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The role of endo--1,4-glucanase in strawberry fruit development

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THE ROLE OF ENDO-β-1,4-GLUCANASE IN STRAWBERRY

FRUIT DEVELOPMENT

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A Thesis Submitted to the Open University
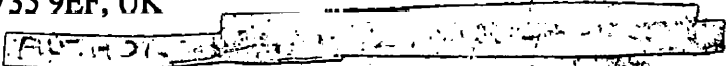
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ABSTRACT

The ripening of strawberry fruit is characterized by changes in the composition and structure of the cell wall polysaccharides leading to textural changes and loss of firmness of the fruit. An endo-β-1,4-glucanase (EGase) was purified from ripe strawberry (*Fragaria x ananassa* Duch.) fruit using cellulose affinity chromatography. The purified enzyme gave a single protein band of 54 kDa on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. A 25 amino acid N-terminal sequence showed strong homology with the proteins encoded by recently identified EGase genes from different strawberry cultivars and from *Arabidopsis*, pepper and tomato. The enzyme specifically cleaved the β-1,4-glucosyl linkages of xyloglucan but was unable to hydrolyze those of insoluble cellulose. The pH optimum and K_m of the enzyme against the artificial substrate carboxymethylcellulose (CMC) were pH 5.0 to 7.0 and 1.3 mg ml^{-1} respectively. A cDNA of the corresponding ripening-enhanced, fruit-specific gene, *cel1*, was isolated from a ripe fruit cDNA library. This was used to down-regulate *cel1* expression in transgenic strawberry plants in order to assess the potential role(s) played by Cel1 during strawberry fruit ripening. In several transgenic lines, *Cel1* mRNA was suppressed to undetectable levels in ripe fruit. However, EGase activity and firmness of these fruit were indistinguishable from control fruit. A second strawberry EGase gene, *cel2*, is also expressed in ripening fruit and this presence has prevented specific down-regulation of *cel1* from revealing its role in fruit softening. Southern analysis of *cel1* and *cel2* revealed the presence of related sequences in the strawberry genome indicating a small multigene family, consistent with the isolation of two different EGase cDNAs from strawberry.

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ABBREVIATIONS AND SYMBOLS

A	adenine
ATP	adenosine triphosphate
AVG	aminoethoxyvinylglycine
AZ	abscission zone
2-BE	2-butoxyethanol
β-Gal	β-galactosidase
bp	base pairs
BSA	bovine serum albumen
C	cytosine
°C	degrees Celsius
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
CAPS	3-[cyclohexylamino]-1-propanesulphonic acid
cDNA	complementary DNA
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
CMC	carboxymethylcellulose
CTAB	cetyltrimethylammonium bromide
C-terminal	carboxy (COO⁻)-terminal
cv	cultivar
d	days
DAA	days after anthesis

dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
DIG	digoxigenin
DMF	dimethylformamide
DMSO	dimethylsulphoxide
dNTP	deoxynucleotide-5'-triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreitol
dTTP	deoxythymidine-5'-triphosphate
dUTP	deoxyuridine-5'-triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid (disodium salt)
EGase	endo-β-1,4-glucanase
FW	fresh weight
G	guanine
h	hour
HCl	hydrochloric acid
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IAA	indole-3-acetic acid (auxin)
IPTG	isopropyl-1-thio-β-D-galactopyranoside

JA	jasmonic acid
JAMe	methyl jasmonate
kb	kilobase pairs
KCl	potassium chloride
kDa	kilodalton
K_m	Michaelis constant
Km	kanamycin
LB	Luria-Bertani
LiCl	lithium chloride
M	molar (concentration)
M13	M13 bacteriophage
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
min	minutes
MOPS	3-[N-morpholino]propanesulphonic acid
M_r	relative molecular weight
mRNA	messenger RNA
MS	Murashige & Skoog
1-NAA	1-naphthaleneacetic acid
NaCl	sodium chloride
NaOH	sodium hydroxide
NBD	norbornadiene
$(NH_4)_2SO_4$	ammonium sulphate
nos	nopaline synthase

npt	neomycin phosphotransferase
N-terminal	amino (NH$_2$)-terminal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PDB	phage dilution buffer
PEG	polyethylene glycol
%	percentage
pfu	plaque forming unit
λ	phage λ
pI	isoelectric point
PG	polygalacturonase
PME	pectinmethylesterase
PMSF	phenylmethanesulphonyl fluoride
POA	phenoxyacetic acid
PVDF	polyvinylidene difluoride
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal RNA
RT	room temperature
SDS	sodium dodecyl sulphate

SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	standard error of the mean
Sm	streptomycin
SSC	saline sodium citrate
SSPE	saline sodium phosphate EDTA
T	thymine
T3/T7	T3/T7 bacteriophage
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TB	Terrific broth
TE	Tris-EDTA
Temed	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
Triton X-100	t-octylphenoxypolyethoxyethanol
U	unit
UV	ultraviolet
V	volt
V_{max}	maximum velocity at saturating substrate concentrations
v/v	volume for volume
w/v	weight for volume
XET	xyloglucan endotransglycosylase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
ZnCl$_2$	zinc chloride

CHAPTER 1.   INTRODUCTION

1.1   STRAWBERRY FRUIT DEVELOPMENT

1.1.1   Commercial strawberry production

The strawberry is grown commercially in all temperate regions of the world. The cultivated strawberry (*Fragaria x ananassa* Duch.) is a complex interspecific hybrid octoploid complex derived from two natural octoploid species *F. chiloensis* and *F. virginiana*. The octoploid *Fragaria* genome is designated AAA'A'BBBB, where the AA genomes are derived from the modern diploids *F. vesca* and *F. viridis* (Senanayake and Bringhurst, 1967). The strawberry is classified botanically as a false fruit, consisting of an enlarged fleshy receptacle with the achenes (seeds) on the surface of the fruit and belongs to the group generally known as soft fruits that includes various berries and currants. Strawberries are amongst the most economically important of the soft fruits, along with raspberries and blackcurrants, with around 2.5 M tonnes grown annually worldwide. Europe is responsible for almost half of the world's production of strawberries and the USA for almost a quarter, the remainder being grown in many countries on a smaller scale. In the UK, strawberry production is more than double that of either raspberries or currants (Manning, 1993) and has an estimated value of around £55 M (Department of Trade and Industry Foresight programme report, 1997).

Strawberries are valued as a fresh fruit crop for their unique flavour, colour, texture and nutritional quality attributes which make them attractive to the consumer. Strawberries

and, as their name implies, soft fruit in general, lack a firm texture and as a result they have a short postharvest shelf life, a characteristic which is undesirable for producers and consumers alike. Tissue firmness is the major factor affecting postharvest deterioration in soft fruit as it renders the fruit susceptible to mechanical damage and subsequent microbial attack. Thus the handling of soft fruits commercially throughout the harvesting, packaging and distribution processes is minimized to reduce damage. The use of refrigeration and modified atmosphere conditions during transportation and storage is widely used to slow down the ripening and senescence of the fruit, reduce dehydration and inhibit microbial growth. Other approaches have been investigated in an attempt to reduce postharvest deterioration of strawberry fruit during storage. Heat treatment of ripe fruit was found to prevent fungal development and decrease the number of damaged fruits during storage. This treatment was also shown to retard the rate of ripening of the fruit as indicated by the reduced softening rate and colour development, suggesting that the method may be useful for extending the postharvest shelf life (Civello *et al.*, 1997). Modified atmosphere packaging systems for strawberries usually involve the use of various plastic films (Garcia *et al.*, 1998b; Sanz *et al.*, 1999) which are designed to produce optimum O_2 and CO_2 concentrations around the fruit and minimize water loss. An alternative method of controlling gaseous exchange has been evaluated in which a semipermeable, plasticized, starch-based coating was applied directly to the fruit. This was found to modify the internal fruit atmosphere and decrease water evaporation and resulted in extended storage life of the fruit (Garcia *et al.*, 1998a). Gamma irradiation has the potential to extend the shelf life of fruit by reducing ripening and microbial spoilage. However, the use of irradiation is limited in strawberries as it has been shown to cause extensive tissue softening by

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partial degradation of cellulose and polygalacturonic acid chains of pectin (d'Amour *et al.*, 1993).

The problem of postharvest deterioration, and hence short shelf life, associated with strawberries and soft fruits in general, results in many of these being processed into products such as frozen or canned whole fruit, jams and fruit juices. The quality of strawberries as a fresh product depends primarily on the underlying textural changes which occur in the fruit during ripening, although there are a number of other biochemical factors affecting colour and flavour during ripening that influence quality. Improvements in the handling and storage characteristics of strawberries and the development of novel ways to maintain fresh fruit quality require a better understanding of the biochemistry and molecular biology of fruit ripening, and in particular softening. The ability to manipulate strawberry fruit ripening in a way that reduces the postharvest deterioration in fruit quality and extends the shelf life of the fruit is likely to have significant commercial importance.

1.1.2 Physiology of strawberry fruit development

Anatomically the strawberry is not a true fruit, since the fleshy part is derived from receptacle tissue rather than the ovary itself. The true fruits, or achenes which arise from fertilized ovules, are located on the outside of the fleshy receptacle and are attached to it by vascular connections. However, physiologically the receptacle exhibits the characteristics of a fruit in that it becomes fleshy, accumulates water and many organic compounds and undergoes ripening. It thus provides a suitable environment for seed

development and a mechanism for the dispersal of the mature seeds by virtue of its attractiveness as a food source.

The development of strawberry fruits shares similarities with a diverse range of both edible and inedible fleshy fruits. Different fruits display variations of the developmental program which can be divided into phases (Gillaspy et al., 1993). The earliest phase after anthesis is referred to as fruit set and marks the commitment to proceed with fruit development. The initial phase of fruit growth is due primarily to cell division, occuring for the first 7 to 10 days after fruit set in strawberry (Woodward, 1972) and tomato (Gillaspy et al., 1993). Once cell division has ceased, fruit growth continues, mostly as a result of cell expansion, until the fruit reaches its final size. Cell number is believed to contribute to the variation in fruit size within a species, but in general the increase in cell volume makes the greatest contribution to the final size of a fruit (Coombe, 1976). The interval from anthesis to fruit maturity varies for different species. For strawberry fruits, the length of this developmental period ranges from 20 to 60 days, with an average of 30 days, and is dependent on temperature (Stutte and Darnell, 1987). Typically, strawberry fruit growth has been shown to follow a single sigmoid growth curve (Woodward, 1972; Coombe, 1976; Mudge et al., 1981; Stutte and Darnell, 1987). However, there are reports of biphasic or double sigmoidal growth curves (Mudge et al., 1981; Archbold and Dennis, 1984; Veluthambi et al., 1985) indicating that the kinetics of strawberry fruit growth may vary with cultivar. Fruit size at maturity is correlated, within a cultivar, with both the position of the fruit on the inflorescence (cyme) and the number and size of developed achenes (Moore et al., 1970; Stutte and Darnell, 1987). Fruits decline in size from primary to secondary to tertiary positions on the cyme. The

smaller size of secondary fruit compared to primary fruit was correlated with a lag period in secondary fruit growth, following fertilization, although the relative growth rates were similar once growth was initiated. This delay was independent of environmental conditions and appears to be physiologically regulated. The lag in secondary fruit growth may be the result of inter-fruit competition as secondary fruit increased in weight if primary fruit exerting the stronger sink activity were removed from the inflorescence. Alternatively, there may be some form of apical regulation occuring within an inflorescence (Stutte and Darnell, 1987) similar to apical dominance in shoots. Differences in size exist not only between fruit from different positions on an inflorescence but also between fruit from different cultivars. Thus, fruit size is also genetically determined and a close relationship is observed between fruit weight and developed achene number and size. Small fruits arising from lower flower positions or from small-fruited cultivars were found to have fewer and smaller achenes than larger fruit (Moore et al., 1970). Indeed, it has been clearly shown that the growth of the receptacle is dependent on, and regulated by, the fertilized achenes and that strawberry receptacle weight is proportional to the number of fertilized ovules or achenes present (Nitsch, 1950).

Following the early stages of growth and development, fruits enter a maturation phase during which they acquire the ability to ripen. The ripening phase can be characterized by a set of distinct biochemical events that result in important changes affecting the quality attributes of fruit. These include the colour, texture and flavour changes characteristic of each fruit. Thus ripening is defined by a programmed series of changes in gene expression and the resultant de novo protein synthesis and /or degradation. It is a highly coordinated phase at the end of a continuous process of fruit

development. As such it is far more complex than the early perception of ripening which was described in terms of the catabolic events associated with the breakdown of cell structural integrity, referred to as 'organizational resistance' (Brady, 1987).

1.1.3 Non-climacteric fruit

Fruits in general can be classified as climacteric or non-climacteric depending on their patterns of respiration and ethylene synthesis during ripening and several reviews exist on this subject (Tucker and Grierson, 1987; Tucker, 1990, 1993). Climacteric fruit are characterized by a burst of autocatalytic ethylene production and a peak of respiratory activity, termed the respiratory climacteric, during ripening. The transient increases in both ethylene synthesis and respiration usually occur at an early stage of ripening but the magnitude of the increases varies considerably between different fruits (Table 1.1). In any particular fruit, the rate of peak ethylene production is generally proportional to the peak respiration rate. Similarly, the timing of the peaks in ethylene production and respiration in relation to each other, and in relation to the stage of ripening, is different depending on the fruit. In most fruits, the increase in ethylene synthesis occurs before, or is coincident with, the respiratory climacteric, although in some the respiratory increase precedes the rise in ethylene. These events may occur prior to optimum eating ripeness, as in the case of tomato and apple, or they may coincide with, or follow ripeness, as observed in banana and avocado.

Table 1.1   Maximum CO_2 and ethylene production of selected climacteric and non-climacteric fruits (after Tucker, 1990)

Fruit	CO_2 (ml kg^{-1} h^{-1})	Ethylene (ppm)
Climacteric		
Avocado	155	500
Banana	60	40
Cherimoya	170	219
Pear	33	40
Tomato	20	27
Non-climacteric		
Cherry	25	-
Grape	20	-
Lemon	5	0.15
Pineapple	17	0.30
Strawberry	21	-

In contrast, the ripening of non-climacteric fruits is not accompanied by an increase in respiratory activity or ethylene production. In most cases there is actually a gradual decline in respiration rate as ripening procedes. Although non-climacteric fruit do produce low levels of ethylene, as do most plant tissues, there is no increase in synthesis as seen for climacteric fruit. Instead they exhibit a slow decline in ethylene levels from

the mature green to the ripe stage of development in parallel with the decline in respiration. However, as with climacteric fruits, both the respiratory rate and level of ethylene production vary between different non-climacteric fruits (Table 1.1). In general, fruits with higher respiratory and ethylene synthesis rates tend to ripen faster and hence have a shorter shelf life. Thus, non-climacteric fruits such as strawberry and cherry are more perishable than climacteric fruits such as tomato since, although they do not show a respiratory peak, their respiration rates are consistently higher (Table 1.1).

The two classes of fruit also differ in their synthesis of endogenous ethylene and response to exogenous ethylene. Non-climacteric fruit respond to exogenous ethylene with an increased respiration rate which is proportional to the concentration of exogenous ethylene and is dependent on its continued presence. The basal level of ethylene production is unaffected. The resulting rise in respiration rate is responsible for a corresponding increase in the rate of ripening. However, the effect of exogenous ethylene on climacteric fruit is to accelerate the onset of the respiratory climacteric without causing any change in the magnitude of the peak respiratory rate. In this case, it is the extent to which the climacteric is brought forward in time that is proportional to the concentration of exogenous ethylene. These differences can be explained by the autocatalytic synthesis of ethylene in climacteric fruit, which is absent in non-climacteric fruit and which led to the suggestion that two control systems for ethylene synthesis exist in fruit (McMurchie *et al.*, 1972). Thus, exogenous ethylene triggers the autocatalytic production of endogenous ethylene by system II in climacteric fruit and advances the respiratory climacteric. In contrast, non-climacteric fruit possess only system I ethylene, which is responsible for both background and wound ethylene

production in all tissues, and hence are unable to respond autocatalytically to exogenous ethylene.

The autocatalytic response of climacteric fruit to exogenous ethylene is believed to be identical to the autocatalytic response to an increase in endogenous ethylene levels that occurs during fruit development and induces ripening in these fruit. Ethylene is synthesized from S-adenosyl methionine (SAM) which is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase and then to ethylene by ACC oxidase. Developmental regulation of ACC synthase and ACC oxidase is believed to initiate the rise in endogenous ethylene levels that in turn activates the autocatalytic production of endogenous ethylene. Thus ethylene is considered to play an essential role in the regulation of ripening in climacteric fruits, but not in non-climacteric fruits, which are considered to be ethylene-independent (Lelievre *et al.*, 1997).

On this basis, ripening of strawberry fruit is classified as being non-climacteric. There is a lack of increased respiration and ethylene production during ripening (Knee *et al.*, 1977; Abeles and Takeda, 1990; Perkins-Veazie *et al.*, 1996). In addition, inhibitors of the ethylene biosynthetic enzyme ACC synthase (aminoethoxyvinylglycine) or ethylene action (silver thiosulphate, norbornadiene) failed to retard the onset of ripening as measured by anthocyanin accumulation (Given *et al.*, 1988c) indicating that ethylene is not required for the induction of ripening in strawberry.

1.1.4 Biochemical changes during strawberry fruit ripening

The ripening phase of fruit development can be characterized by a set of physico-chemical changes characteristic of each fruit. These result in important changes

affecting the main quality attributes of flavour, colour and texture. The biochemical events that occur in many fleshy fruits include the production of flavours and aromas which affect the palatability of the fruit, the production of new pigments which affect the aesthetic quality and texture changes resulting in tissue softening which affect the postharvest shelf life.

1.1.4.1 Flavour

Flavour is one of the most important factors affecting strawberry fruit quality and its perception can be separated into two components, taste and aroma. Flavour changes result from a combination of the accumulation of various sugars, organic acids and phenolics and the synthesis and interaction of complex mixtures of volatile compounds.

Sugars are one of the major soluble components in strawberry fruit. The fruit are strong sinks for photosynthetic assimilate from the leaves and accumulate sugars throughout their development, including the ripening phase. This requires that the fruit has to ripen on the plant if acceptable flavour is to develop. Consequently, the harvesting of strawberry fruits prior to ripening in order to prolong shelf life results in inferior flavour in contrast to fruits such as tomato which accumulate most of their sugars before the onset of ripening.

In strawberry, sucrose is the main carbohydrate translocated to the fruit. Experiments using ^{14}C-labelled sugars have indicated that sucrose is unloaded apoplastically and is hydrolyzed in the free space before uptake as hexoses into the fruit. Some intact sucrose may also accumulate in the fruit (Forney and Breen, 1986). The presence of a cell wall bound invertase has been reported in strawberry fruit, the activity

of which increases during the initial growth of the receptacle, suggesting that it could be important in establishing a strong sink activity in the growing fruit (Poovaiah and Veluthambi, 1985). Hydrolysis of sucrose in the free space by invertase can also account for the greater accumulation of the hexoses, glucose and fructose, over sucrose observed in strawberry fruit (Forney and Breen, 1986). Sucrose levels are very low in strawberry fruit for the first 10 days after anthesis but then rapidly increase to reach a maximum at the turning stage before declining as fruit ripen (Forney and Breen, 1986). This pattern was accounted for by the activity of a soluble invertase (Poovaiah and Veluthambi, 1985) that declined after an initial high activity at anthesis and then increased again at the time of ripening. Glucose, fructose and sucrose account for more than 99% of the total sugars in ripe fruit, as is also the case for raspberry and blackcurrant. Sorbitol, xylitol and xylose are other sugars which are present in trace amounts (Makinen and Soderling, 1980).

Organic acids also constitute a significant proportion of the soluble solids in strawberry and are important components of flavour. The balance between sugar and acid levels is considered to be of importance in determining the level of flavour acceptance of the fruit. Acids can also affect flavour directly and may influence the processing quality of the fruit since they affect the formation of off-flavours and the gelling properties of pectin (Manning, 1993). The major acids found in strawberry fruit, as in many fruit, are citric and malic acids, with several others, including shikimic and quinic acids, present in trace amounts (Montero et al., 1996). Total acid content has been shown to increase on a per fruit basis during strawberry fruit development with a decline in over-ripe fruit (Woodward, 1972). This is in contrast to most fruits and to raspberry fruits in particular

which exhibited a decline in total acid levels per unit FW throughout development (Perkins-Veazie and Nonnecke, 1992). The most important acid in fruits from the point of nutritional quality is ascorbic acid (vitamin C) and strawberries have been shown to contain considerable levels of this compound (Montero *et al.*, 1996).

Phenolics, although present at relatively low levels in ripe fruits, are important contributors to their taste, palatability and nutritional value. Several different phenolic compounds have been isolated from fruit and they represent a diverse range of substances including the secondary plant metabolites polyphenols (tannins), proanthocyanidins (condensed tannins) and esters of hydroxybenzoic and hydroxycinnamic acids. Phenolics are responsible for the astringency of fruits, which generally decreases as fruit ripen and may ensure that the fruit are only consumed when they are ripe and the seeds are ready for dispersal. Astringency is thought to be caused by the interaction of phenolics with the proteins and mucopolysaccharides in saliva. It has been suggested that loss of astringency during ripening may be due to interactions between soluble pectins and polyphenols, disrupting the binding of polyphenols to proteins (Ozawa *et al.*, 1987). In addition, a reduction in proanthocyanidin phenolic compounds and a concomitant loss of astringency is likely to occur as they are the precursors of the anthocyanin pigments synthesized in ripe fruit.

