/a
	sp12 (*LeNCED1*)	55.2	2.45
	G28 (*LeNCED1*/ *LeBCH2*)	82.1	3.64