The sugars, organic acids and phenolics found in fruits contribute greatly to determining the taste component of flavour. However, the aroma is a result of the presence of a complex range of volatile compounds. Some of these compounds are common to many fruits whereas others are specific to a particular fruit and are responsible for the unique

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and characteristic aroma and flavour of the fruit. In strawberry fruit over two hundred different volatile compounds have been identified comprising alcohols, aldehydes, esters, carbonyls, sulphur compounds and furanone-derived compounds. Only a small proportion of these are likely to contribute to aroma, with furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone) being one of the most important (Pickenhagen *et al.*, 1981). Fatty acids are quantitatively the major precursors of volatile compounds responsible for aroma in plants. Many of the volatile alcohols, aldehydes, acids and esters are generated from the oxidative degradation of linoleic and linolenic acids by lipoxygenase and hydroperoxide lyase (Perez *et al.*, 1999). Both enzymes have been identified in strawberry fruit and their sequential activities are responsible for the production of the C-6 aldehydes hexenal and hexanal. Hexenal levels are relatively constant throughout ripening whereas hexanal levels increase sharply in over-ripe fruit. This rise could be attributable to a change in the substrate specificity of the hydroperoxide lyase during ripening, yielding a different product (Perez *et al.*, 1999). Indeed, it seems likely that enzymes with a broad substrate specificity could account for the diverse range of volatile compounds produced (Manning, 1993). Hexenal and hexanal are also the precursors of hexyl and hexenyl esters formed by the action of alcohol acyltransferase, which has also been isolated from strawberry fruit (Perez *et al.*, 1999). Lipoxygenase activity is also involved in the production of hexenal in tomato. It has been suggested that of the two lipoxygenase genes identified in tomato, the gene *tomloxB* may be more specifically involved in this reaction as its expression is fruit specific and highest in ripe fruit (Ferrie *et al.*, 1994).

1.1.4.2   *Colour*

For many fruits a change of colour is a natural indicator of fruit ripeness. Colour is an important aesthetic component of ripe fruits and results from a combination of the degradation of existing pigments, such as chlorophyll, and the synthesis of new pigments such as carotenoids and/or anthocyanins (Tucker, 1993). During the ripening of strawberry fruit there is degradation of chlorophyll, consistent with the disappearance of chloroplasts, and a decrease in the content of carotenoids (Gross, 1982). The reddening of the fruit as they ripen is a result of the increased synthesis and accumulation of anthocyanins (Woodward, 1972). Anthocyanins, the glycosidic derivatives of anthocyanidins, comprise a diverse range of pigments that are localized within the vacuole of the plant cell (Tucker, 1993). In strawberry they are inherently unstable during processing with the result that fruit colour is adversely affected by heat and freezing treatments. Anthocyanins are derived from flavonoid compounds synthesized from the primary metabolic precursor phenylalanine, an aromatic amino acid. In strawberry fruit, the accumulation of anthocyanins coincides with the induction of phenylalanine ammonia-lyase (PAL) activity (Given *et al.*, 1988a). This is the first and key enzyme in phenylpropanoid metabolism and as such may be involved in the synthesis of several classes of compounds other than flavonoids. The increase in PAL activity was shown to be due to *de novo* synthesis of the enzyme rather than activation of pre-existing enzyme (Given *et al.*, 1988b). However, the activity of uridine diphosphate glucose:flavonoid O^3-transferase (UDPGFT), the terminal enzyme in the synthesis of pelargonidin-3-glucoside, the principal anthocyanin in strawberry, was also induced as anthocyanin content increased, suggesting that both enzymes regulate

anthocyanin synthesis (Given *et al.*, 1988a). The competitive inhibitor of PAL, L-α-aminoxy-β-phenylpropionic acid (L-AOPP) inhibited anthocyanin synthesis demonstrating that PAL activity is necessary for the accumulation of anthocyanins in ripening strawberry fruit (Given et al., 1988a).

1.1.4.3   *Texture*

The majority of fruits undergo textural changes during ripening leading to extensive softening. Tissue firmness is a major factor determining fruit quality and postharvest shelf life and can vary widely between fruits from different species and within cultivars of the same species. The variation in the extent and rate of softening is likely to reflect different underlying mechanisms causing the textural changes. Turgor loss is associated with postharvest dehydration of fruit and may contribute to textural changes during fruit storage. Starch degradation may account for considerable textural changes in fruit such as banana, where starch comprises a high percentage of the fresh weight. However, it is generally considered that softening and textural changes during ripening of fruits are the result of modification and degradation of the fruit cell walls. Strawberry fruit undergo remarkable softening throughout development and particularly during ripening eventually resulting in almost total liquefaction and a correspondingly short postharvest shelf life. Of the soft fruits, the strawberry has been the most extensively studied in relation to textural changes, although the accompanying alterations in the composition and structure of the cell wall are still not well characterized.

Most work on strawberry has focused on the pectic fraction of the cell wall. During ripening there is swelling and hydration of the cell wall and middle lamella associated with an increase in soluble polyuronides (Woodward, 1972; Knee *et al.*, 1977; Huber, 1984). There is a loss of the neutral sugars arabinose, galactose and rhamnose from the cell wall consistent with higher levels of these sugars in the soluble polyuronide fraction (Knee *et al.*, 1977; Huber, 1984). Huber (1984) suggested that the increased levels of soluble polyuronides may be due to increased synthesis of a modified, more soluble form of polyuronide during ripening, and that the higher proportion of arabinose, galactose and rhamnose present may contribute to this. Arabinose and galactose can be linked to the polygalacturonan backbone via the rhamnosyl moiety, which itself can influence the conformation of the polymer and hence its interaction with other polysaccharides. This addition of less firmly bound, more freely soluble polyuronides may affect the structural integrity of the cell wall resulting in fruit softening. This idea is supported further by the observation that there is no detectable depolymerization of polyuronide as evidenced by the constant average molecular size of these soluble polymers during ripening (Huber, 1984). Polyuronide solublization in strawberry has not been attributed to the enzymic hydrolysis of pectin polymers as many studies show that strawberries lack endopolygalacturonase (PG) activity (Neal, 1965; Barnes and Patchett, 1976; Huber, 1984; Abeles and Takeda, 1990), contrary to one early report of its presence (Gizis, 1964). Alternatively, it has been suggested that increased methylation of strawberry polyuronides during ripening is responsible for their solubilization by removing the sites available for Ca^{2+} cross-linking (Neal, 1965). This is consistent with the observed reduction in pectinmethylesterase (PME) activity in the later stages of ripening (Barnes and Patchett, 1976). Recently, the ripening-enhanced expression of a

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gene with sequence homology to pectate lyase from higher plants has been reported in strawberry (Medina-Escobar *et al.*, 1997b). Pectate lyases randomly cleave β-1,4-linked galacturonosyl residues of pectins. Thus it is possible that pectate lyase activity may contribute to pectin solubilization in strawberry. However, to be consistent with the constant molecular size of strawberry pectin polymers throughout ripening, its mode of action would be unlikely to involve hydrolysis in the middle of pectin polymers which would result in depolymerization of the polyuronide fraction.

In contrast to the pectic fraction of the wall, the hemicellulose fraction undergoes marked depolymerization during ripeness as evidenced by a significant reduction in the molecular weight of hemicellulosic polymers extracted from strawberry fruit cell walls. This change is temporally related to fruit softening and has been attributed to enzymic degradation, although there is little alteration in the neutral sugar content of the hemicellulose polymers (Huber, 1984). Knee *et al.* (1977) also suggested that hemicelluloses are degraded during ripening as an increase in xylose, mannose and glucose, residues characteristic of hemicellulosic polymers, occurs in soluble cell wall fractions. The possibilty that the endo-β-1,4-glucanase (EGase) activity detected in ripe strawberry fruit is involved in hemicellulose degradation has been suggested as it was unable to degrade insoluble cellulose (Barnes and Patchett, 1976). This idea is supported by the observation that the content of cellulose is essentially constant in strawberry fruit throughout development. The composition and structure of cell walls and their modification during fruit ripening is discussed more generally in section 1.2.

1.1.5   Gene expression during strawberry fruit development

The biochemical events that characterize ripening fruits are the result of a developmentally regulated series of changes in gene expression and the resultant *de novo* protein synthesis and/or degradation. Labelling experiments have clearly demonstrated that both protein and mRNA synthesis occur in fruit during ripening. Fractionation by 2-D polyacrylamide gel electrophoresis (2-D PAGE) of the labelled *in vitro* translation products of mRNAs from tomato fruit has revealed considerable differences between unripe and ripe fruit. Some mRNAs persisted as the fruit ripened, whereas the synthesis of others declined or ceased, whilst some new mRNAs appeared as ripening progressed (Tucker and Grierson, 1987).

The changes in gene expression that occur during the ripening of strawberry fruit have been studied by a variety of approaches. The qualitative changes in mRNA have been studied by the *in vitro* translation of total RNA isolated from strawberry receptacle tissue at various stages from immature green to over-ripe (Manning, 1994). The translation products were analyzed by 2D-PAGE and a change in the abundance of more than 50 mRNAs was observed throughout this period of development. The most prominent changes were observed at or just before the onset of ripening (when anthocyanins accumulate) and involved both the increase and decrease of specific mRNAs. The translated products of a number of mRNAs undetectable in immature green fruit increased as the fruit matured and ripened. Another group decreased before the onset of ripening and yet others were prominent in both immature green and ripe fruit, but were reduced or undetectable in fruits between these two stages. Protein

synthesis at different stages of strawberry fruit ripening has also been analyzed by electrophoresis of labelled proteins extracted from receptacle tissue incubated with ^{35}S-methionine (Civello *et al.*, 1996). Whilst many proteins were present at all ripening stages, some either increased or decreased during ripening as subsequently observed for mRNA levels.

Polymerase chain reaction (PCR) differential display has been used to compare differences in gene expression between white (unripe) and red (ripe) strawberry fruit (Wilkinson *et al.*, 1995). Five mRNAs with ripening-enhanced expression were identified by PCR amplification of cDNA subpopulations using a specific set of oligonucleotide primers. Three of these had homology to known proteins including chalcone synthase, an enzyme involved in anthocyanin biosynthesis. A cDNA subtractive library representing genes expressed in red but not green fruit has been used to successfully isolate ripening-specific cDNAs (Medina-Escobar *et al.*, 1997a). Conventional differential screening of the library followed by PCR-Southern blot differential screening (PCR-SBDS) identified eight genes that were expressed only in red fruit.

However, standard differential screening of a ripe fruit cDNA library using cDNA from ripe and white fruits has identified the greatest number of ripening-related genes from strawberry (Manning, 1998a). More than 100 ripening-enhanced clones were isolated, representing 26 different gene families. Those identified with putative functions related to quality traits are shown in Table 1.2.

Table 1.2   Selected strawberry ripening-related genes and their putative functions

Gene	Function
O-methyltransferase Chalcone synthase Chalcone reductase Flavonoid-3-hydroxylase UDPG-glucosyl transferase	Phenylpropanoid metabolism (colour, astringency)
Sucrose transporter	Sugar accumulation (taste)
Endo-β-1,4-glucanase	Cell wall metabolism (texture)
Acyl carrier protein	Lipid biosynthesis (aroma)

Five of the genes appear to encode enzymes involved in phenylpropanoid metabolism. Anthocyanins are derived from secondary metabolites of phenylpropanoid metabolism and the coordinated upregulation of the expression of these genes may be necessary for anthocyanin accumulation. Two of the genes, chalcone synthase and flavonoid-3-hydroxylase, are also expressed in immature strawberry fruit. The products of these genes act at intermediate steps in the phenylpropanoid pathway, which produces many phenolic compounds besides anthocyanins. The expression patterns of these genes suggest they may initially be involved in the synthesis of the astringent phenolics found in unripe fruit, after which synthesis is redirected towards anthocyanin production as the fruit mature and their phenolic content declines (Manning, 1998b). Sugars are an important component of flavour in fruits and have to be imported into these sink organs. Sucrose accumulates in strawberry fruit along with the hexoses glucose and fructose. The increased expression of genes encoding two cell wall invertases (isolated

independently of the differential screen) and a sucrose transporter in ripe fruit is consistent with the accumulation of the hexose products of sucrose hydrolysis and the uptake of intact sucrose into the fruit. The expression of a gene encoding acyl carrier protein, an essential component of the fatty acid synthetase complex catalysing lipid biosynthesis, is also upregulated in ripe fruit. Although strawberry fruit do not accumulate fatty acids and the lipid composition changes little during development, fatty acids are one of the main precursors of the numerous flavour volatiles produced in ripe strawberry fruit. Thus it may be expected that fatty acid synthesis must be maintained to sustain the production of these volatile flavour compounds that are lost from the fruit. The ripening-enhanced endo-β-1,4-glucanase (EGase) gene identified from this differential screen was the basis for the research on the role of EGases in cell wall metabolism during strawberry fruit development described in this thesis. The approach of differentially screening a ripe fruit cDNA library has also been applied to the wild strawberry *F. vesca* (Nam *et al.*, 1999). Of the 8 ripening-induced cDNAs isolated, two had putative identities that were the same as those of the cDNAs isolated from the cultivated strawberry, namely acyl carrier protein and *O*-methyltransferase.

Ripening-enhanced cDNAs have also been isolated from grape (Davies and Robinson, 2000), melon (Aggelis *et al.*, 1997; Hadfield *et al.*, 2000), peach (Callahan *et al.*, 1993), raspberry (Jones *et al.*, 1998a), blackcurrant (Woodhead *et al.*, 1998) and kiwifruit (Ledger and Gardner, 1994) by differential screening, showing that this technique can be used effectively for studying climacteric and non-climacteric fruit, particularly when there are numerous changes in gene expression during development.

1.1.6   Regulation of strawberry fruit development

The ripening of strawberry fruit is typically non-climacteric in that it is independent of

the hormone ethylene. Early work revealed that the hormone auxin is essential for

strawberry fruit growth (Nitsch, 1950). Since then the hormonal regulation of strawberry

fruit development has been studied in detail and a principal role for auxin has emerged.

1.1.6.1   Auxin

The work of Nitsch (1950, 1955) demonstrated that i) the growth of the strawberry

receptacle is regulated by the achenes, ii) the growth of receptacles from which the

achenes have been removed can be restored by the application of synthetic auxins and

iii) the achenes are a source of the biologically active free auxin indole-3-acetic acid

(IAA). The level of free IAA in the achenes has been shown to reach a maximum 10-12

days after pollination, after which time the level declines, whereas in the receptacle, free

IAA was found to be absent or only present in trace amounts (Nitsch, 1955; Dreher and

Poovaiah, 1982). In contrast, conjugated forms of IAA, in which IAA is attached

through either an ester or amide linkage to a sugar or amino acid moiety, were found to

be predominant in the receptacle but were present at only a small proportion of the free

IAA levels in the achenes (Dreher and Poovaiah, 1982). However, other workers have

reported much higher levels of both free IAA in the receptacle, reaching a maximum

concurrently with the maximum level in the achenes, and conjugated IAA in the achenes

(Archbold and Dennis, 1984). The inter-relationships between the free and conjugated

forms of IAA are not well understood. Conjugated IAA may serve as a source of free

IAA in the achenes in the early stages of development in addition to IAA synthesized *de novo*. Conjugates of IAA may also represent temporary storage forms of IAA or inactivated end-products of IAA metabolism (Archbold and Dennis, 1984). Studies using labelled auxins have shown that strawberry fruit are able to conjugate free IAA and metabolize conjugated IAA (Darnell and Martin, 1987).

Thus it is generally accepted that the achenes are able to synthesize free auxin which they export to the receptacle. A continuous supply of auxin is required to maintain receptacle expansion. Several reports describe the inhibition of fruit growth by the removal of the achenes which can be restored by the application of various auxin analogues (Mudge *et al.*, 1981; Archbold and Dennis, 1985). The effectiveness of particular auxins in stimulating growth varied between cultivars suggesting that there were differences either in auxin specificity or in the transport and metabolism of auxins between cultivars (Mudge *et al.*, 1981; Darnell *et al.*, 1987).

The achenes not only have an essential role in fruit growth during the early stages of strawberry fruit development, they also play a role in ripening. For example, removal of the achenes from one half of a mature green fruit accelerated ripening in the de-achened half, as evidenced by increased anthocyanin content and PAL activity and decreased firmness and chlorophyll content compared to the intact control half (Given *et al.*, 1988c). In addition, this accelerated ripening was prevented by the application of synthetic auxins such as 1-naphthaleneacetic acid (1-NAA), but not by the inactive auxin analogue phenoxyacetic acid (POA), indicating an auxin-specific effect. These results lead to the hypothesis that auxin produced by the achenes inhibits ripening in green fruit and that the declining level of auxin in the achenes as the fruit continues to develop (Dreher and Poovaiah, 1982) modulates the rate of ripening (Given *et al.*,

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1988c). Thus auxin appears to be the principal hormone regulating all stages of strawberry fruit development. High concentrations of auxin in the early stages of fruit development are required for receptacle growth and also suppress ripening. As fruit (and achenes) mature auxin levels decline in the receptacle allowing ripening to proceed.

The regulation of strawberry fruit development by auxin has been investigated at the level of gene expression. The patterns of polypeptides obtained from *in vitro* translation of total RNA extracted from de-achened receptacle tissue treated either with water (control) or with auxin indicates that auxin regulates gene expression in ripening strawberry (Manning, 1994). The pattern produced from the de-achened receptacle treated with auxin which did not ripen was similar to that for normal intact unripe fruit. The ripened de-achened control receptacle produced a translation profile similar to that of normal ripe fruit as did the de-achened receptacle treated with POA which ripened normally indicating that active auxin is a repressor of ripening in strawberry.

Changes in the abundance of specific polypeptides during strawberry fruit development have been reported (Veluthambi and Poovaiah, 1984). Removal of the achenes from small green fruit retarded growth and suppressed the appearance of polypeptides of 81, 76 and 37 kDa. Application of auxin to the de-achened fruit restored growth and the formation of these polypeptides indicating that they are induced by auxin. In contrast, two polypeptides of 52 and 57 kDa were present in de-achened fruit but absent in auxin-treated de-achened fruit indicating that they are repressed by auxin and may inhibit fruit growth in the absence of auxin. This is consistent with the correlation shown between a lack of receptacle growth and the accumulation of a 52 kDa polypeptide in a strawberry variant genotype (Veluthambi *et al.*, 1985). Receptacle

growth of the variant genotype required the application of auxin which also abolished the 52 kDa polypeptide.

The cDNA (λSAR5) of an auxin-repressed gene has been identified in strawberry (Reddy and Poovaiah, 1990). The expression of this gene is blocked by endogenous auxin during normal fruit development. In the variant genotype referred to above, transcripts were 50-fold more abundant in the untreated fruit which did not grow, than in the auxin-treated fruit which did grow, suggesting that the product of this gene is required for fruit growth. In addition, the cDNAs (λSAR1 and λSAR2) of two auxin-inducible genes have been isolated, which show the opposite patterns of expression to the auxin-repressed gene in normal fruit and in fruit of the variant genotype (Reddy *et al.*, 1990). Thus, auxin regulates strawberry fruit development by both inducing and repressing the expression of specific genes.

1.1.6.2 Ethylene

A principal role for auxin in the regulation of strawberry fruit development contrasts with the apparent lack of any involvement of ethylene in this process. As a typical non-climacteric fruit (section 1.1.3), the strawberry does not exhibit increased respiration and ethylene production during ripening (Knee *et al.*, 1977; Abeles and Takeda, 1990). Its ripening is unaffected by exogenous ethylene and by inhibitors of ethylene synthesis or action (Given *et al.*, 1988c), indicating that ethylene does not have a role in the regulation of strawberry fruit development. The results of a study on the effect of ethylene on RNA metabolism in strawberry fruit after harvest appear to contradict this idea (Luo and Liu, 1994). The authors found that fruit treated with ethylene had

increased total and polyA RNA content, although the identity of the induced mRNAs was not investigated. However, non-climacteric fruit are known to respond to exogenous ethylene with an increased respiration rate, possibly as a result of a general increase in metabolism including increased gene expression. Conversely, a reduced respiration rate and slowed ripening may also account for the extended shelf life of strawberries which were stored in a reduced ethylene environment (Wills and Kim, 1995).

However, studies on other non-climacteric fruit have implicated endogenous ethylene in the regulation of certain aspects of ripening. In citrus fruit, endogenous ethylene appears to regulate the degreening process. Ethylene antagonists prevent colour changes of the flavedo tissue and both chlorophyll degradation and carotenoid biosynthesis, processes that result in degreening, are stimulated by ethylene (Lelievre et al., 1997). Several cDNAs corresponding to ethylene-inducible mRNAs have been isolated from citrus fruit and three of these showed increased expression in fruit between the green and fully coloured stages of ripening (Alonso et al., 1995). It is also interesting that anthocyanin accumulation is unaffected by ethylene in strawberry (Given et al., 1988c) and cherry whereas anthocyanin biosynthesis is stimulated by exogenous ethylene in grape (Lelievre et al., 1997).

Most studies on the regulation of strawberry fruit development have focused on the role

of auxin. However, there are a few reports on the effects of other growth regulators on

strawberry development *in vitro*.

The effects of gibberellin and cytokinin in conjunction with auxin were studied

on intact fruit cultured *in vitro* (Kano and Asahira, 1978). Gibberellin was found to act

synergistically with auxin to promote growth and ripening, whilst cytokinin repressed

growth and ripening. This work also indicated that the achenes are a source of cytokinin

in addition to auxin and that the promotive effect of auxin on fruit growth is balanced by

an inhibitory effect of cytokinin. It was concluded that auxin was the dominant hormone

regulating growth and ripening, but that its effects could be modulated by other growth

regulators. The reported effect of exogenous gibberellin and auxin together on ripening

contrasts with the effect of gibberellin alone. When gibberellin was applied to whole

and de-achened mature green strawberry fruit it delayed ripening as measured by a

reduction in the anthocyanin production and chlorophyll degradation (Martinez *et al.*,

1994). This achene removal did not affect the response to gibberellin, indicating that

auxin was not involved. Gibberellin was also found to decrease respiratory activity in

ripening fruit, leading to the conclusion that gibberellin inhibits general metabolic

activity rather than the ripening process itself.

Abscisic acid (ABA) was found to decline in the achenes in the early stages of

fruit development before accumulating during ripening (Archbold and Dennis, 1984).

The concentration of ABA was consistently higher in the achenes than the receptacle

throughout development and the increase in ABA levels during ripening was much less

in the receptacle. Thus there is a declining ratio of auxin to ABA in the achenes as fruit ripen although no significant correlation with fruit growth or ripening was found. In a separate study, exogenous ABA stimulated ripening in receptacles cultured *in vitro* (Kano and Asahira, 1981). However, as with all *in vitro* studies with growth regulators, the results should be interpreted carefully as detached fruit develop more slowly than fruit on the plant and the levels of growth regulators present in them may not represent the true levels present in intact fruit (Manning, 1993).

One of the more recently identified endogenous plant growth regulators is jasmonic acid (JA) and its volatile methyl ester, methyl jasmonate (JAMe) (Staswick, 1996), the main activities of which include the promotion of senescence. The effect of JAMe on strawberry fruit ripening has been studied *in vitro* (Perez *et al.*, 1997). A significant increase in respiration and ethylene production was observed in immature fruit as was a transitory induction of anthocyanin biosynthesis and chlorophyll degradation. A role for JAMe as an inducer of ripening in strawberry was suggested. However, as with gibberellin which appears to inhibit ripening, it may be that the effects on ripening are the result of the altered respiration rate and hence altered general metabolic activity rather than a specific effect on aspects of ripening.

The possible involvement of polyamines in strawberry fruit development has been investigated after reports that polyamines affect fruit growth (Ponappa and Miller, 1996). Application of auxin to de-achened strawberry receptacles not only re-initiated growth of the receptacle but also caused an increase in the polyamine concentration in the receptacle. However, in normal fruit development total polyamine concentration decreased from a maximum at the early stages of receptacle development to a minimum in ripe receptacles. A similar pattern was observed in the achenes, although the

concentration of polyamines was higher than in receptacles. In addition, application of exogenous polyamines did not induce receptacle growth. Thus it appears that polyamines do not act as hormonal regulators of strawberry fruit growth, although their biosynthesis is auxin-inducible. Polyamines are implicated in cell division and the high polyamine levels coincide with the period of cell division that occurs early in strawberry fruit development and is induced by auxin treatment. The high polyamine concentration may also influence the early development of the achenes and in doing so may indirectly affect development of the whole fruit.

1.2   CELL WALL STRUCTURE AND FRUIT TEXTURE

The plant cell wall has many functions, the most important being related to structural properties. The cell wall determines the size and shape of the cell, attaches it to its neighbours and provides rigidity to the cell. In doing so, the wall makes a key contribution to the structural strength of the whole plant. Selective reversible weakening of the wall allows controlled growth of the cell while maintaining the overall structural integrity of the wall and cell. The structure of the cell wall and its net negative charge are an obstacle to the movement of large and positively charged molecules into and out of the cell. Water and low molecular weight molecules are able to pass between cells across the cell wall, as are cell signalling compounds which are generally small and either neutral or negatively charged. Certain wall components may themselves act as regulatory molecules with a role in cell-cell communication. The walls of neighbouring cells are in direct contact and together with the intercellular space, they constitute the apoplast, a major transport pathway that allows movement of materials external to the cell cytoplasm. The nature of the wall that causes it to be a barrier becomes an advantage in the protection of the cell from attack by pathogens. Thus cell walls are actively involved in a wide range of metabolic processes. There is active synthesis and modification or degradation of the cell wall during cell growth and in response to attack and specific degradation of the wall during particular developmental processes such as fruit ripening, abcission and senescence. The variety of roles that the cell wall fulfils may explain its complex structure and the tight control exerted over the structural changes that occur in the wall throughout development.

Cell structure and the nature of the polymers in the cell wall determine the physical characteristics of plant tissues. Thus the texture of fruit is determined by the underlying composition and structural integrity of the fruit cell walls. Turgor pressure acting against the cell wall also contributes to tissue firmness. During ripening, physical changes occur in the structure of fruit cell walls leading to a loss in their integrity often resulting in separation of the cells. Hence, the composition of fruit cell walls, the changes they undergo during ripening and the mechanisms responsible for these changes have all been studied in an attempt to understand what determines the texture of ripe fruits.

1.2.1 Composition and structure of the plant primary cell wall

1.2.1.1 Structural components of the cell wall

The plant cell wall is deposited as a series of layers with the earliest layers being on the exterior, adjacent to the neighbouring cells. The layer between adjacent cells is known as the middle lamella and occupies the site of the cell plate that was laid down at cell division. The primary cell wall is deposited on the middle lamella during the growth of the cell that follows cell division and continues to be deposited as long as the cell continues to grow. Primary cell walls are generally of a similar thickness in most cell types ranging between 0.1 µm and 1.0 µm. In contrast, some specialized cells go on to produce a thicker secondary cell wall, which is internal to the primary wall, after cell growth has ceased and the cell begins to differentiate.

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The structural components of a typical growing primary cell wall comprise 90-95% carbohydrate polymers and 5-10% glycoprotein. The types of polysaccharide polymers present in the primary wall fall into three classes, cellulose, hemicelluloses and pectins, and are found in all higher plants. However, the proportion of each type of polysaccharide present varies with cell type and between species. This variation is most marked in grasses where the pectin and hemicellulose content differs considerably from that typical of other higher plants (Fry, 1988). The classification of the polysaccharides was originally based on the methods used for their extraction from the cell wall. The pectic fraction can be obtained by extraction with a hot, aqueous solution of a chelating agent or hot, dilute acid, the hemicellulose fraction with alkaline solutions and the remaining insoluble fraction is cellulose. However, in reality not all the various polysaccharides within a particular class will extract equally under the same conditions and some cross-contamination of fractions may occur. Water can also be considered as a structural component of cell walls. Growing primary walls are composed of about 65% water which influences the conformation of certain polymers in the wall (Fry, 1988).

Cellulose is usually about 30% of the dry weight of the primary wall and is an unbranched polymer of D-glucose residues joined by β-(1$\rightarrow$4) linkages. The linear chains of β-(1$\rightarrow$4)-D-glucan associate with each other via hydrogen bonds to form fibrillar structures called microfibrils of about 10 nm diameter. X-ray diffraction and chemical studies indicate that the bulk of the microfibril is made up of cellulose chains organized in a crystalline lattice giving the microfibril considerable strength. Less crystalline regions may exist around the crystalline core of the microfibril (Brett and Waldron, 1996).

Hemicelluloses, unlike cellulose, are comprised of a variety of different sugars, the major ones being glucose, xylose, arabinose and glucuronic acid, and are able to form hydrogen bonds with cellulose. A variety of different hemicellulosic polymers exist including xylans, xyloglucans, glucomannans and β-(1$\rightarrow$3)(1$\rightarrow$4)-glucans. Depending on the plant or cell type, one type of hemicellulose usually predominates in the primary wall with others present in lesser amounts. Xyloglucan is the major hemicellulose in dicots accounting for about 20% of the dry weight of the primary wall, with glucomannans and galactoglucomannans also present (Tucker, 1993). Xyloglucans are neutral polysaccharides and have a backbone of β-1,4-linked glucose residues as found in cellulose. Xylose-containing side chains are attached to the majority of the glucose residues in the backbone by α-(1$\rightarrow$6) bonds. The most common side chain consists solely of D-xylose but in some xyloglucans some of the xylose residues may be further substituted by the disaccharide fucose-α-(1$\rightarrow$2)-galactose-β-(1$\rightarrow$2) or arabinose-(1$\rightarrow$2) (Fry, 1988). Glucomannans consist of a backbone of β-1,4-linked glucose and mannose residues with a slightly higher proportion of mannose than glucose. In some cases single galactose residues are present as side chains to produce galactoglucomannans (Brett and Waldron, 1996). Xylans and β-(1$\rightarrow$3)(1$\rightarrow$4)-glucans are found predominantly in grasses. Arabinogalactan II has been classed as a hemicellulose, but as described later, these molecules may be the polysaccharide component of arabinogalactan proteins.

Pectins are polysaccharides rich in D-galacturonic acid as well as arabinose, galactose and rhamnose. They can be subdivided into the neutral pectins comprising arabinans, galactans and arabinogalactans and the acidic pectins consisting of rhamnogalacturonans and homogalacturonans (polygalacturonic acid, PGA). Pectins have been described as

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having a block structure with homogalacturonan and rhamnogalacturonan covalently linked in the same molecule (Fry, 1988). The 'smooth' regions of homogalacturonan consist mainly of an unbranched backbone of D-galacturonic acid residues joined by α-(1→4) linkages punctuated by the occasional α-1,2-linked rhamnose residue. These regions are covalently joined to 'hairy' regions of rhamnogalacturonan (RG) which exists in two forms, RG I and RG II. RG I consists of a backbone of alternating α-1,4-linked galacturonic acid and α-1,2-linked rhamnose residues with arabinose and galactose-rich side chains ('hairs') attached to the 4-position of the rhamnose. RG II contains a greater variety of sugars and appears to be built up of a galacturonic acid-rich core with very specific side chains. The neutral arabinan, galactan and arabinogalactan pectins may also be linked to the rhamnogalacturonan backbone via the rhamnose residues as further side chains in the 'hairy' regions. Arabinans are highly branched molecules containing a backbone of α-1,5-linked arabinose with side chains of single arabinose residues or α-1,5-linked arabinose oligosaccharides linked by α-(1→2) or α-(1→3) bonds to the main chain. Galactans consist mainly of β-1,4-linked galactose residues whilst arabinogalactan I polymers have short α-1,5-linked arabinose side chains attached to the β-(1→4)-galactan backbone. In addition to being attached to the acidic pectin backbone, neutral pectins also exist as independent molecules (Brett and Waldron, 1996).

Some of the galacturonic acid residues in the 'smooth' homogalacturonan regions may be methyl esterified. It is not clear whether distinct blocks of methylated galacturonic acid residues exist interspersed with regions that are not esterified or if the distribution of methyl ester groups is more random (Fry, 1988). Regions of un-esterified residues can chelate calcium ions allowing adjacent pectin molecules to be cross-linked

together non-covalently by Ca^{2+} bridges forming PGA 'junction zones' and producing an 'egg-box' structure.

In addition to polysaccharide polymers, structural proteins are an important part of the primary cell wall. The majority are glycosylated and frequently have an unusual amino acid composition rich in hydroxyproline. For this reason they are often referred to as HRGPs (hydroxyproline-rich glycoproteins). One of the best studied families of cell wall glycoproteins is the extensin family. Extensins contain high proportions of hydroxyproline, serine and lysine residues with the sequence Ser-(Hyp)$_4$ repeated throughout the molecule. The hydroxyproline residues are attachment sites for tri- and tetra-arabinose oligosaccharides and the serine residues are linked to single galactose residues. Tyrosine residues are also present which are able to cross-link to form intramolecular and possibly intermolecular covalent bonds (Brett and Waldron, 1996).

A second class of cell wall glycoproteins is the arabinogalactan proteins (AGPs). The polypeptide backbone is rich in hydroxyproline, serine, alanine and glycine. Long polysaccharide side chains are attached to the hydroxyproline residues via a β-galactose linkage. These polysaccharides are similar to arabinogalactan II with their galactan backbones joined by β-(1→3) and β-(1→6) linkages and side chains containing arabinose and smaller amounts of glucuronic acid and galacturonic acid (Fry, 1988).

Phenolic compounds are present in much smaller amounts in the cell wall and the most abundant, lignin, is mainly confined to specialized secondary cell walls. Ferulic acid may be present in primary cell walls, usually esterified to arabinose and galactose

residues in neutral pectins. It may have a role in cross-linking these pectins via covalent diferulic acid bonds (Brett and Waldron, 1996).

1.2.1.2 Structure of the primary cell wall

Early models of plant primary cell wall structure envisaged cellulose microfibrils coated with xyloglucan which was also attached to arabinogalactans, with further links between arabinogalactans and acidic pectins, and pectins and extensin (Keegstra *et al.*, 1973). This separate layered construction was based on the polysaccharides and proteins that had been characterized at the time. In this model, xyloglucans were hydrogen bonded to the cellulose microfibrils and all other polymers were linked by covalent bonds, although little evidence was available to confirm the type of interaction or bonding between the different components. The current model of the primary cell wall (Figure 1.1, Carpita and Gibeaut, 1993) describes a far less static structure and accounts for growth of the wall during cell expansion. In addition, it is now clear that non-covalent bonding, such as hydrogen bonding and ionic bonding, plays a significant role in linking the different polymers in the wall together, rather than the predominance of covalent bonds postulated by the early model. It is difficult to distinguish intra- and inter-molecular covalent linkages in cell wall polymers and so relatively few types of covalent cross-link have been characterized. Covalent linkages also probably exist between other polymers in the wall, for example pectin and protein (Brett and Waldron, 1996). Essentially the primary cell wall consists of cellulose microfibrils coated with hemicellulose embedded in a matrix of pectins and structural proteins. More specifically, the wall comprises three structurally independent but interacting networks

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or domains which interlink to form the overall complex structure that is the primary cell wall (Carpita and Gibeaut, 1993; Brett and Waldron, 1996).
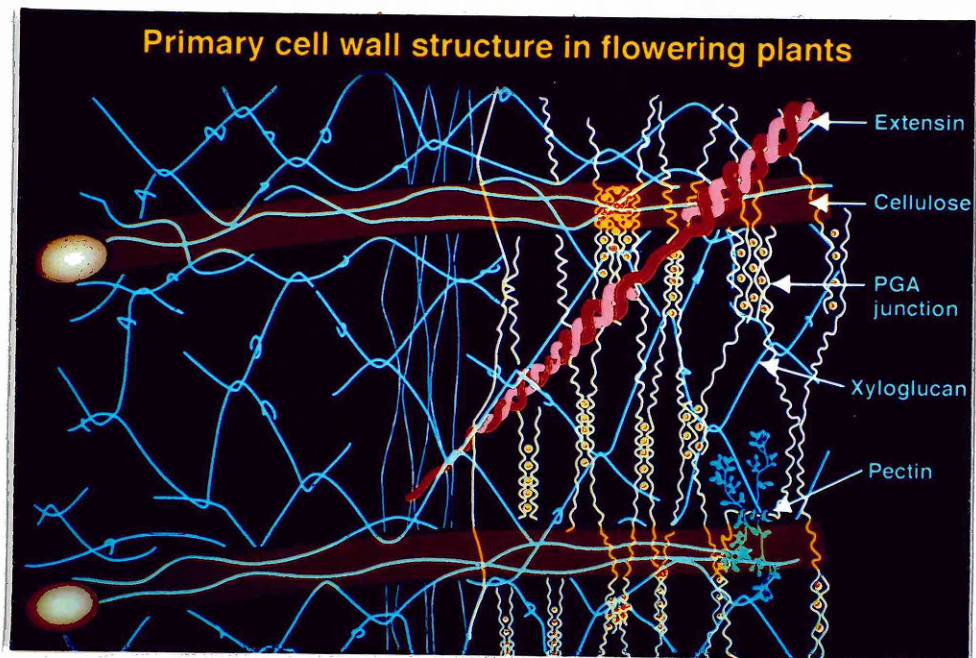
Figure 1.1   Structural model of the primary cell wall of most flowering plants (after Carpita and Gibeaut, 1993)

The first and most fundamental of these networks is the cellulose-hemicellulose framework. In most higher plants (Type I walls) this is a cellulose-xyloglucan network, xyloglucan being the predominant hemicellulose in these plants. In grasses (Type II walls), the major hemicellulose is different and the resulting structure will not be considered here. Xyloglucans in Type I walls are able to hydrogen bond to cellulose and these polymers coat the surface of the microfibrils. The coating is restricted to a single layer as hydrogen bonds can only be formed on one side of the xyloglucan molecule. However, as cellulose and xyloglucan are present in the primary wall in about equal amounts, not all of the xyloglucan can exist as a monolayer coating the microfibrils. The remainder is thought to span the gaps between microfibrils and these cross-links have been observed by electron microscopy. In this way the xyloglucan molecules act as 'molecular tethers' to interlock the microfibrils and they may also bind to other matrix components. It has also been suggested that in coating the microfibrils the xyloglucan prevents them from hydrogen bonding to each other (Hayashi, 1989) and in doing so allows the microfibrils to move as necessary during cell growth. In dividing cells and during isodiametric cell expansion, the microfibrils are wound around the cell randomly. When cell elongation begins, the microfibrils are wound transversely or in a shallow helix around the longitudinal axis of the cell, restricting cell expansion to one dimension. These move further apart as the cell expands and can then be finally locked back into place once growth has stopped (Carpita and Gibeaut, 1993). The substantial number of hydrogen bonds that form between the surface of microfibrils and xyloglucan molecules is likely to be structurally significant in anchoring the microfibrils into the matrix of the wall. Thus the cellulose-xyloglucan network is considered to be the dominant load-bearing structure in the growing cell wall. Hemicelluloses are also likely

to hydrogen bond to each other. Xyloglucan molecules may bind pairwise, since it is not possible for several molecules to stack together because the xylosyl residues protrude from one side of the molecule. The differential extraction of three structurally distinct xyloglucan fractions from cell walls isolated from pea has extended the existing model of the cellulose-xyloglucan network (Pauly *et al.*, 1999). These authors propose the existence of three different macromolecular domains of xyloglucan. The first comprises the xyloglucan cross-links between microfibrils and any exposed regions of xyloglucan molecules that extend away from the microfibril surface. This domain is covalently attached to the second domain, which consists of xyloglucan hydrogen bonded to the surface of the microfibrils as described by current cell wall models. In addition, a third unlinked xyloglucan domain is believed to be entrapped within or between cellulose microfibrils in relatively non-crystalline regions of the microfibril. The xyloglucan cross-link domain is predicted to be the only one accessible to cell wall enzymes and hence likely to be the domain modified during changes in the cell wall.

The cellulose-xyloglucan network is embedded in the second network formed by the pectin matrix, which although independent, probably interacts with the cellulose-xyloglucan framework (Carpita and Gibeaut, 1993). There is some evidence for covalent bonding between xyloglucan and pectin polymers (Brett and Waldron, 1996). Xyloglucan may be linked to the arabinogalactan side chains attached to rhamnogalacturonan I. It is possible that the linkage is a glycosidic bond between the reducing group (=O) of the xyloglucan and a hydroxyl group (-OH) in the pectin side chain (Fry, 1988). Non-covalent hydrogen bonds may also form between hemicellulosic and pectic polysaccharides in the matrix (Fry, 1988). Strong cross-links can be formed

between pectin molecules in regions termed 'junction zones'. Stretches of unmethylated polygalacturonic acid in the pectin backbone can bind Ca^{2+} ions allowing multiple pectin molecules to be linked together by non-covalent ionic Ca^{2+} bridges to produce 'egg-box' like structures. When regions of pectin are heavily methylated or calcium levels in the wall are low the junction zones may be held together by hydrogen bonds instead (Brett and Waldron, 1996). Some pectins in the matrix may also form covalent cross-links with other pectins if ferulic acid is present in their side chains. Ferulic acid is usually found esterified to arabinose and galactose residues and can form diferulic acid bonds linking two molecules together. Whilst the cellulose-xyloglucan network provides the main structural strength of the growing cell wall, the pectin matrix is believed to determine the porosity of the wall. The location of de-esterified polygalacturonic acid, the size and abundance of junction zones and the size and conformation of the attached side chains could all influence the nature of the gel formed by the pectin and hence determine the pore size (Carpita and Gibeaut, 1993). Water is also important in the formation of gels in the pectin matrix (Fry, 1988). By affecting the movement of macromolecules through the wall the pectin network may control the access of cell wall modifying enzymes to the different components of the wall embedded within it and thus indirectly influence the mechanical properties of the wall.

The third network is that of the structural proteins. The major contributor is extensin and this forms an insoluble network that is structurally independent of the polysaccharide networks (Carpita and Gibeaut, 1993). The nature of the cross-links between extensin molecules and other structural proteins is not clear. Tyrosine residues form covalent isodityrosine bonds within an extensin molecule and may also link these molecules

together. Cross-links between tyrosine and lysine are another possibility. The arrangement of the extensin network is the key to its contribution to cell wall structure. The cellulose microfibrils are laid down parallel to the plasma membrane in the transverse axis of the cell and are cross-linked by xyloglucan molecules in the longitudinal axis. The extensin network is believed to be oriented radially, that is, perpendicular to the plasma membrane and the cellulose-xyloglucan framework (Carpita and Gibeaut, 1993). In this way extensins are proposed to interlock and hold the layers of the cellulose-xyloglucan network together, fixing the shape of the cell. The extensin network may also form non-covalent and covalent bonds with the polysaccharide networks, for example ionic bonds may form with pectins.

Thus the primary cell wall is composed of three structurally independent, yet interacting domains. The components of each of these domains can change independently such that the cell wall is a dynamic, highly ordered, developmentally regulated network.

1.2.2   Changes in the cell wall during fruit ripening

Structurally, fruit cell walls appear to be very similar to the generalized model of a typical primary cell wall. Analysis of the monomer composition of apple and strawberry fruit cell walls revealed low amounts of xylose and mannose and higher levels of galacturonic acid, arabinose and galactose (Knee and Bartley, 1982). This may indicate that fruit cell walls contain relatively more pectic polysaccharides and less hemicellulosic polymers and proteins than other plant cell walls. This suggests that fruit

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cell walls may have a higher ratio of pectin-rich middle lamella to primary wall which is likely to be important in the softening process.

Textural changes and extensive tissue softening accompany the ripening of the majority of fruits and this is predominantly due to the modification and degradation of the fruit cell wall structure, including the middle lamella. The different extents to which different fruits soften reflects the underlying variations in the specific composition of their cell walls and hence the changes these polymers undergo during ripening. The specific cell wall changes that occur during the ripening of strawberry fruit have already been discussed in detail (section 1.1.4). Generally, fruits undergo similar types of modification but to varying degrees, and these usually involve the solubilization and/or depolymerization of the pectic and hemicellulosic polymers in the wall, resulting in softening of the fruit tissue.

Changes in cell wall structure during ripening have been observed under both the light and electron microscope in many fruit. The most apparent change is the dissolution of the pectin-rich middle lamella which is seen as a loss of electron density in this region for example in the ultrastructural studies of avocado (Platt-Aloia et al., 1980). Breakdown of the middle lamella also occurs in tomato, strawberry, plum and persimmon where swelling of the wall in the middle lamella region has been observed under the light microscope (Redgwell et al., 1997). In extreme cases, swelling of the cell wall and dissolution of the middle lamella results in complete separation of adjacent cells. Swelling of the wall appears to be linked to pectin solubilization and increased hydration of the wall.

Ultrastructural studies also often show disorganization of the fibrillar structures of the wall under the electron microscope. This has been observed in ripening avocado, pear and apple (Fischer and Bennett, 1991) and was attributed to degradation of the cellulose microfibrils. However, the relatively constant cellulose content of most fruits during ripening does not support this view. In avocado, a loss of the organization and density of the wall striations was observed representing a loss of fibrillar material (Pesis et al., 1978; Platt-Aloia et al., 1980). O'Donoghue et al. (1994) suggested that the observed loss of cohesiveness of cellulose microfibrils was due to limited degradation of the non-crystalline regions of the microfibrils, resulting in disorganization within the fibril structure and disruption of the binding of the associated matrix polysaccharides. Thus, the ultrastructural changes in microfibrillar organization may also result from the disruption of other, non-cellulosic components associated with the microfibrils (Fischer and Bennett, 1991).

Changes in the pectic fraction of fruit cell walls have been the most commonly studied. In many fruits, there is a net loss or solubilization of non-cellulosic neutral sugars from the wall during ripening. These sugars comprise mainly galactose and arabinose, both of which are major components of neutral pectins (Gross and Sams, 1984), which may exist as side chains on the acidic pectin backbone or as independent polymers. Whilst in strawberry fruit the loss of these sugars from the cell wall corresponds to an increase in the soluble polyuronide fraction (Knee et al., 1977; Huber, 1984), this is not the case for tomato, where the loss of galactose and arabinose is associated with an increase in the total free sugars (Tucker and Grierson, 1987). Differences in the texture of 'crisp' and 'soft' cherry fruit are thought to be related to their neutral sugar content (Batisse et al.,

1996). The higher content of neutral sugars in the crisp fruit compared with the soft fruit may indicate that the pectins are more highly branched in crisp fruit. This could result in a greater interaction between polymers, leading to a stronger cell wall structure. Another major change in the pectins observed during the softening of tomato fruit is an increase in the solubility of the polyuronides comprising the acidic pectin fraction (Tucker and Grierson, 1987). This increased polyuronide solubilization is believed to be independent of the loss of neutral sugars, which is not associated with softening of the fruit (Tucker and Grierson, 1987). Evidence supporting this is found in fruits of the tomato ripening mutant *rin*, which have lower levels of cell wall galactose and arabinose than wild type fruits and exhibit little pectin solubilization or softening (Huber, 1983). The increase in polyuronide solubility indicates that covalent cross-links to insoluble polymers have been cleaved, releasing pectin polymers that are subsequently water- or chelator-soluble (Fischer and Bennett, 1991). However, in strawberry it has been suggested that the increase in soluble polyuronides may result from the synthesis of new, more soluble polymers (Huber, 1984).

Size fractionation of extracted pectins by gel filtration has revealed that in many fruits the average molecular size of the pectins decreases dramatically during ripening. This indicates that in addition to the breakage of cross-links which releases soluble pectin polymers, the rhamnogalacturonan backbone is also cleaved to produce lower molecular weight polymers (Fischer and Bennett, 1991). It is possible that the removal of the neutral side chains and the disruption of cross-links makes the pectin backbone more accessible to degradation by cell wall hydrolases, resulting in the overall depolymerization of the pectic fraction (Tucker and Grierson, 1987). Avocado polyuronides exhibited a marked downshift in molecular weight during ripening,

indicating that substantial depolymerization of the solubilized pectin had occurred (Sakurai and Nevins, 1997). Less rapid and less extensive depolymerization was observed in tomato (Huber and O'Donoghue, 1993) and also in pear (Yoshioka *et al.*, 1992). In contrast, soluble polyuronides of apple (Yoshioka *et al.*, 1992) and strawberry (Huber, 1984) do not undergo depolymerization.

The degree and pattern of methyl esterification of the polyuronide fraction may also change during ripening (Tucker, 1993). Only unesterified pectin is available to bind Ca^{2+} ions and form cross-links with adjacent pectin molecules via Ca^{2+} bridges. Changes in the esterification of polyuronides and in the level of Ca^{2+} ions in the wall may alter pectin stability. The increase in soluble pectins during the softening of apple fruit could be partly explained by an increase in the degree of polyuronide esterification resulting in fewer ionic cross-links between pectin molecules (Huber, 1983). However, in tomato there is a decline in esterified polyuronide during ripening (Tucker, 1993). Similarly, a reduction in the levels of Ca^{2+} in the wall reported in ripening fruit could destabilize the pectin matrix (Huber, 1983). Calcium treatment of apple fruits was found to preserve the structural integrity of the cell wall and maintain cell cohesiveness (Seymour and Gross, 1996). Unesterified pectin and Ca^{2+} levels were found to be highest in the middle lamella and corner junctions of intercellular spaces in tomato fruit cell walls (Seymour and Gross, 1996). Destabilization of the pectin in these areas is consistent with the observed dissolution of the middle lamella and loss of cell cohesiveness that occurs as fruit ripen.

Changes in the hemicellulose fraction of the cell wall also occur during the ripening of most fruits, although they are not as well characterized as those of the pectic fraction. In

general, the hemicellulose content of the wall is not significantly altered during ripening (Fischer and Bennett, 1991) and neither is the content of the major hemicellulose monomers, xylose, glucose and mannose (Tucker and Grierson, 1987). However, gel filtration demonstrates a significant reduction in the average molecular weight of hemicellulose polymers extracted from the cell walls of many fruits during ripening (Seymour and Gross, 1996) which is taken as evidence that they undergo depolymerization. Hemicelluloses from avocado fruit exhibited a broad range of polymer sizes and an overall decrease in molecular weight during ripening, as did those from strawberry fruit (Huber, 1984). The pattern observed for total hemicelluloses was also evident for xyloglucan in avocado fruit. This molecular weight downshift was shown to be the consequence of depolymerization and was associated with significant changes in fruit texture (O'Donoghue and Huber, 1992; Sakurai and Nevins, 1997). A similar situation occurs in ripening tomato fruit where the molecular mass of hemicelluloses from red fruit walls was 50% of that from green fruit (Sakurai and Nevins, 1993). This difference was associated primarily with the degradation of xyloglucans. However, in the non-softening tomato mutant *rin*, no significant change in hemicellulose molecular weight was reported (Seymour and Gross, 1996). It has been suggested that the molecular weight shift of hemicelluloses may also involve synthesis of small polymers enriched in mannosyl and glucosyl residues, which may be glucomannans (Fischer and Bennett, 1991). Changes in the hemicellulose fraction have also been studied in the fruit of peach (Hegde and Maness, 1998), hot pepper (Gross *et al.*, 1986), papaya (Paull *et al.*, 1999), kiwifruit (Miceli *et al.*, 1995) and melon (Rose *et al.*, 1998), all of which exhibit depolymerization of hemicelluloses during ripening. In

contrast, apple fruits show no change in the molecular weight profile of hemicellulose fractions or xyloglucan polymers during ripening (Percy *et al.*, 1997).

Other cell wall changes that may occur during fruit ripening include alterations in wall pH and altered biosynthesis of wall components. The pH of the plant cell wall is generally thought to be in the range of 4.5 to 6.0. The desterification of pectins may contribute to the acidity of the wall and changes in pH are likely to alter the activity of cell wall bound enzymes, including cell wall modifying enzymes. Wall integrity could be altered not only as a result of degradation by cell wall hydrolytic enzymes or modification of existing polymers, but also by changes in the synthesis of cell wall polymers (Seymour and Gross, 1996). Cell wall synthesis and degradation have been shown to occur simultaneously in ripening strawberry fruit (Knee *et al.*, 1977) and it has been suggested that increased polyuronide solubility in strawberry may involve the synthesis of more soluble forms of this polymer during ripening (Huber, 1984). Radiolabelling has been used to demonstrate an increase in the synthesis of cell wall components in tomato fruit during ripening and softening that does not occur in tomato ripening mutants. Analysis of the hemicellulose fraction of tomato fruit cell walls indicated there may be an increased synthesis of glucomannans during ripening (Seymour and Gross, 1996). Thus, it may be that the changes that occur as fruit soften are the result of altered cell wall turnover during ripening, that is a combination of degradation, modification and synthesis of cell wall polymers rather than just degradative processes.

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1.2.3 Cell wall modifying enzymes and proteins

It has been well established that softening and textural changes associated with fruit ripening occur largely as the result of modification and degradation of the fruit cell walls. These changes are considered to result from the action of cell wall modifying enzymes and proteins. The complexity of the wall suggests that there are likely to be a number of activities involved and the major classes into which these activities fall are described below.

1.2.3.1 *Nomenclature*

The majority of the cell wall degrading enzymes are hydrolases, breaking glycosidic, ester and peptide bonds (Fry, 1988, 1995). Most hydrolases are glycosidases (*O*-glycosylhydrolases, EC 3.2.1.-), that is they hydrolyse glycosidic bonds. Glycosidic bonds form between the reducing terminus (C=O) of a monosaccharide (glycose) and another molecule to give the glycosyl (non-reducing) residue and its joined aglycone. Glycosidases can be exo- or endo- acting. Exo-glycosidases attack polysaccharides from the non-reducing termini, generally releasing monosaccharides. Thus their activity can be detected by a substantial increase in the release of reducing sugars from a polysaccharide substrate. They are usually specific for the glycosyl residue hydrolysed and require that it is not substituted, but relatively non-specific for the aglycone. Exo-glycosidases are unlikely to have much impact on the chain length of wall polymers as they only hydrolyze from the ends of the molecules, but they may have a substantial effect on the physical properties of a polymer by removing short side chains from the

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backbone. They are also likely to be responsible for the release of neutral sugars that occurs during fruit ripening. However, endo-glycosidases (endoglycanases) attack polysaccharides at any position except at or near the termini and hence can have a large impact on the molecular weight of the polymer and the structural integrity of the cell wall. They are likely to be responsible for the depolymerization of pectic and hemicellulosic polysaccharides that occurs during ripening. Their activity can be detected by a rapid decrease in the viscosity of a soluble polysaccharide substrate and also by the release of reducing end-groups. Hydrolase activities that act on carboxy-ester bonds found in esterified pectins are also present in cell walls.

Transglycosylase activity (EC 2.4.-.-) has also been detected in plant cell walls (Fry, 1988, 1995). In this case, the glycosidic bond is cleaved and the glycosyl residue, instead of being transferred to water as in hydrolysis, is transferred to the alcohol group of another sugar. Many glycosidases also function as transglycosylases when the concentration of acceptor substrate (alcohol) is high. Only those enzymes exhibiting high levels of transglycosylase activity at low acceptor substrate concentrations are classified as true transglycosylases. Depending on the location within the polymer of the glycosidic bond that is cleaved, transglycosylases are classed as exo- or endo- as for glycosidases. Endo-transglycosylation can attach a section of one polysaccharide onto another. The total number of glycosidic bonds is conserved so there is no change in average molecular weight of the polymers. This type of activity is likely to be important in the molecular rearrangements that lead to loosening of the cell wall that occurs during cell expansion and fruit ripening.

Finally, loosening of the cell wall also results from the action of the most recently discovered cell wall modifying proteins, expansins. Although the number of

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enzymes and modifying proteins present in the cell wall is likely to be large, most studies have focused on the relatively few enzymes that accumulate to high levels in particular fruit, such as tomato and avocado. Only the characteristics of those cell wall modifiying enzymes and proteins believed to have important roles in fruit softening are summarized here.

1.2.3.2   *Pectin-degrading and modifying enzymes*

Endopolygalacturonase (PG)

Endopolygalacturonase (EC 3.2.1.15) catalyses the hydrolysis of internal α-$(1\rightarrow4)$ linkages between unesterified D-galacturonic acid residues in the polygalacturonic acid backbone of pectin polymers. This enzyme is the most thoroughly studied of the cell wall hydrolases and has been characterized from a number of different fruits. In many fruits, for example, tomato and avocado (Huber and O'Donoghue, 1993), pear (Yoshioka *et al.*, 1992), papaya (Paull *et al.*, 1999) and melon (Rose *et al.*, 1998) PG activity can be correlated with fruit softening and pectin depolymerization. The levels of PG activity found in these fruits were low or undetectable at the unripe stage and increased dramatically during ripening concomitant with a decrease in the average molecular weight of the polyuronide fraction of the cell wall. In contrast, PG activity is undetectable throughout ripening in the fruit of strawberry (Neal, 1965; Barnes and Patchett, 1976; Huber, 1984; Abeles and Takeda, 1990), pepper (Gross *et al.*, 1986) and apple (Yoshioka *et al.*, 1992) and this correlates with the lack of any depolymerization of the polyuronides of these fruits. However, an apparent absence of PG activity in

persimmon fruit is not consistent with the observed depolymerization of pectic polysaccharides extracted from the fruit (Cutillas-Iturralde *et al.*, 1993).

PG has been most extensively studied in tomato fruit where it is synthesized *de novo* at the onset of ripening as a result of increased PG gene expression. It exists as two main isoforms PG1 and PG2, where PG1 is composed of PG2 plus a non-catalytic β-subunit, and PG cDNA and genomic clones have been characterized (Fischer and Bennett, 1991). PG activity is highly correlated with softening in wild type tomato fruits. In addition, in the *Nr* tomato mutant which has a slow rate of softening, PG activity is only 10% of wild type activity and is absent in the *rin* tomato mutant which does not soften at all (Tucker and Grierson, 1987). However, in transgenic plants where PG levels were reduced to 1% of wild type, fruits apparently softened normally and pectins were solubilized, although they did show reduced depolymerization (Smith *et al.*, 1990a). In other transgenic experiments, the over-expression of PG in the non-softening mutant *rin* failed to induce softening, although pectin solubilization and depolymerization did occur (Giovannoni *et al.*, 1989). These results suggest that PG is involved in the depolymerization but not the solubilization of pectins. Thus, although there is evidence supporting a primary role for PG in pectin degradation and fruit softening (Fischer and Bennett, 1991), results from experiments using transgenic plants suggest that PG alone is not sufficient to cause softening (Seymour and Gross, 1996). Furthermore, although *in vitro* degradation of tomato fruit cell walls by purified tomato PG mimics *in vivo* pectin degradation, it does not proceed to the same extent suggesting that other enzymes may be involved *in vivo* (Tucker and Grierson, 1987). A recent study has looked at the effect of purified avocado PG on polyuronides extracted from avocado fruit at various stages of ripening compared to the *in vivo* changes in these polymers

(Wakabayashi *et al.*, 2000). The results indicated that although PG plays the central role in polyuronide degradation in ripening avocado fruit cell walls, the prior action of the enzyme pectin methylesterase (PME) is required to increase the susceptibility of the polyuronide to degradation by PG in the later stages of softening. Thus, PG activity combined with that of other pectin-degrading enzymes may be required for extensive polyuronide depolymerization. A comparison of the extent of polyuronide depolymerization in avocado and tomato fruit relative to their levels of PG and PME activity has led to the suggestion that the full capacity for polyuronide depolymerization in tomato fruit is restricted *in vivo* (Huber and O'Donoghue, 1993; Wakabayashi *et al.*, 2000). It may be that apoplastic pH and ionic conditions in avocado fruit are inherently more conducive to enzymic hydrolysis or reduced Ca^{2+} bridges than in tomato fruit. The glycoprotein β-subunit of PG1 may also have a role in limiting PG action *in vivo*, as transgenic plants in which the β-subunit was drastically down-regulated showed enhanced pectin solubilization and depolymerization (Watson *et al.*, 1994). Irrespective of the enzymes or conditions required for pectin depolymerization, the depolymerization itself is not a prime determinant of softening in fruit as evidenced by fruits that do not exhibit pectin depolymerization, but do soften, such as strawberry, and by fruit of transgenic *rin* tomato mutants that do not soften despite the over-expression of PG causing pectin depolymerization.

Pectin methylesterase (PME)

Pectin methylesterase (EC 3.1.1.11), also referred to as pectinesterase, catalyzes the demethylation of the C6 carboxyl group of galacturonosyl residues. PME activity has been detected in a wide range of fruits including strawberry, tomato, avocado, banana

and mango and is generally present at a high level in unripe fruit (Tucker and Grierson, 1987). In strawberry, PME activity increases as fruit ripen, but then declines in the ripe and over-ripe stages (Barnes and Patchett, 1976). Two isoforms were detected, PE I and PE II, with PE II being the predominant form in the later stages of ripening. In tomato, PME activity is present throughout fruit development, with activity increasing slightly during ripening (Tucker and Grierson, 1987). Two groups of isoforms have been identified in this fruit and various isoenzymes have been isolated. As in strawberry, one isoform predominates over the other as ripening progresses. Since PG degrades demethylated pectin it has been suggested that PME may act to de-esterify the acidic pectin polymers prior to the action of PG. Thus, PME may play an important role in determining the extent to which pectin is accessible to depolymerization by PG. Transgenic tomato fruit in which PME activity was reduced by up to 93% apparently softened to the same extent as wild type fruit although their pectin had a higher degree of esterification (Hall *et al.*, 1993). In a separate study, pectins from low PME tomato fruit also showed reduced depolymerization and solubility (Seymour and Gross, 1996). Thus, PME has a role in pectin de-esterification *in vivo*, consistent with the reduced degree of pectin esterification found in ripe tomato fruit relative to mature green fruit. The low PME transgenic tomato fruit were also more susceptible to a loss of tissue integrity (Tieman and Handa, 1994), presumably due to the increased methylation level of their pectins resulting in fewer Ca^{2+} cross-links between pectin polymers. This is not consistent with the increasing PME levels detected in wild type fruit as they ripen, although it has been suggested that the decline in PME activity in ripe strawberry fruit may be responsible for the increased pectin solubility (Neal, 1965).

β-Galactosidase (β-Gal)

β-Galactosidase (EC 3.2.1.23) activity is characterized by the ability to release galactose

from *p*-nitrophenyl-β-D-galactopyranoside. All β-Gal activities detected in fruit so far

have been exo-acting and some are believed to be capable of removing galactosyl

residues from cell wall polymers. In many fruits, there is a net loss of wall-bound

neutral sugar residues, particularly galactose, during ripening (Gross and Sams, 1984). It

has been proposed that β-Gal activity is responsible for this solubilization of galactose

observed during ripening (Fischer and Bennett, 1991). Galactosyl residues are lost

mainly from pectic polymers, and more specifically from the neutral galactan-rich

pectins. This has led to the suggestion that β-Gal may be involved in enhancing pectin

solubilization and depolymerization (Seymour and Gross, 1996). Three β-Gal

isoenzymes have been identified in tomato fruit, although only one isoform, β-Gal II,

was found to be capable of hydrolyzing a (1→4)-β-D-galactan isolated from tomato

fruit cell walls (Carey *et al.*, 1995). The activity of β-Gal II increases during ripening,

which is consistent with its potential role as an exo-(1→4)-β-D-galactanase causing

galactose solubilization from a galactan-rich polymer. Analysis of cell wall

polysaccharide fragments revealed that the loss of galactose from the cell wall resulted

from the removal of β-(1→4)-linked galactose, which could have been due to the action

of an exo-(1→4)-β-D-galactanase (Fischer and Bennett, 1991). Thus, β-Gal II may also

play a key part in tomato fruit softening, an idea which is supported by the finding that

its activity does not increase in the non-ripening tomato mutants *nor* and *rin*.

β-Gal isoforms have also been isolated from the fruit of papaya (Ali *et al.*,

1998), Japanese pear (Kitagawa *et al.*, 1995), kiwifruit (Ross *et al.*, 1993) and apple

(Ross *et al.*, 1994). The enzyme from kiwifruit was found to release galactose from a

variety of kiwifruit pectic and hemicellulosic wall polymers. The apple enzyme was shown to be active against polysaccharides extracted from apple fruit cell walls, indicating that it could degrade native β-(1→4)-linked galactans and hence may have an effect on pectin solubility by reducing the size of the pectin side chains. The apple and tomato β-Gal enzymes are encoded by multigene families and it may be that these genes encode isoenzymes with differing substrate specificities (Seymour and Gross, 1996).

1.2.3.3 Hemicellulose-degrading and modifying enzymes and proteins

Endo-β-1,4-glucanase (EGase)

Endo-β-1,4-glucanase (EC 3.2.1.4), also known as cellulase, catalyzes the hydrolysis of internal β-(1→4) linkages between adjacent glucose residues which occur in cellulose, xyloglucans and mixed-linkage glucans. EGase activity is generally characterized by the ability to degrade the synthetic substrate carboxymethylcellulose (CMC). In dicots, xyloglucans are considered to be the most likely endogenous substrate of EGases (Cosgrove, 1999) given the apparent inability of EGases to degrade insoluble or crystalline forms of cellulose (Fischer and Bennett, 1991) and that cellulose is not significantly modified during ripening (Seymour and Gross, 1996). EGase activity increases dramatically during the ripening of many fruits and is often correlated with hemicellulose degradation and softening. The properties and functions of plant EGases are discussed further in section 1.3.

Xyloglucan endotransglycosylase (XET)

Xyloglucan endotransglycosylase catalyzes the cleavage of the backbone of a xyloglucan molecule and the subsequent transfer of the generated reducing end onto the non-reducing end of another xyloglucan molecule. The new bond formed is identical to the β-$(1\rightarrow4)$-glucosyl bond broken in the donor molecule, thus the number of bonds is conserved (Fry, 1995). XET activity could contribute to xyloglucan rearrangement and/or incorporation of newly synthesized xyloglucan polymers into the cell wall during ripening. In addition, if XET acted to join a cleaved xyloglucan molecule to a smaller fragment, a reduction in xyloglucan molecular weight would result, and this could be a mechanism for xyloglucan depolymerization.

XET activity has been detected in many dicots. Activity is generally high in growing stems and some fruits (Fry, 1995), including persimmon (Cutillas-Iturralde *et al.*, 1994) and apple (Percy *et al.*, 1996), suggesting a role for XET in the cell wall loosening associated with cell expansion and fruit softening. XET activity was found to increase during the ripening of tomato fruit concomitant with a decline in the molecular weight of the xyloglucan fraction of the cell wall (Maclachlan and Brady, 1994). In the ripening mutant *rin*, there was neither a decrease in xyloglucan molecular weight nor an increase in XET activity as in the wild type. It was suggested that XET and EGase are the enzymes most likely to be responsible for the observed depolymerization of xyloglucan in ripening tomato fruit. Two tomato fruit XET cDNAs have been cloned and are believed to be members of a small multigene family (Arrowsmith and de Silva, 1995). One of these clones was expressed in *E. coli* and the recombinant protein demonstrated XET activity with no detectable hydrolytic activity. XET activity has also been shown to increase during the ripening of kiwifruit when there is a decrease in the

molecular weight of xyloglucan (Redgwell and Fry, 1993). Kiwifruit XET was found to be active against xyloglucan and insoluble cell wall material purified from kiwifruit cell walls, suggesting a potential role in modifying the fruit cell wall during ripening. The authors suggested that oligosaccharides produced by the enzymic hydrolysis of xyloglucan polymers could act as acceptor molecules in the XET transglycosylation reaction, resulting in a decrease in the molecular weight of the xyloglucan. An XET has been purified from ripe kiwifruit and shown to have both hydrolytic and endotransglycosylase activity (Schroder et al., 1998). Again, it was suggested that, depending on the nature of the available substrates, the XET could depolymerize xyloglucan by acting as a hydrolase in the presence of undegraded xyloglucan and as an endotransglycosylase in the presence of xyloglucan-derived oligosaccharides. Thus XET transglycosylase activity could play a role in xyloglucan degradation during fruit ripening, acting in conjunction with other xyloglucan degrading activities. The isolation of six XET cDNA clones from ripe kiwifruit indicates, in common with tomato, the presence of a multigene family (Schroder et al., 1998), and different members may show specific expression patterns and preferred substrates.

Expansins

Expansins are a recently discovered class of wall-modifying proteins isolated as the mediators of acid-growth of cell walls. Their proposed action as cell wall-loosening agents has led to considerable interest in them and many recent reviews have been published (McQueen-Mason, 1995; Cosgrove, 1998, 1999, 2000). Acid growth refers to the increase in growth rate of plant cells when placed in acidic solutions as a result of increased wall extensibility. Plant cell walls that were treated to denature wall proteins

showed reduced extensibility and were insensitive to pH. Addition of the plant cell wall proteins now termed expansins restored acid-inducible extension of the walls and expansins are now considered as primary cell wall loosening agents (Cosgrove, 1999). Most work has been carried out on expansins isolated from cucumber hypocotyls which have been shown to bind tightly to the cell wall, apparently to the non-crystalline regions of cellulose microfibrils or at the interface between cellulose and tightly bound hemicellulose (Cosgrove, 1998). The cloning of different expansins has identified two families, α-expansins and β-expansins, of which the α-expansin family has been studied in greatest detail (Cosgrove, 2000).

Expansins do not exhibit hydrolytic activity against cell wall components and indeed α-expansin does not lead to a progressive, time-dependent weakening of the cell wall as would be expected of a hydrolase (Cosgrove, 1999). Instead, expansins have been shown to weaken pure cellulose paper without detectable hydrolysis, suggesting that they may disrupt the non-covalent hydrogen bonds between glucan molecules (Cosgrove,1998). Thus the proposed model of expansin action hypothesizes that the expansin protein is anchored to the surface of the microfibril by a putative binding domain. The putative catalytic domain would then be able to interact with hemicellulose at the microfibril surface or in the matrix between microfibrils to disrupt the hydrogen bonds between the two polymers (Cosgrove, 2000). In this way, expansins may render the hemicellulose polymers more susceptible to attack by wall enzymes, thus weakening the hemicellulose interactions. In a cell wall under tension, such as found in a growing cell, this loosening would allow the hemicellulose polymers to creep, presumably dragging other attached polymers with them and allowing cell expansion. This model has been developed to explain how expansins might induce cell wall loosening leading

to cell expansion. However, there is evidence that expansins have roles in other processes such as fruit ripening, where their ability to loosen the cell wall and render it more susceptible to attack by wall enzymes could be important in contributing to the cell wall disassembly that occurs during fruit softening.

A fruit-specific, ripening-regulated expansin (*LeExp1*) has been identified in tomato (Rose *et al.*, 1997). *LeExp1* was specifically expressed at high levels in ripening tomato fruit. In contrast, transcript abundance in the fruit of the non-softening tomato mutants *nor* and *rin* was severely reduced compared to wild type levels. cDNAs closely related to *LeExp1* were also identified in ripening melon and strawberry fruit, suggesting that expansins are a common feature of fruit undergoing rapid softening and may contribute to the process of cell wall disassembly. The production of transgenic tomato plants in which LeExp1 accumulation was both suppressed and overexpressed confirmed a role for expansins in fruit softening (Brummell *et al.*, 1999b). Fruits with reduced *LeExp1* expression were firmer than controls while fruit overexpressing *LeExp1* were softer and had altered depolymerization of their polyuronide and hemicellulose polymers. These data indicate that the cell wall-loosening action of expansin is required for fruit softening in addition to the action of cell wall-modifying enzymes, and that some of these enzymes require the prior action of expansins to be fully effective. This is consistent with the suggestion that the synergistic action of a suite of cell wall-modifying enzymes and proteins, such as expansins, EGases and XETs, is required for coordinated cell wall modification in both growing vegetative tissues and expanding and ripening fruit (Rose and Bennett, 1999).

Seven unique expansin genes in total have now been identified in tomato (Rose *et al.*, 1997; Brummell *et al.*, 1999c; Catala *et al.*, 2000). The specific expression

patterns of each of these indicate that expansins are involved in cell wall-loosening during growth and softening of fruit in addition to cell expansion. A family of expansin genes is also present in strawberry (Harrison, McQueen-Mason and Manning, personal communication), each one having its own specific pattern of expression. A cDNA encoding a ripening-related expansin, *FaExp2*, isolated from strawberry fruit was most closely related to an expansin expressed in early tomato fruit development (*LeExp5*) rather than the ripening-specific tomato expansin *LeExp1* and also to expansins expressed in apricot fruit (Civello *et al.*, 1999).

1.3 PLANT ENDO-β-1,4-GLUCANASES

Plants have long been known to possess enzyme activities capable of the *in vitro* hydrolysis of soluble cellulose derivatives such as carboxymethylcellulose (CMC). The enzymes responsible for this activity were called cellulases but are now known to differ considerably from their microbial counterparts. Microbial cellulases are of the C_1 type which can degrade native crystalline cellulose. Plant cellulases are of the C_x type capable of hydrolyzing only soluble substituted cellulose derivatives (Brummell *et al.*, 1994). The microbial true cellulases are usually multi-enzyme complexes composed of endo-β-1,4-glucanases, cellobiohydrolases and cellobiases which act in a synergistic manner to degrade insoluble cellulose (Bayer *et al.*, 1998). Since plant cellulases have not been shown to possess this type of activity, they are now more correctly termed endo-β-1,4-glucanases, a name which refers to the bond hydrolyzed rather than to the substrate. To date, EGases have been characterized from many species and tissues at both the level of gene expression and enzyme activity. A summary of the properties and functions of plant EGases is given here. More detailed information on specific EGases and their relationships to each other can be found in the individual chapter discussions.

1.3.1 Properties of plant endo-β-1,4-glucanases

Endo-β-1,4-glucanase activity has been reported in numerous plant species and tissues and is usually associated with developmental processes where the cell wall is undergoing modification or degradation. Such processes occur in growing and senescing tissues and include cell growth and expansion, organ abscission and fruit ripening.

Several EGases have been purified and this has allowed characterization of their biochemical properties (Table 1.3). The enzymes from avocado and pepper fruit, bean leaf, sweet pea anthers and tobacco callus were all effectively purified by affinity chromatography on cellulose. Most of the purified EGases exhibited pH optima between pH 5 and 7 and this is likely to reflect the pH of the cell wall space where their substrates are located (Brummell et al., 1994). The majority of the enzymes had molecular masses in the range 46-70 kDa, with the exception of the much smaller EGases from pea and periwinkle.

Characterization of the substrate specificities of the purifed EGases confirms that plant EGases are unable to significantly degrade the crystalline cellulose found in the cell wall. The exceptions appear to be the EGases isolated from pea epicotyls (Wong et al., 1977) and periwinkle (Smriti and Sanwal, 1999) which have been reported to hydrolyze insoluble and swollen forms of cellulose. However, the rates of hydrolysis were much lower than those towards CMC and neither report has been confirmed. In addition, ultrastructural studies of the cell walls of ripening avocado have shown disruption of cellulose microfibrils (Platt-Aloia et al., 1980). In the absence of any activity of avocado EGase towards avocado xyloglucan in vitro (O'Donoghue and Huber, 1992), it was suggested that avocado fruit EGase may cause limited hydrolysis of microfibrils at accessible sites in the non-crystalline regions, resulting in disruption of microfibril structure and organization (O'Donoghue et al., 1994).

Table 1.3   Biochemical properties of purified plant EGases

(- denotes that values were not determined; AZ, abscission zone)

Plant	Tissue	M_r (kDa)	pH optimum	pI	Reference
Peach	Leaf AZ	54	-	9.5	Bonghi et al. (1998)
Elder	Leaf AZ	54	7.0	-	Webb et al. (1993)
Bean	Leaf AZ	51	6.0-8.5	9.5	Durbin and Lewis (1988)
Bean	Cotyledon	70	4.8-5.6	4.8	Lew and Lewis (1974)
		70	5.7-6.2	4.5	
Coleus	Leaf AZ	56	5.0, 7.2	4.7	Wang et al. (1994b)
		62		4.7	
Avocado	Fruit	49	-	4.7	Awad and Lewis (1980)
Pepper	Fruit	54	-	8.5	Ferrarese et al. (1995)
Apple	Fruit	67	-	-	Abeles and Biles (1991)
Sweet pea	Anther	49	6.5-6.8	8.0	Sexton et al. (1990)
		51		7.8	
Poplar	Cell	50	6.0	5.5	Nakamura and Hayashi (1993)
	culture	47	6.5	5.6	Ohmiya et al. (1995)
Tobacco	Callus	50, 52	5.5-6.5	8.2	Truelsen and Wyndaele (1991)
Periwinkle	Stem	25	5.2	-	Smriti and Sanwal (1999)
Pea	Stem	46	-	-	Hayashi and Ohsumi (1994)
Pea	Epicotyl	15	5.5-6.0	5.2	Byrne et al. (1975)
		70	5.5-6.0	6.9	

However, xyloglucan is thought to be the most likely *in vivo* substrate of EGases (Hayashi *et al.*, 1989). Activity against xyloglucan has been demonstrated for the EGases from tobacco callus (Truelsen and Wyndaele, 1991), pea epicotyls (Hayashi *et al.*, 1994) and poplar (Nakamura and Hayashi, 1993; Ohmiya *et al.*, 1995). Avocado EGase, although apparently inactive against avocado xyloglucan, showed limited activity towards soybean xyloglucan (Hatfield and Nevins, 1986). EGases appear to show differing activities towards xyloglucans isolated from different sources. This suggests that they each have their own specific substrate requirements, as xyloglucan polymers exhibit differing structures and substitution patterns (Hayashi, 1989). It is also possible that the extraction of xyloglucan polymers from the cell wall may alter their structure so that the level of activity of EGases determined against isolated substrates may not reflect the true situation *in vivo*.

Multiple EGase isoforms are common in plants, for example the pI 9.5 and 4.5 isoforms present in bean leaf abscission zones (Durbin and Lewis, 1988), the pI 9.5 and 6.5 isoforms in peach fruit and leaf and fruit abscission zones (Bonghi *et al.*, 1998) and the buffer-soluble and insoluble forms identified in pea (Byrne *et al.*, 1975). Multiple forms of EGase with differing pIs have been detected in ripening avocado fruit (De Francesco *et al.*, 1989; Kanellis and Kalaitzis, 1992) and these result from transcript heterogeneity. This was explained by the existence of a small multigene family of EGases in avocado for which there is some evidence (Tucker *et al.*, 1987). However, Cass *et al.* (1990) demonstrated that despite the identification of two genes encoding EGase, a single gene was responsible for all the EGase transcripts in ripe fruit, which led to an alternative explanation of posttranscriptional processing to account for the multiple forms of EGase

(De Francesco *et al.*, 1989). EGases in most species appear to be encoded by multigene families and many members of these families have been cloned (Table 1.4). Analysis of the expression patterns of each of the family members generally reveals that each exhibits a specific pattern of temporal and spatial expression. This differential expression of a set of genes within a tissue suggests each has a distinct physiological function which may be coordinated with that of other family members (Fischer and Bennett, 1991). In addition, EGase genes may be differentially regulated by the plant hormones ethylene and auxin thereby allowing specific regulation of their expression in different developmental processes. The presence of multiple, differentially expressed and regulated EGase genes not only reflects the wide range of physiological processes involving cell wall modification in which EGases participate but also reflects the underlying complexity of the cell wall structure requiring these different activities.

Table 1.4   EGase genes characterized from higher plants

(AZ, abscission zones; FR, fruit ripening; CS, cell separation events; CE, cell growth and expansion)

Plant	Gene	Major areas of expression	Putative functions	Reference
Tomato	*cel1*	Fruit, AZ	FR, CS	Lashbrook *et al.* (1994)
	cel2	Fruit, AZ	FR, CS	Lashbrook *et al.* (1994)
	cel3	Expanding tissues	CE	Brummell *et al.* (1997a)
	cel4	Expanding tissues	CE	Brummell *et al.* (1997b)
	cel5	Fruit, AZ	FR, CS	del Campillo and Bennett (1996); Kalaitzis *et al.* (1999)
	cel6	AZ	CS	del Campillo & Bennett (1996)
	cel7	Expanding tissues	CE	Catala *et al.* (1997)
Pepper	*PCEL1*	Fruit	FR	Harpster *et al.* (1997)
	cCel2	AZ	CS	Trainotti *et al.* (1998a)
	cCel3	AZ	CS	Trainotti *et al.* (1998b)
Arabidopsis	*cel1*	Expanding tissues	CE	Shani *et al.* (1997)
Peach	*ppEG1*	AZ	CS	Trainotti *et al.* (1997)
Elder	*JET1*	AZ	CS	Taylor *et al.* (1994)
Avocado	*cel1*	Fruit, fruit AZ	FR, CS	Cass *et al.* (1990); Tonutti *et al.* (1995)
	cel2	Not known		
Bean	*BAC10*	AZ	CS	Tucker & Milligan (1991)
Soybean	*pSAC1*	AZ	CS	Kemmerer & Tucker (1994)
Pea	*pEGL1*	Expanding tissues	CE	Wu *et al.* (1996)
Orange	*CEL-a1*	AZ	CS	Burns *et al.* (1998)
	CEL-b1			
Sweet pea	*pLAC*	Anthers	CS	Neelam & Sexton (1995)
Pine	*PrCel1*	Expanding tissues	CE	Loopstra *et al.* (1998)
	PrCel2			

1.3.2 Role of endo-β-1,4-glucanases in plant development

Endo-β-1,4-glucanases participate in developmental processes where modification or degradation of the cell wall is required or complete cell separation occurs. The three main processes with which EGases are associated are cell growth and expansion in growing tissues, fruit ripening and abscission. EGases are also involved in the development of flower reproductive organs (del Campillo *et al.*, 1992; Milligan and Gasser, 1995; Neelam and Sexton, 1996), adventitious root initiation (Kemmerer and Tucker, 1994) and differentiating tissues (Brummell *et al.*, 1994). In many cases a particular EGase may participate in more than one developmental process within the plant indicating the need for complex regulatory control and differential expression. Examples of EGases involved in the three main physiological processes are described here, in particular with reference to their hormonal control.

1.3.2.1 Cell expansion

In order for irreversible cell expansion to take place, plants must partially and selectively weaken or loosen the structural integrity of their primary cell walls to allow incorporation of newly synthesized polymers into the wall and permit growth. Thus EGases could potentially be involved in modification of the xyloglucan fraction of the wall allowing loosening of the cellulose-xyloglucan framework. In growing tissues, induction of EGase transcript and activity levels by auxin have been observed. The two EGases purified from pea were isolated from auxin-treated epicotyls. Within five days of auxin treatment a 100-fold increase in their activities was observed (Byrne *et al.*,

1975). A 10-fold increase in transcript levels of the buffer-soluble isoform was induced in auxin-treated epicotyls within 48 h of treatment and there was no evidence for any pre-existing untranslated mRNA for this form in untreated control epicotyls (Verma *et al.*, 1975). In another study, auxin treatment of pea epicotyls *in vivo* resulted in the induction of EGase activity, a reduction in the average degree of polymerization of the xyloglucan fraction and an increase in soluble xyloglucan (Hayashi *et al.*, 1984). The pea EGases were found to be capable of hydrolyzing pea xyloglucan *in vitro* suggesting that xyloglucan is the substrate for these EGases associated with cell expansion in growing tissue. Treatment of azuki bean epicotyls with auxin caused a similar decrease in the average molecular mass of xyloglucans and this was attributed in part to the action of an EGase (Hoson *et al.*, 1995). Auxin has also been shown to induce a 10-fold increase in the accumulation of transcripts of the pea EGase gene *EGL1* in rapidly elongating epicotyls (Wu *et al.*, 1996) indicating that auxin induces the *de novo* synthesis of EGase.

Auxin was shown to induce the expression of two members of the tomato EGase gene family, *cel4* (Brummell *et al.*, 1997b) and *cel7* (Catala *et al.*, 1997) in the expanding hypocotyls of intact seedlings. Expression of *cel4*, which is found predominantly in the apical region of the hypocotyl, was also induced by ethylene, whereas *cel7*, which is expressed equally in the apical and basal regions, showed a decrease in transcript accumulation in response to ethylene. However, auxin has been shown to induce ethylene biosynthesis in tomato hypocotyls and it may be that *cel4* is not regulated by auxin itself, only by the ethylene synthesized as a result of the applied auxin (Brummell *et al.*, 1997b). A third gene, *cel3*, also expressed in expanding hypocotyls, was not induced by auxin or ethylene in intact seedlings (Brummell *et al.*,

1997a), demonstrating the differential regulation of divergent EGase genes in the same tissue.

Thus these EGase genes are potential candidates for mediating the xyloglucan modification that occurs during auxin-induced cell expansion. Analysis of the time-course for tomato *cel7* induction after auxin treatment compared to the induction of hypocotyl elongation indicated that this EGase may be involved in sustained cell expansion rather than the rapid growth responses to auxin (Catala *et al.*, 1997). This may also be true for other plants in which rapid changes in growth and xyloglucan are followed by a later increase in EGase activity (Brummell *et al.*, 1994; Maclachlan and Carrington, 1991).

1.3.2.2 *Fruit Ripening*

Increasing EGase activity in ripening fruit is often correlated with fruit softening and hemicellulose degradation indicating that EGases are likely to be involved in the cell wall modification leading to texture changes and fruit softening. Once again, multiple EGase genes are often differentially expressed during ripening and may be subject to distinct regulatory control (Gonzalez-Bosch *et al.*, 1996) allowing the coordinated disassembly of the cell wall during ripening.

The EGase that increases in activity most dramatically during ripening is that from avocado fruit. The increase was found to be directly correlated with the climacteric rise in respiration, ethylene synthesis and fruit softening (Pesis *et al.*, 1978; Awad and Young, 1979). The polypeptide for EGase has also been shown to appear during the climacteric rise in respiration (Christoffersen *et al.*, 1984). Preclimacteric avocado fruit

lack both EGase activity and detectable antigen indicating *de novo* synthesis of EGase protein, which occurs as a result of a 50-fold increase in EGase transcript levels (Christoffersen *et al.*, 1984). Avocado EGase is synthesized as a high molecular weight precursor which undergoes glycosylation and carbohydrate trimming on its way to the cell wall (Bennett and Christoffersen, 1986).

Ethylene has been shown to induce EGase activity in avocado fruit (Pesis *et al.*, 1978) at the same time as accelerating the onset of ripening. Ethylene treatment also induced EGase gene expression, with an increase in transcript being detected after 8 h (De Francesco *et al.*, 1989). Similarly, in ripening tomato fruit, the expression of the EGase genes *cel1* and *cel2* follows the initiation of ethylene synthesis. The accumulation of transcripts from both *cel1* and *cel2* was severely inhibited in fruit treated with the ethylene action inhibitor 2,4-norbornadiene (NBD) indicating that the expression of *cel1* and *cel2* is regulated by ethylene (Lashbrook *et al.*, 1994). A similar inhibition of fruit softening, EGase transcript accumulation and EGase activity by NBD was observed in peach fruit. Two isoforms exist in peach, the pI 9.5 EGase being most abundant during ripening and the pI 6.5 EGase being the only form present during the early stages of growth (Bonghi *et al.*, 1998). Propylene treatment reduced the pI 6.5 EGase activity during early fruit development but increased pI 9.5 EGase activity and transcript accumulation and accelerated the loss of firmness during ripening. Thus ethylene is again found to induce ripening-related EGase gene expression.

Although the ripening of pepper fruit is accompanied by a respiratory climacteric, the rise in CO_2 production is minor and only very low levels of endogenous ethylene can be detected in ripening fruit. Despite this, a role for ethylene in ripening has been suggested (Harpster *et al.*, 1997). Immature green fruit and nearly ripe fruit

appear to be unresponsive to ethylene but the ripening of mature green fruit is accelerated in the presence of ethylene. However, prolonged ethylene treatment was found to induce accumulation of *PCEL1* transcripts and the corresponding PCEL1 protein in addition to EGase activity in immature green fruit. Although the induced levels were not as high as those found in untreated mature red fruit, the results indicated that ethylene may act as a regulator of pepper fruit ripening (Harpster *et al.*, 1997). Ferrarese *et al.* (1995) also demonstrated a strong promotitive effect of exogenous ethylene on the ripening and induction of EGase activity in mature green fruit and showed that the increase was the result of *de novo* synthesis of the protein.

In contrast, the increase in EGase activity during non-climacteric strawberry fruit ripening is not induced by exogenous ethylene (Abeles and Takeda, 1990). This is consistent with earlier findings that ethylene does not appear to regulate strawberry fruit ripening, which may also be the case for other non-climacteric fruit.

1.3.2.3   Abscission

During the shedding of plant organs, abscission zones develop in which localized cell wall breakdown leads to cell separation resulting in abscission of the organ. An increase in EGase activity in abscising tissue has been reported in many plants. The accumulation of EGase activity and the abscission process itself are stimulated by exogenous ethylene and retarded by auxin and inhibitors of ethylene synthesis or action (Brummell *et al.*, 1994). EGases associated with the abscission of leaves, flowers and fruit have been characterized from different plants, the EGase involved in bean leaf abscission being the most studied.

In bean leaf abscission zones several forms of EGase have been detected. Two acidic forms with isoelectric points of 4.5 and 4.8 (Lew and Lewis, 1974) and one basic form with a pI of 9.5 (Koehler et al., 1981) have been purified. Using antibodies specific for the pI 9.5 isoform it has been shown that this isoform is undetectable in the abscission zone prior to induction of abscission (Durbin et al., 1981). Following induction with ethylene, there was an increase in the activity of this isoform prior to a decrease in integrity of the abscission zone cell walls. Thus de novo synthesis of the pI 9.5 isoform is induced by ethylene. Levels of the pI 4.5 isoform decreased in response to ethylene indicating that it is not involved in the abscission process. The presence of this form in young tissues throughout the plant suggests it may be involved in cell growth and expansion. The pI 4.5 isoform is also responsible for the low levels of EGase activity detected in uninduced abscission zones (Durbin et al., 1981). Specific localization of the pI 9.5 EGase in activated leaf abscission zones has also been demonstrated in bean (del Campillo et al., 1990). Nitrocellulose tissue prints immunoblotted with the pI 9.5 EGase antibody showed that ethylene induced the pI 9.5 isoform in the separation layers of the two abscission zones, which comprise a narrow band of cells at the site of the fracture. Expression of the gene encoding the pI 9.5 EGase was also regulated by ethylene. After 24 h exposure to ethylene there was an accumulation of the pI 9.5 EGase mRNA which corresponded with the increases in EGase activity and immunodetectable pI 9.5 protein (Tucker et al., 1988). Tissue exposed to ethylene to initiate abscission was subsequently treated with NBD to inhibit ethylene action due to endogenous ethylene. The level of mRNA and EGase activity declined demonstrating that the continued presence of ethylene is necessary to maintain EGase expression (Tucker et al., 1988). Auxin was shown to be a negative regulator of

abscission as tissue exposed to auxin prior to exposure to ethylene failed to accumulate pI 9.5 EGase transcripts and abscission was inhibited (Tucker *et al.*, 1988). Localization of pI 9.5 EGase transcripts in bean leaf abscission zones showed accumulation in the separation layers, a distribution that is consistent with immunolocalization of the corresponding protein in the same tissue (Tucker *et al.*, 1991). An EGase immunologically similar to the bean pI 9.5 isoform has also been detected in soybean leaf, flower and pod abscission zones after induction of abscission with ethylene (Kemmerer and Tucker, 1994). However, the form of EGase that is induced in abscission zones of coleus is different to the bean abscission EGase (Wang *et al.*, 1994a). The coleus enzyme has an acidic pI of 4.7, more similar to the bean pI 4.5 isoform that is not associated with abscission. The hormonal regulation of the two is different though, with the coleus enzyme activity being inhibited by auxin and promoted by ethylene, consistent with its involvement in the abscission process.

In tomato, multiple EGase transcripts are found in abscising tissues. The expression of *cel5* increased in flower and leaf abscission zones in response to ethylene treatment and was inhibited by pretreatment with auxin and silver thiosulphate, an inhibitor of ethylene action (Kalaitzis *et al.*, 1999). Ethylene-induced abscission of tomato flowers was correlated with increased expression of tomato *cel1* and *cel2* EGase genes, which were shown to be regulated by ethylene (Lashbrook *et al.*, 1994). Breakstrength and EGase gene expression were studied during tomato flower abscission. The expression of *cel1*, *cel5* and *cel6* was found to correlate with flower shedding (del Campillo and Bennett, 1996). While *cel1* and *cel5* expression appeared to be affected by auxin and ethylene in a manner consistent with their involvement in the abscission process, *cel6* expression increased after auxin treatment. This response, together with

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the observation that *cel6* transcript accumulation declined in the final stage of abscission, suggests that abscission of tomato flowers requires the differential expression of divergent EGase genes brought about by distinct regulatory control. Antibodies raised against a fusion protein encoding a region of the Cel1 polypeptide cross-reacted with proteins in flower abscission zones induced to abscise by ethylene, further confirming Cel1 involvement in abscission (Gonzalez-Bosch *et al.*, 1997).

Multiple EGases involved in abscission have also been characterized from abscission zones of pepper leaves (Ferrarese *et al.*, 1995) and flowers (Trainotti *et al.*, 1998a), peach leáves and fruit (Trainotti *et al.*, 1997) and orange fruit, leaves and flowers (Burns *et al.*, 1998) and found to be regulated by ethylene.

RESEARCH OBJECTIVES

The ripening of many fruits is characterized by textural changes which lead to softening. This results in an edible product, but also has significant implications for post-harvest fruit quality, since excessive softening results in increased susceptibility to mechanical damage and disease during handling and storage and drastically reduces shelf-life. Changes in texture and firmness are considered to be a consequence of alterations in the composition and structure of cell wall polysaccharides brought about by a combination of cell wall hydrolases and wall-modifying proteins. EGase has been one of the most studied of the hydrolytic enzymes in relation to fruit softening because its activity increases to relatively high levels and is temporally correlated with loss of firmness in many fruits, including strawberry, suggesting an important role in fruit softening.

The overall aim of the present work was to determine more precisely what role EGase might have in strawberry fruit development and this was approached in two ways. The modification of gene expression in transgenic plants has been used extensively in the study of fruit ripening in tomato, particularly with respect to understanding the changes in cell wall metabolism. The aim here was to use transgenic plants to study the role of EGase in strawberry fruit ripening and in particular to evaluate its effect on fruit texture. The first requirement therefore was to isolate a ripening-related EGase cDNA clone. This would enable the expression of the corresponding EGase gene to be characterized. In addition, it would allow the genetic manipulation of EGase levels by antisense and sense suppression in transgenic strawberry plants. Transgenic fruits exhibiting reduced expression of the endogenous EGase gene would be analyzed for changes in their ripening behaviour in order to assess

the physiological function of the corresponding enzyme. In addition to the molecular studies, the aim was to biochemically characterize the corresponding ripening-related EGase enzyme to elucidate the *in vivo* role of EGase in strawberry fruit softening. This required purification of the enzyme from ripe fruit and determination of its properties. Determination of the preferred substrate of the purified EGase *in vitro* would provide information on the cell wall component(s) most likely to be the *in vivo* target of the enzyme. This would give an indication of its role in the modification of cell wall structure that results in fruit softening.

CHAPTER 2.   MATERIALS AND METHODS

2.1   CHEMICALS AND ENZYMES

All chemicals were of analytical, tissue culture or molecular biology grade and were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK) or Merck Ltd. (Leics, UK) unless otherwise stated. Bacterial media were supplied by Oxoid (Unipath Ltd., Hampshire, UK) and radiochemicals by Amersham Pharmacia Biotech (Herts, UK). Enzymes were purchased from Boehringer Mannheim (East Sussex, UK) or Promega UK (Southampton, UK) unless otherwise stated.

All media, buffers and solutions were made up in reverse-osmosed, deionized, double distilled water as described in Appendix A. Where required, they were sterilized by autoclaving at 101 kPa for 20 min.

2.2   PLANT MATERIAL

The strawberry (*Fragaria x ananassa* Duch.) cultivars used in this work were the day neutral cultivar (cv) Brighton, cv Elsanta and cv Calypso. The cDNA library screened in this work was constructed from fruit of cv Brighton. Plants were obtained from micropropagated material and grown in a compost mixture consisting of Richmoor Mix 1, Osmocote Plus and Suscon Green (900 litres : 4 kg : 550 g). They were maintained in a glasshouse with night/day temperatures of 12°C/18°C. The large amount of ripe fruit required for the protein purification was obtained from cv Elsanta, grown commercially.

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The cultivar Calypso was used for transformation. This cultivar was chosen because of its relatively high transformation efficiency compared with other cultivars (D. J. James, personal communication). The plants used were micropropagated aseptically from meristems. Initially meristems were placed on S5 proliferation medium to produce shoots. Shoots were transferred to R13 rooting medium for 4 d and then onto R37 rooting medium in honey jars to produce small plants. A supply of plants was maintained by subculturing the innermost petiole with the growing tip onto fresh R37 medium every 6 weeks to 3 months when the plants had filled the jars. The plants were grown under controlled environmental conditions at 20-22°C, 16 h day and at a light intensity of 70 μmol m^{-2} s^{-1} (Phillips 70 W Type 84 fluorescent tubes sited 25 cm above the shelf).

2.3   MOLECULAR BIOLOGY

2.3.1   Preparation of plasmid DNA

Individual cDNAs cloned into the vector pBK-CMV (Stratagene Ltd.) were stored at -70°C as bacterial glycerol stocks prepared from single, isolated colonies using the host bacterium *E. coli* XL1-Blue MRF' (Stratagene Ltd.). Plasmids were prepared using an alkaline lysis method. A scrape of the glycerol stock was resuspended in sterile Terrific Broth (TB) medium containing 50 μg ml^{-1} kanamycin and incubated at 37°C overnight with shaking (250 rpm). The cells from 1.5 ml of culture were pelleted by centrifugation at 12 000xg for 5 min in a microcentrifuge and the supernatant discarded. The pellet was resuspended in 100 μl resuspension buffer. After incubation on ice for 10 min, 200 μl

lysis buffer were added and the tube inverted gently several times to mix. After a further

10 min on ice, 150 μl 5 M potassium acetate, pH 4.8 was added and the tube mixed.

Precipitated proteins, chromosomal DNA and cellular debris were pelleted by

centrifugation at 12 000xg for 15 min and the supernatant containing the plasmid DNA

was collected. An equal volume of isopropanol was added followed by centrifugation at

12 000xg for 10 min. The pellet was washed consecutively with 1 ml each of 70% and

100% ice-cold ethanol and dried under vacuum. To remove contaminating RNA the

pellet was dissolved in 0.5 ml TE, pH 8.0, and incubated with 1 Unit of RNase ONE™

(Promega UK) at 37°C for 1 h. To pellet the plasmid DNA, leaving the digested RNA in

solution, an equal volume of 13% (w/v) PEG in 1.6 M NaCl was added, mixed by

vortexing, incubated at room temperature (RT) for 5 min and centrifuged at 12 000 g for

10 min. The final plasmid DNA pellet was washed with ethanol as before, dried under

vacuum and redissolved in 25 μl TE, pH 8.0. For larger scale preparations of plasmid

DNA volumes were adjusted accordingly.

2.3.2   Determination of RNA and DNA concentration

The concentration of RNA and DNA in solution was determined by measuring the

absorbance at 230, 260, and 280 nm in a 10 mm cuvette. An absorbance value at 260 nm

of 1.0 corresponds to approximately 40 μg ml^{-1} for RNA and single-stranded DNA and

50 μg ml^{-1} for double-stranded DNA. The absorbance ratios at 260/280 nm and 260/230

nm provide an indication of the purity of the nucleic acid sample. A ratio at 260/280 nm

≥ 1.8 indicates no significant protein contamination and that at 260/230 nm ≥ 2.0 shows

no significant polyphenol contamination.

2.3.3   Digestion of DNA with restriction endonucleases

DNA was digested with the appropriate restriction enzyme in 1 x reaction buffer for that enzyme. Up to 10 μg of DNA was digested in a total reaction volume of 50 μl. For the enzymes *Eco*R I and *Hin*d III, 4 mM spermidine was included for optimal digestion (Bouche, 1981). Reactions containing 5 Units enzyme μg^{-1} DNA were incubated at the optimum temperature for the enzyme (usually 37°C) for 1 h then stopped by the addition of 0.5 M EDTA, pH 8.0 to give a final concentration of 10 mM. The digested DNA was precipitated free of salts by adding 0.5 volume 6 M ammonium acetate, 1 μl glycogen and 2 volumes ice-cold 100% ethanol. Incubation on ice for 20 min was followed by centrifugation at 12 000xg for 10 min. The pellet was washed with ice-cold 100% ethanol and dried.

2.3.4   Agarose gel electrophoresis

Agarose gels for the elecrophoresis of DNA prepared with 0.8% or 1% (w/v) agarose in 1 x TAE buffer containing 0.5 μg ml^{-1} ethidium bromide were cast in a horizontal gel tank. Samples were made up in 1 x DNA sample loading buffer, loaded into the wells and the gel was run at 5 V cm^{-1} in 1 x TAE buffer. The DNA was visualized under UV light and the sizes of the bands were determined by comparison with markers obtained by restricting λ DNA with *Sty* I.

2.3.5 Purification of DNA from agarose gels

Gel slices containing fragments of DNA were excised with a clean scalpel blade and the DNA purified using the QIAEX II gel extraction kit (QIAGEN Ltd., West Sussex, UK). Three volumes of Buffer QX1 were added to 1 volume of gel (300 µl QX1 to 100 mg gel). QIAEX II particles were resuspended and 30 µl added to the gel and buffer. The gel was dissolved by incubating at 50°C for 10 min with mixing every 2 min to keep the particles in suspension. The DNA adsorbs to the QIAEX II particles in the high salt conditions. The particles and DNA were pelleted by centrifugation at 12 000xg for 30 s and the supernatant removed. To remove agarose contaminants, the pellet was washed with 500 µl Buffer QX1 by resuspending, centrifugation and removal of the supernatant as before. Residual salt contaminants were removed by washing with 2 x 500 µl Buffer PE as before. The pellet containing the purified DNA was then air dried for 30 min or until the pellet became white. The DNA was eluted by resuspension in 20 µl TE, pH 8.0 and incubation at RT for 5 min. A final centrifugation pelleted the particles and the supernatant containing the DNA was recovered. For sizes of DNA fragments or amounts outside the range given above, addition volumes and incubation times were adjusted as described in the kit protocol.

2.3.6 Preparation of digoxigenin-11-dUTP labelled cDNA probes

A non-radioactive DNA labelling kit (Boehringer Mannheim) was used to incorporate the nucleotide analogue digoxigenin-11-dUTP (DIG-11-dUTP) into DNA by the random primed labelling technique. The DNA to be labelled (20 ng in a total volume of

15 μl) was denatured for 10 min at 100°C and cooled immediately on ice/ethanol. The denatured DNA was mixed with 2 μl hexanucleotide mixture in 10 x reaction buffer, 2 μl 10 x dNTP labelling mixture (1 mM each dATP, dCTP, dGTP, 0.65 mM dTTP and 0.35 mM DIG-dUTP, pH 6.5) and 1 μl Klenow enzyme (2 U) and incubated at 37°C overnight. The reaction was stopped by adding 2 μl 0.2 M EDTA, pH 8.0.

2.3.7 Preparation of radiolabelled cDNA probes

A random primer labelling kit (Prime-It II, Stratagene Ltd.) was used to radioactively label cDNA with [α^{32}P]-dCTP. The DNA (25 ng in 24 μl) plus 10 μl random oligonucleotide primers were denatured for 5 min at 100°C, cooled to RT and mixed with 10 μl 5 x dCTP buffer followed by the addition of 5 μl [α^{32}P]-dCTP (3000 Ci mmol^{-1}) and 1 μl Exo(-)Klenow enzyme (5 U). The reaction was incubated at 37°C for 10 min and 2 μl stop mix was added. Unincorporated nucleotides were removed by gel filtration chromatography on Probe-Quant G-50 Micro columns (Amersham Pharmacia Biotech) in STE buffer (0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). The column matrix was resuspended by vortexing and the buffer was removed by centrifugation at 735xg for 1 min. The labelled probe was carefully loaded onto the top of the column and centrifuged at 735 g for 2 min to remove unincorporated nucleotides.

2.3.8 Isolation of a full-length cDNA

For isolation of a full-length ripening-related cDNA encoding endo-β-1,4-glucanase (EGase), an amplified cDNA library prepared from ripe receptacle tissue of strawberry

(*Fragaria x ananassa* Duch. cv Brighton) was screened. The library was constructed in the cloning vector λgt10 with *Eco*R I as the cloning site (Manning, 1998a). The bacterial host for the library was *E. coli* C600 *hfl*A150.

2.3.8.1 Library plating

For screening the cDNA library, 100 mm^2 TYN plates were prepared. Sterile TYN medium containing 0.2% (w/v) maltose was inoculated with host cells from a frozen glycerol stock and cultured overnight at 37°C. The titre of the amplified cDNA library was 1.33 x 10^9 pfu ml^{-1} and 60 000 pfu were used for the primary screen. Six aliquots of 10 000 pfu in 100 μl phage dilution buffer (PDB) were incubated at RT for 20-30 min with 200 μl of the overnight culture to allow the phage to adhere to the bacterial cell walls. The cell/phage suspension was then rapidly mixed with 5 ml molten TYN top agarose at 50°C and immediately poured onto a TYN plate pre-incubated at 37°C. The plates were swirled to ensure an even coverage without bubbles and after the top agarose had set were incubated inverted at 37°C overnight.

2.3.8.2 Plaque lifts

After the clear plaques formed by lysis of the plating cells by the phage had become visible overnight, the plates were chilled at 4°C to allow the top agarose to harden. Hybond-N nylon membrane (Amersham Pharmacia Biotech) 100 mm^2 was placed onto the surface of the plate ensuring there were no air bubbles or movement of the membrane once in position. Duplicate lifts were taken for each plate; the first was left in

contact with the top agarose for 1 min and the second for 1.5 min for equivalent transfer of plaques. After the lifts were taken the plates were stored at 4°C to minimize diffusion of phage out of the plaques. The membranes were placed in denaturing solution for 1 min to denature the phage DNA, then transferred to neutralizing solution for 5 min and finally washed in 2 x SSC for at least 5 min. After drying in air the DNA was fixed to the membranes by baking at 120°C for 30 min.

2.3.8.3   *Probing with partial-length EGase cDNA (FAN R97)*

The membranes were pre-hybridized in 0.2 μm filtered, boiled HYBSOL buffer (Yang *et al.*, 1993) at 65°C for ≥ 4 h. Hybridization was carried out overnight at 65°C in fresh HYBSOL buffer containing approximately 10 ng ml^{-1} denatured DIG-labelled FAN R97 cDNA insert (as described in section 2.3.6) as probe. The membranes were washed for 2 x 15 min at RT in 2 x SSC, 0.1% (w/v) SDS and then for 2 x 15 min at 65°C in 0.1 x SSC, 0.1% (w/v) SDS.

2.3.8.4   *Chemiluminescent detection of hybridized probe*

The detection was carried out using a DIG nucleic acid detection kit (Boehringer Mannheim) with modifications. The membranes were washed in Buffer 1 for 5 min at RT. They were then incubated for ≥ 60 min at RT in Blocking buffer 2. The anti-DIG-alkaline phosphatase conjugate was diluted 10 000-fold in Blocking buffer 2 and incubated with the membranes at RT for 30 min. The membranes were then washed for 4 x 10 min at RT in Buffer 1. Finally they were equilbrated for 5 min at RT in Buffer 3.

The chemiluminescent substrate CDP-Star (Boehringer Mannheim) was diluted 500-fold in Buffer 3 and the membranes were incubated briefly in the solution so the surfaces were covered. The membranes were enclosed in Saran wrap to prevent them from drying out and exposed to Kodak X-Omat AR film (Sigma). By aligning the film with the membranes and plates positive plaque regions could be identified. The density of plating for the primary screen did not allow individual plaques to be identified so the wide end of a glass pasteur pipette was used to remove an agar plug from the region of a positive plaque. From the primary screen, 12 of the strongest hybridizing plaques were chosen, 2 from each of the 6 plates. Each agar plug was placed in 500 μl PDB containing 50 μl chloroform at 4°C to allow the phage to diffuse out of the agar into the buffer. Each of the positives was then re-plated at a 500 000-fold dilution to give 20-30 well isolated plaques per plate for the secondary screen. This was carried out exactly as for the primary screen except that the hybridization and second wash temperatures were increased from 65°C to 68°C to increase the stringency. The single, well-isolated positive plaques (7 out of the original 12) were each removed into 100 μl PDB plus 10 μl chloroform and stored at 4°C.

2.3.8.5   Estimation of insert size by polymerase chain reaction (PCR)

The sizes of the cDNA inserts in the positive clones were estimated by PCR. The isolated plaques in 100 μl PDB plus 10 μl chloroform described above were used as the template DNA. Standard λgt10 primers were obtained from Sigma. The dNTP mix (Amersham Pharmacia Biotech) contained 10 mM each of dATP, dTTP, dGTP and dCTP in water, pH 7.5. The 10 x *Taq* Extender™ buffer (Stratagene Ltd.) contained 200

mM Tris-HCl, pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg ml^{-1} bovine serum albumen (BSA). All the components were mixed together in a 0.5 ml microfuge tube and kept on ice. The DNA polymerase (Flowgen) was added last and the mixture overlaid with one drop of mineral oil.

Each reaction was set up as follows :

Component	Volume	Final Amount
λgt10 forward primer (0.8 pmol μl^{-1})	12.5 μl	10 pmol
λgt10 reverse primer (1.0 pmol μl^{-1})	10 μl	10 pmol
Ultrapure dNTP mix (10 mM)	1 μl	200 μM each
10 x Taq Extender™ buffer	5 μl	1 x
sterile distilled water	19.5 μl	
template DNA	1 μl	
Taq Extender™ (5 U μl^{-1})	0.5 μl	2.5 U
Dynazyme™ DNA polymerase (5 U μl^{-1})	0.5 μl	2.5 U
Total volume	50 μl	

The PCR was carried out in a thermal cycler (Omn-E, Hybaid Ltd.) with the following cycle program:

95°C for 5 min	1 cycle
Denature at 95°C for 0.8 min] Anneal at 58°C for 1 min] Extend at 72°C for 1.5 min]	35 cycles
Final extension at 72°C for 6 min	1 cycle

The sizes of the PCR products were analyzed by electrophoresis on 1.5% (w/v) agarose gels. The clones with the longest length cDNA inserts were amplified in *E. coli* to prepare λ DNA as described below.

2.3.8.6   *Phage λ DNA preparation - scraped plate lysates*

To prepare λ DNA from the chosen positive clones, the phage were grown to confluent lysis on 90 mm diameter TYN plates. A 10 μl aliquot of each isolated plaque in PDB was made up to 100 μl in PDB, mixed with 200 μl *E. coli* plating cells and plated as described in section *2.3.8.1*, except that only 2.5 ml top agarose was used. After incubation at 37°C overnight the plates were cooled to 4°C for 1 h. The top agarose was scraped into 7 ml PDB plus 140 μl chloroform. After vigorous shaking the lysate was incubated at RT for 1 h with occasional shaking. The lysate was centrifuged at 10 000xg at 4°C for 10 min and 6 ml of the supernatant containing the phage particles was collected. Stocks of the phage were prepared at this stage by mixing 93 μl supernatant with 7 μl dimethylsulphoxide (DMSO), freezing in liquid nitrogen and storage at -70°C.

The collected lysate was incubated with 6 µl each of 1 mg ml^{-1} DNase I and 10 mg ml^{-1} RNase A at 37°C for 30 min to digest bacterial DNA and RNA. To precipitate the phage particles 6 ml 20% (w/v) PEG in 2M NaCl were added followed by incubation on ice for 1 h. The phage were collected by centrifugation at 10 000xg at 4°C for 20 min, resuspended in 0.5 ml TE, pH 8.0 and transferred to a microfuge tube. The phage were incubated at 68°C for 5 min after the addition of 5 µl 10% (w/v) SDS then 10 µl 5 M NaCl were added. An equal volume of phenol:chloroform:isoamyl alcohol (IAA) (25:24:1) was added, mixed by vortexing and the phases separated by centrifugation at 12 000 g for 30 s. The upper aqueous layer was collected and the extraction repeated. The extraction was repeated a third time with chloroform only. An equal volume of - 20°C isopropanol was added followed by incubation at -70°C for 15 min. The phage nucleic acid was precipitated by centrifugation at 12 000xg for 15 min at 4°C and washed with 70% ethanol at -20°C. The pellet was dried under vacuum and redissolved in 0.5 ml TE, pH 7.5 at RT for approximately 30 min. Ten units of RNase ONE^{TM} (Promega UK) were added and incubated at 37°C for 30 min to digest residual RNA. An equal volume of 13% (w/v) PEG in 1.6 M NaCl was added, incubated at RT for 5 min and centrifuged at 12 000xg for 10 min. The pellet of phage DNA was washed twice with 70% ethanol at RT, once with 100% ethanol at RT and dried under vacuum. The phage λ DNA was finally redissolved in up to 50 µl 10 mM Tris, pH 8.0 at 4°C overnight.

2.3.9   Sub-cloning of isolated cDNAs

2.3.9.1   *Preparation of cDNA insert and vector DNA*

The cDNA insert for cloning was released from the λ DNA by restriction with *Eco*R I, separated on a 1% (w/v) agarose gel and purified from the gel as described previously. The insert was dephosphorylated using calf intestinal alkaline phosphatase (CIAP) to prevent cloning of multiple inserts in the same vector molecule. Insert DNA (up to 10 pmol ends) was incubated with 5 µl 10 x CIAP buffer (0.5 M Tris-HCl, pH9.0, 10 mM MgCl$_2$, 1 mM ZnCl$_2$, 10 mM spermidine) and 0.5 U CIAP in a total volume of 50 µl at 37°C for 30 min. A further 0.5 U CIAP was added and incubated as before. The reaction was terminated by adding 300 µl stop buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 7.5, 200 mM NaCl, 0.5% SDS). An equal volume of phenol:chloroform (1:1) was added, mixed by vortexing and centrifuged at 12 000xg for 30 s. The upper aqueous phase was transferred to a clean tube. The extraction was repeated with chloroform only. The DNA was precipitated by adding 0.5 volume of 6 M ammonium acetate, 1 µl glycogen, 2 volumes of 100% ethanol at -20°C and incubated at -70°C for 15 min. The DNA was pelleted by centrifugation at 12 000xg for 15 min and washed once with each of 70% and 100% ethanol at -20°C. After drying under vacuum the dephosphorylated insert DNA was redissolved in 7.5 µl 5 mM Tris, pH 7.4, 0.1 mM EDTA ready for ligation into the vector.

The cDNA insert was sub-cloned into the vector pBK-CMV (Stratagene Ltd.). The pBK vector DNA was supplied digested and ready to use.

2.3.9.2   *Ligation of insert*

It was determined from test ligations that the ratio of insert-to-vector DNA that gave the highest ligation efficiency was 10:1, measured in picomole ends. The amount of pBK vector DNA used in the ligation reaction was 50 ng which corresponds to approximately 0.03 pmol ends. The following equation was used to calculate the amount of insert DNA required to give the desired ratio :

$$\text{pmol ends} / \mu\text{g DNA} = (2 \times 10^{6}) / (\text{length in bp} \times 660)$$

The 10 x ligase buffer contained 300 mM Tris-HCl, pH 7.8, 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 5 mM ATP.

The ligation reaction was set up as follows :

Component	Volume	Final Amount
digested pBK-CMV vector (50 ng μl^{-1})	1 μl	0.03 pmol ends
dephosphorylated cDNA insert	7.5 μl	0.3 pmol ends
10 x ligase buffer	1 μl	1 x
T4 DNA ligase (4 Weiss U μl^{-1})	0.5 μl	2 U
Total volume	10 μl	

The reaction was incubated at 16°C overnight and then stored at 4°C until ready for transformation.

2.3.9.3 Transformation into competent bacterial cells

The ligation mixture was transformed into supercompetent *E. coli* XL1-Blue MRF' cells using the protocol supplied (Stratagene Ltd.). These cells allow blue-white colour selection for transformants (pBK vector containing cDNA insert). The cells were thawed on ice and gently mixed by hand. Falcon 2059 polypropylene tubes were placed on ice to chill. A 100 µl aliquot of cells was added to a pre-chilled tube and mixed gently with 1.7 µl 1.42 M β-mercaptoethanol. The cells were incubated on ice for 10 min with gentle swirling every 2 min before 2 µl of the ligation reaction were added. A control transformation was set up using 1 µl of the pUC18 control plasmid supplied with the cells. The cells and DNA were mixed gently by swirling and incubated on ice for 30 min. A 45 s heat-pulse at 42°C was immediately followed by incubation on ice for 2 min to transform the cells with the ligated DNA. SOC medium was pre-heated to 42°C and 0.9 ml was added to the transformed cells. The cells were finally incubated at 37°C for 1 h with shaking at 225-250 rpm. During this time, LB plates containing 50 µg ml^{-1} kanamycin and 12.5 µg ml^{-1} tetracycline and an LB plate containing 50 µg ml^{-1} ampicillin were spread with 40 µl each of 2% (w/v) IPTG and 2% (w/v) X-gal for blue-white colour selection and allowed to dry at 37°C. After the 1 h incubation the transformation mix was plated onto the appropriate plates. The control transformation mix was plated on the LB-ampicillin plate using 5 µl diluted with 200 µl SOC medium

and spread evenly over the plate with a sterile spreader. For the ligation reaction transformations, 50, 100, 150 and 200 µl aliquots of transformation mix were made up to a volume of 200 µl with SOC medium as necessary and spread on separate LB-tetracycline-kanamycin plates. The plates were then incubated inverted at 37°C overnight. Transformed cells with inserts grew into white colonies which were picked off individually into 200 µl SOB medium. The resuspended cells were re-streaked onto fresh plates and well isolated colonies were selected for analysis.

2.3.9.4   *Confirmation of transformation*

Confirmation that the transformed colonies contained a cDNA insert was by PCR and restriction analysis. White colonies were picked off into 50 µl TB, resuspended by vortexing and 5 µl was used as the template DNA for PCR. The reaction mixture was as described in section 2.3.8.5 except that 10 pmol each of T3 and T7 primers (Promega UK) were used. The cycle programme was also as described in section 2.3.8.5 but with the annealing step carried out at 55°C. The reactions were analyzed on a 1% (w/v) agarose gel to confirm the presence of a cDNA insert of the correct size. For confirmed transformants, the remaining resuspended colony was used to grow an overnight culture of the cells in TB plus 50 µg ml^{-1} kanamycin as described in section 2.3.1. A glycerol stock for long-term storage of the cells at -70°C was prepared by taking 0.85 ml culture plus 0.15 ml glycerol, vortexing to mix and rapidly freezing in liquid nitrogen. To determine the cDNA insert size accurately, plasmid DNA was prepared (section 2.3.1) and restricted with *Eco*R I. The plasmid containing the longest insert was sequenced.

2.3.10 Isolation of a cDNA fragment by reverse transcription-polymerase chain reaction (RT-PCR)

A short cDNA fragment encoding a second form of EGase (*cel2*) in strawberry was obtained by RT-PCR for use as a probe. Degenerate oligonucleotide primers were designed to the C-terminal region of the published sequence (Llop-Tous *et al.*, 1999) and used for first strand cDNA synthesis and subsequent PCR. First strand cDNA was synthesized from 10 µg total RNA isolated from ripe receptacle tissue of strawberry (*Fragaria x ananassa* Duch. cv Calypso) using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Superscript II, Life Technologies Ltd., Paisley, UK) and Cel2 reverse primer (5' TGC/TTGA/GTCA/GCAA/GTTA/GTGA/GAA 3', nucleotide 1784 to 1765) according to the manufacturer's instructions. Amplification by PCR was performed in a total reaction volume of 20 µl containing single-stranded cDNA synthesized from 0.5 µg total RNA, 400 nM each of reverse and forward (5' GAC/TAAC/TTAC/TGAA/GCAA/GACNGA 3', nucleotide 1522 to 1541) Cel2 primers, 100 µM dNTPs and 0.5 U Dynazyme II (Flowgen). The PCR was carried out in a PCT200 Thermal Cycler (MJ Research) with the following cycle program:

95°C for 5 min, 45°C for 1 min, 72°C for 2 min   1 cycle

    Denature at 94°C for 45 s   ]
    Anneal at 45°C for 1 min   ]   34 cycles
    Extend at 72°C for 2 min   ]

Final extension at 72°C for 10 min   1 cycle

The reaction products were analyzed by gel electrophoresis and a fragment of the expected size was cloned into pCR-Script™ SK(+) (Stratagene Ltd.) and sequenced.

2.3.11 DNA sequencing

A fluorescence-based dideoxynucleotide chain termination method (Sanger *et al.*, 1977) was used to obtain DNA sequence by cycle sequencing using dye-labelled terminators. Sequencing reactions were performed using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) according to the protocol (Revision A). The sequenced samples were analyzed on an automated DNA sequencer (PE Applied Biosystems). The templates used for sequencing were double-stranded (ds) plasmid DNA prepared as described in section 2.3.1. Appropriate primers were used depending on the nature of the templates. The terminator premix contained A, C, T and G-Dye Terminators, dATP, dCTP, dTTP, and dITP in place of dGTP to minimize band compressions, Tris-HCl (pH 9.0), $MgCl_2$, a thermal stable pyrophosphatase and AmpliTaq DNA polymerase FS. The components were mixed together in a 0.5 ml microfuge tube and overlaid with one drop of mineral oil.

The sequencing reactions were set up as follows :

Component	Volume	Final Amount
Terminator premix	8 μl	
ds plasmid DNA template + dH$_2$O	8.8 μl	250-500 ng
Primer (1 pmol μl^{-1})	3.2 μl	3.2 pmol
Total volume	20 μl	

The sequencing reaction was carried out in a thermal cycler (Omn-E, Hybaid Ltd.) with the following cycle program:

Pre-heat to 96°C

96°C for 30 s   ]
50°C for 15 s   ]   25 cycles
60°C for 4 min   ]

Hold at 4°C

The extension products were purified by ethanol precipitation to remove excess dye terminators. For each reaction, 2 μl 3 M sodium acetate, pH 4.6 and 50 μl 100% ethanol were added to a 1.5 ml microfuge tube. The entire 20 μl sequencing reaction was added, mixed by vortexing and incubated on ice for 10 min. The extension products were then pelleted by centrifugation at 12 000xg for 30 min. The pellet was rinsed with 250 μl 70% ethanol and then allowed to air dry. The dried pellet was sequenced by Durham

University Sequencing Service on an automated DNA sequencer (PE Applied Biosystems).

2.3.12 Computer analysis of sequence data

Sequence data was analyzed using the University of Wisconsin Genetics Computer Group (GCG) software package (Devereux *et al.*, 1984). Raw sequence data was converted into GCG format using the program REFORMAT and edited as necessary using SEQED. The EMBL and Genbank nucleic acid databases were searched for related sequences using FASTA and BLAST programs. The derived amino acid sequence was obtained from the DNA sequence data using the program TRANSLATE. This derived protein sequence was then compared against the translated EMBL and Genbank databases using TFASTA and BLAST programs. Comparison of two nucleic acid or protein sequences was conducted using the BESTFIT and GAP programs. Multiple sequence alignments were conducted using the programs GCLUSTALW and PILEUP. A restriction map of a sequence for all or specified restriction enzymes was created using the program MAP.

2.3.13 Extraction of total RNA

Total RNA was extracted from strawberry receptacle, leaf, petiole and root as described by Manning (1991). Solutions were autoclaved where appropriate and labware was baked at 160°C overnight. All tissues were frozen in liquid nitrogen immediately after harvest and stored at -70°C until extraction. Frozen tissue (typically 5 g FW) was ground

to a fine powder in a pre-chilled mortar. Three volumes of RNA extraction buffer at RT were added with further grinding. The homogenate was extracted immediately with an equal volume of phenol:chloroform (1:1) at RT and mixed by shaking. The phases were separated by centrifugation at 20 000xg for 10 min at RT and the upper aqueous phase was transferred to a clean tube. The interphase and lower phase were shaken with a further 3 volumes of extraction buffer, centrifuged as before and the upper phase was combined with the first one. The combined upper phases were extracted with an equal volume of phenol:chloroform (1:1) as before. Differential precipitation of nucleic acids was started by adding 1.4 volumes water, 0.1 volume 1 M sodium acetate/acetic acid buffer, pH 4.5 and 0.4 volumes 2-butoxyethanol (2-BE) to 1 volume upper phenol phase. Following incubation on ice for 30 min, the contaminating polysaccharides were precipitated and pelleted by centrifugation at 20 000xg for 10 min at 0°C. The supernatant was collected and 0.6 volumes (with respect to the diluted aqueous phenol phase) 2-BE was added. After a further 30 min on ice the nucleic acids were precipitated by centrifugation as before. The pellet was washed consecutively with extraction buffer:2-BE (1:1 (v/v)) to remove traces of polyphenols, 70% (v/v) ethanol containing 0.1 M potassium acetate/acetic acid, pH 6.0 and 100% ethanol. The pellet was then dried under vacuum and redissolved in sterile distilled water on ice to give a concentration not less than 500 µg ml^{-1}. This nucleic acid solution was adjusted to 3 M LiCl by adding 0.25 volume 0.2 µm sterile filtered 12 M LiCl and incubated on ice for 1 h to precipitate the RNA. The RNA was pelleted by centrifugation at 11 600xg for 10 min at 4°C and washed with 2 x 1 ml 3 M LiCl, 1 ml 70% (v/v) ethanol and finally 1 ml 100% ethanol. The ethanol was removed and the pellet then dried under vacuum. The final RNA pellet was redissolved in sterile distilled water to give a concentration of

approximately 4 μg μl^{-1}. Aliquots of 5 μl were taken for determination of RNA

concentration by spectrophotometry (section 2.3.2). The RNA was then stored either

dissolved in RNA loading buffer at -70° (short-term) or precipitated as a pellet and

stored under 100% ethanol at -70° C (long-term).

2.3.14 Northern analysis

2.3.14.1 *Denaturing RNA gel electrophoresis*

Denaturing formaldehyde-agarose gels for electrophoresis of RNA were prepared with

1% (w/v) agarose in 1 x MOPS buffer containing 0.22 M formaldehyde. The gel was

cast in a horizontal gel tank and submerged in the same buffer. Samples of RNA (20 μg)

and RNA markers (Promega Ltd.) were made up in RNA loading buffer and heated at

65°C for 15 min prior to loading. The gel was run first at 2.5 V cm^{-1} until the samples

had entered the wells and then at 5 V cm^{-1} until the bromophenol blue front was

approximately 1 cm from the end of the gel.

2.3.14.2 *Northern blotting*

After electrophoresis, the RNA was transferred onto Hybond-N nylon membrane

(Amersham Pharmacia Biotech) using a capillary blotting unit (BIOS). The wick of the

blotting unit was wetted with ethanol and rinsed well with sterile water. A sheet of

Whatman 3MM filter paper the same size as the gel was placed on the wick and the gel

placed upside-down on top. The Hybond-N membrane was pre-wetted in 20 x SSC then

placed on the gel ensuring no air bubbles were trapped. Three pieces of pre-wetted 3MM filter paper followed by a stack of blotting pads (Sigma) were placed on top of the gel. The wick around the gel stack was masked with Saran wrap to prevent short-circuiting of the blotting buffer from the unit to the blotting pads. Transfer was carried out overnight at RT in 500 ml 20 x SSC which was added to the wick tray of the blotting unit. Following transfer, the membrane was rinsed in 2 x SSC for 2 min and air dried. The membrane and gel were visualized under UV light to check that efficient transfer had occurred and the RNA was cross-linked to the membrane by UV irradiation for 5 min. During this time the positions of the RNA markers and wells were marked on the membrane.

2.3.14.3   *Probing northern blots with radiolabelled cDNA probes*

The membrane was pre-hybridized in 5 x SSPE, 5 x Denhardt's solution, 1% (w/v) SDS and 100 μg ml^{-1} denatured salmon sperm DNA at 65°C for at least 4 h. Hybridization was carried out overnight at 65°C in fresh solution containing the ^{32}P-labelled cDNA probe prepared as described in section 2.3.7. Immediately before use the probe was denatured by heating for 5 min at 100°C followed by cooling on ice/ethanol. The membrane was washed for 2 x 20 min at 65°C in 3 x SSC, 0.1% (w/v) SDS followed by 1 x 20 min at 65°C in 0.3 x SSC, 0.1% (w/v) SDS. The membrane was placed in Saran wrap and exposed to Kodak X-Omat AR film (Sigma) with an intensifying screen at -70°C.

2.3.15 Extraction of genomic DNA

Genomic DNA was extracted from young leaf tissue in a small-scale method using CTAB (H.Y. Yang, personal communication) with minor modifications. A leaf disc was collected into a microfuge tube using the lid to cut out the disc and then frozen in liquid nitrogen. The disc was quickly powdered using a microfuge pestle and 0.5 ml CTAB extraction buffer, pre-warmed to 65°C, was added. After homogenization using the pestle the extract was incubated at 65°C for 10 min to disrupt the cell membranes. The extract was emulsified by adding 0.5 ml dichloromethane:isoamyl alcohol (24:1) and centrifuged at 12 000xg in a microfuge for 2 min at RT. The upper phase was collected into a clean tube and 300 µl isopropanol was added to precipitate the DNA. The DNA pellet was collected by centrifugation for 2 min at RT as before. The supernatant was discarded and the pellet was washed for 2 min with 0.5 ml wash buffer. The pellet was collected by centrifugation and allowed to air dry. The dried DNA pellet was dissolved in 30 µl sterile distilled water. An aliquot of 5 µl was taken for determination of DNA concentration by spectrophotometry (section 2.3.2).

2.3.16 Southern analysis

2.3.16.1 Digestion of DNA and gel electrophoresis

Genomic DNA samples (10 µg) were incubated with 1 Unit RNase ONE™ (Promega UK) at 37°C for 30 min to digest contaminating RNA. Samples were then digested with the required restriction enzyme. Reactions (100 µl) containing the DNA, 50 Units of

enzyme and 1 x reaction buffer were incubated overnight at the temperature required by the enzyme. The digested DNA was precipitated (section 2.3.3), dissolved in 15 μl TE, pH 8.0 and heated at 56°C for 3 min before separation on a 1% TAE-agarose gel (section 2.3.4).

2.3.16.2   *Southern blotting*

Following electrophoresis, the gel was photographed under UV light to record the positions of the DNA markers. The DNA was depurinated by submerging the gel in 0.25 M HCl for 15 min. After rinsing in sterile distilled water the DNA was denatured in 0.5 M NaOH, 1.5 M NaCl for 30 min. The gel was then neutralized in 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl for 30 min. A final incubation in 20 x SSC for 20 min was carried out before the DNA was transferred to positively-charged nylon membrane (Boehringer Mannheim) by blotting overnight in 20 x SSC as described in section *2.3.14.2*. Following transfer, the membrane was rinsed in 2 x SSC for 2 min and air dried. The DNA was fixed to the membrane by baking at 120°C for 30 min and irradiated by UV light for 3 min.

2.3.16.3   *Probing Southern blots with radiolabelled cDNA probes*

The membrane was pre-hybridized and hybridized with the ^{32}P-labelled cDNA probe as described in section *2.3.14.3*. The membrane was washed at high stringency twice in 0.25 x SSC, 0.1% (w/v) SDS at RT for 5 min followed by twice in 0.25 x SSC, 0.1%

(w/v) SDS at 65°C for 15 min. The membrane was placed in Saran wrap and exposed to Kodak X-Omat AR film (Sigma) with an intensifying screen at -70°C.

2.4   PROTEIN PURIFICATION AND CHARACTERIZATION

2.4.1   Rapid enzyme extraction from strawberry for endo-β-1,4-glucanase (EGase) assay

Strawberry fruit were frozen in liquid nitrogen and stored at -70°C until use. Frozen fruit were ground to a fine powder in a pestle and mortar and mixed with 5 volumes Buffer A (CTAB extraction buffer, Appendix A2) at RT. The extract was centrifuged at 10 000xg for 10 min. The supernatant was filtered through Miracloth to remove insoluble material and 3 volumes of acetone at -20°C were added. After incubation on ice for 5 min the protein was precipitated by centrifugation at 3 300xg at 4°C for 10 min. The pellet was washed with acetone at -20°C and allowed to air dry. At this stage the protein pellet could be stored at -20°C for future use or used immediately to assay EGase activity. The pellet was dissolved in Buffer B (Appendix A2) at a concentration equivalent to 2 g FW ml^{-1}. In most cases 1 ml (2 g FW) of the enzyme solution was used per assay.

2.4.2   Viscometric assay of EGase activity

2.4.2.1   Theory

Endoglucanase activity in crude extracts is usually assayed by viscometric methods which provide a rapid, precise and sensitive assay and which are not affected by endogenous reducing substances that may be present. Enzyme is incubated with a solution of a high molecular weight cellulose derivative (carboxymethylcellulose, CMC) as the substrate and the resulting reduction in viscosity of the substrate is used as a measure of the EGase activity in the reaction. The viscosity is determined at intervals after the start of the reaction by measuring the time taken for a fixed volume of the reaction solution to flow through a suspended-level (Ubbelhode) capillary viscometer. An arbitrary but linear relationship is found between inverse specific viscosity (η_{sp}^{-1}) and time-point of the reaction (T). The gradient of this relationship, the increase in inverse specific viscosity with time, is linearly related to EGase activity over a wide range and is proportional to the amount of enzyme added.

2.4.2.2   Calibration of viscometers

Prior to their first use, viscometers were cleaned with chromic acid and thoroughly rinsed in distilled water. The Hagenbach-Couette equation, $\eta = \rho (At - B / t)$ corrects for non-ideal flow in capillaries.

It can be rearranged to give $\eta t / \rho = At^2 - B$ where :

A, B viscometer constants

t flow-through time of assay solution

ρ density of assay solution

η viscosity of assay solution

From this equation, plotting t^2 against $\eta t / \rho$ gives a linear relationship, where the gradient gives the constant A and the intercept on the y-axis gives the constant B. The viscometers were individually calibrated by measuring the flow-through time, t, for three Newtonian fluids (water, 50% (w/w) sucrose and 60% (w/w) sucrose) of known viscosity and calculating the constants A and B.

In practice, each viscometer was filled with 20 ml of each standard solution in turn. The flow-through time was measured at 30°C for each of the solutions until three readings within 0.1 s were obtained. The average of the three readings was calculated for each solution and used with the ρ and η values (Table 2.1A) to determine t^2 and $\eta t / \rho$. For each viscometer, the three values for each of t^2 and $\eta t / \rho$ were plotted and the gradient and intercept determined to give the constants A and B, respectively (Table 2.1B). These values were then used in the calculation of EGase activities in assays.

Table 2.1A   Viscosity and density of standard solutions used in calibration of

viscometers (taken from National Bureau of Standards Circular C440,

Table 132, 673 (1942) ed. F. J. Bates)

Standard solution	Viscosity, η (centipoise at 30°C)	Density, ρ (g ml^{-1} at 30°C)
Water	0.7975	0.99565
50% (w/w) sucrose	10.18	1.22495
60% (w/w) sucrose	34.07	1.28144

Table 2.1B   Calculated values of the constants A and B for individual viscometers

Viscometer number	Constant A	Constant B
4153	1.0668	-3.4342
4149	1.0375	-2.7144
5457	1.0542	-3.192
1866	1.0468	-2.4247
5576	1.0753	-3.0775
5430	1.0606	-2.9368
5443	1.0403	-2.0598
6784	1.1145	-1.825

2.4.2.3   Assay

Viscometry was used to assay EGase in crude enzyme extracts from fruit and during the purification of EGase. The reaction mixture (20 ml) contained 1.5% (w/v) CMC and enzyme in 50 mM acetic acid/NaOH, pH 5.0 (Buffer B) in a viscometer suspended in a water-bath at 30°C. The enzyme was added last, the assay solution mixed and the start time of the assay (E + S) recorded. The flow-through time (t) of the CMC solution was measured to 0.01 s with a stop-watch at recorded time-points (T) over a 2 h incubation.

Using the Hagenbach-Couette equation, $\eta_{cmc} = \rho_{cmc} (At - B / t)$

where ρ_{cmc} density of 1.5% CMC substrate = 1.0085

               A, B   viscometer constants determined from calibration

               t   measured flow-through time of assay solution in seconds

η_{cmc}, the viscosity of the solution, was calculated.

η_r, the relative viscosity, was calculated by $\eta_r = \eta_{cmc} / \eta_{water}$ where $\eta_{water} = 0.7975$ centipoise at 30°C.

η_{sp}, the specific viscosity, was calculated as $\eta_{sp} = \eta_r - 1$ and this value was used to give η_{sp}^{-1}, the inverse specific viscosity of the assay solution.

The accurate time-point (T + 0.5t) of each flow-through measurement was calculated as the time-point of the measurement (T) minus the assay start time (E + S) plus half of the flow-through time (t) in seconds. This takes account of the change in viscosity occurring during the measurement.

The time-point of reaction (T + 0.5t) in seconds was plotted against the inverse specific viscosity (η_{sp}^{-1}), to give a linear relationship (Figure 2.1). The gradient was calculated as

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the change (increase) in inverse specific viscosity with time ($\Delta\eta_{sp}^{-1}$ s^{-1}), and this value was used as a measure of the EGase activity in the assay.
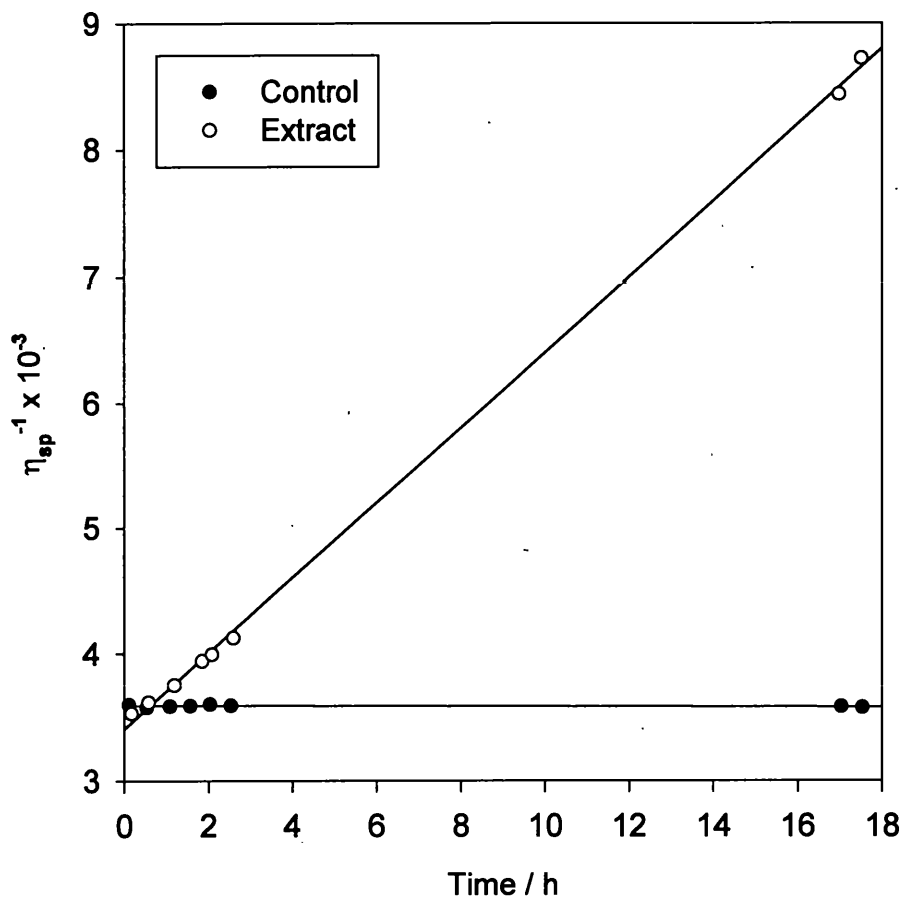
Figure 2.1   An example of a plot of inverse specific viscosity against reaction time-point used in the viscometric determination of EGase activity

2.4.3 Determination of EGase activity by reducing sugar assay

Activity of purified EGase was determined by the release of reducing groups from the substrate. This method, used for purified enzyme, enabled substrates that were either insoluble or did not produce a suitably viscous solution to be examined. The assay was adapted from Schales and Schales (1945) and Kidby and Davidson (1973) to allow reactions to be run in microfuge tubes. For assay 100 μl of sample was mixed with 25 μl ferricyanide reagent. The reactions were heated at 100°C for 5 min, cooled rapidly in cold water and 0.875 ml water was added to each. The reactions were then mixed and the absorbance at 237 nm was read. Standards were prepared using 0 - 20 nmoles glucose. Assays were conducted in duplicate and the amount of reducing sugar in the sample was determined from a standard curve (Figure 2.2).
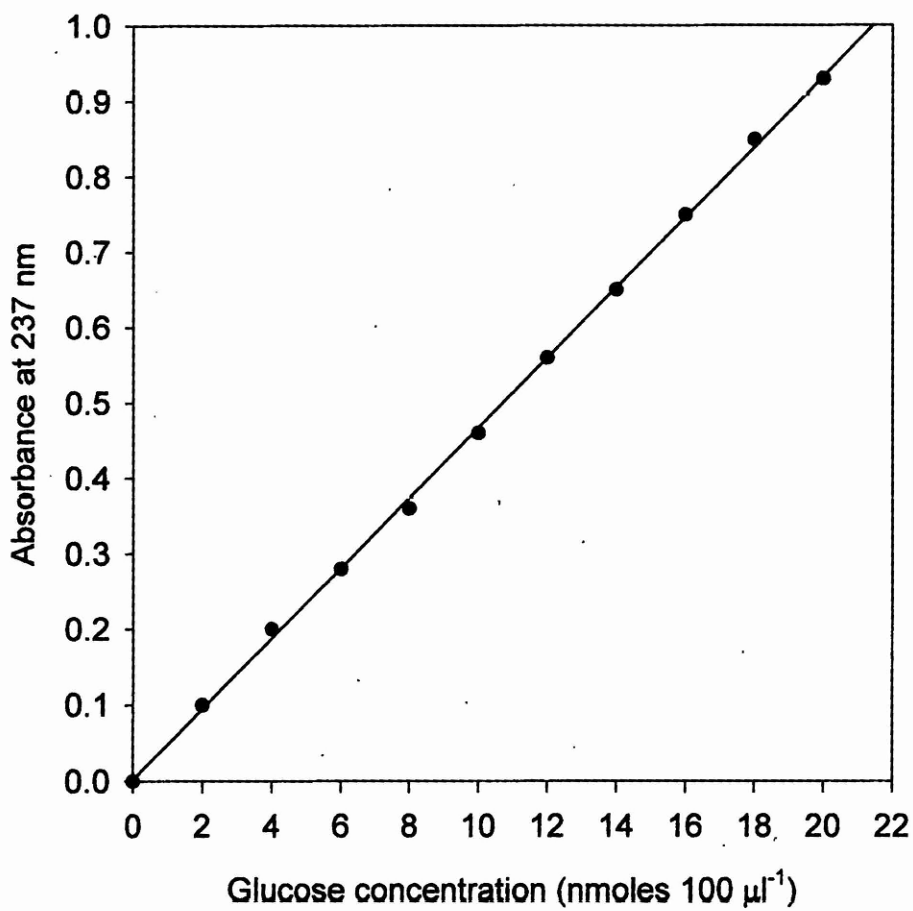
Figure 2.2   A typical standard curve of glucose for the determination of EGase

activity by the release of reducing groups from CMC substrate

2.4.4 Assay of EGase activity in strawberry fruit throughout development

Fruit were harvested at the following stages according to their receptacle colour and size: small green, white with green achenes, white with yellow achenes, turning, orange, red ripe and over-ripe. The fruit were frozen in liquid nitrogen and the achenes were removed. Proteins were extracted from powdered frozen receptacle tissue prepared from 5 g FW of tissue as described in section 2.4.1. EGase activity in the crude extracts was assayed in duplicate by viscometry using the equivalent of 2 g FW per assay. Activity was expressed on a g FW basis.

2.4.5 Measurement of fruit firmness

Strawberry fruit firmness was measured at RT using a motorized penetrometer (Stevens CR Analyzer). Fruit were harvested at the stages described in Section 2.4.4 and stored at $4^{\circ}C$ until measurement, which was carried out as soon as possible after harvesting. Individual fruit were cut in half longitudinally and halved fruit placed with the cut surface on the measurement platform. A thin layer of skin was removed with a new scalpel blade from the upper side of the fruit parallel with the platform to remove the achenes and provide a flat surface of receptacle tissue for measurement. For each sample, measurements were taken on both halves of each of five fruit and the average of these ten readings was used as a measure of receptacle firmness. The penetrometer was equipped with a cylindrical probe with a diameter of 5 mm. The test speed was set to 5 mm min^{-1} and the probe was pushed 5 mm into the fruit. The maximum force (N)

recorded during the measurement from 0 to 5 mm was used as a measure of fruit firmness.

2.4.6   Assay of EGase activity in fruit of other species

Endoglucanase activity was measured in a range of different fruits; ripe avocado mesocarp, ripe tomato (cv Ailsa Craig) pericarp, ripe apple (cv Golden Delicious) cortex, red pepper fruit and ripe raspberry fruit. As before, the tissue was frozen in liquid nitrogen and powdered. Proteins were extracted from 10 g powdered frozen tissue as described in section 2.4.1. EGase enzyme assays were conducted on the crude extract in duplicate. The amount of protein in the extract was determined (section *2.4.8.1*) and used to calculate the specific activity of EGase.

2.4.7   Isolation of a strawberry EGase

2.4.7.1   Extraction of soluble proteins from strawberry fruit

Soluble enzymes were extracted by the acetone powder method as described by Given *et al*. (1988a) with minor alterations. All steps were carried out at $4^{o}C$ unless otherwise stated.

Ripe strawberry (cv Elsanta) fruit were frozen in liquid nitrogen and stored at $-70^{o}C$ until use. Frozen fruit were ground to a fine powder in a coffee grinder. The powder was added to 10 volumes (with respect to fruit FW) of acetone at $-20^{o}C$ and ground briefly in a pestle and mortar before filtering under vacuum onto Whatman 541 filter paper. The

residue was washed with a further 10 volumes of acetone at -20°C and dried under vacuum for at least 1 h. The resulting acetone powder was stored at -70°C until use. The acetone powder was added to 3 volumes (with respect to fruit FW) of enzyme extraction buffer and extracted with stirring for 1 h. The extract was filtered through a nylon mesh bag then through Miracloth (Calbiochem-Novabiochem (UK) Ltd.) and centrifuged at 22 500xg for 30 min. The supernatant was collected and adjusted with 1 M CaCl$_2$ to a final concentration of 50 mM. Solid ammonium sulphate was added to 20% (w/v) saturation (114 g l^{-1} at 25°C) and dissolved by stirring for 1 h. The precipitated pectin was pelleted by centrifugation at 10 000xg for 10 min. The supernatant was collected and solid ammonium sulphate was added to 80% (w/v) saturation (538 g l^{-1} at 25°C) and dissolved by stirring overnight. Precipitated proteins were pelleted by centrifugation at 10 000xg for 10 min and as much of the supernatant as possible was removed. The protein pellet was allowed to air dry for at least 2 h and stored at -20°C until use.

2.4.7.2   *Purification of a strawberry EGase*

Soluble protein extracted from ripe fruit of cv Elsanta was used in the purification of EGase. All procedures were carried out at RT. The protein pellets obtained by ammonium sulphate precipitation from 300 g FW tissue were dissolved in 20 ml Buffer B (Appendix A2) to a concentration of 15 g FW ml^{-1}. The protein extract was applied to a 10 ml (5.7 cm x 1.5 cm diameter) column of CF11 cellulose (Whatman) which had first been equilibrated in Buffer B and the flow rate adjusted to 1 ml min^{-1}. One column volume (10 ml) of Buffer B was added to displace the remaining extract from the column. The column was then washed sequentially with 5 column volumes of Buffer B

to remove unbound protein. The adsorbed EGase protein was eluted with 3 column volumes of elution buffer (50 mM citrate-phosphate-Tris buffer (CPT), pH 9.0 containing 1 M NaCl and 0.1 M cellobiose) and the eluate containing the EGase protein was collected. The eluate was reduced to a volume of 1.0-1.5 ml by ultrafiltration on PM10 membranes (Amicon) and made up to 10 ml in Buffer B to sufficiently dilute the cellobiose present to enable the EGase protein to bind again to cellulose. The 10 ml of eluate was then applied to a 5 ml (2.8 cm x 1.5 cm diameter) column of CF11 cellulose in Buffer B and the procedure repeated as for the first column, adjusting the volumes accordingly for the smaller column. The EGase was eluted with 3 column volumes of elution buffer and ultrafiltrated as before. The eluate from the second column was then applied to a third and final CF11 cellulose column exactly as for the second column. The final eluate after a final ultrafiltration step to remove the cellobiose was designated the purified EGase protein. For characterization of the purified enzyme the final eluate was concentrated to a volume of approximately 1 ml.

2.4.8   Characterization of the purified EGase enzyme

2.4.8.1   *Protein assay*

Protein concentrations were determined by the protein-dye binding method of Bradford (1976) using microtitre plates. Samples were diluted as necessary and standards were prepared using bovine serum albumen (BSA) diluted in the same buffer as the sample at concentrations of 0 - 150 µg 100 µl^{-1}. For assay, 100 µl of each standard or sample were mixed with 100 µl Coomassie protein reagent in a microtitre plate well, incubated at RT

for 5 min and the absorbance was read at 600 nm. All assays were conducted in

duplicate and the amount of protein in the samples was determined from a standard

curve of protein concentration (Figure 2.3).
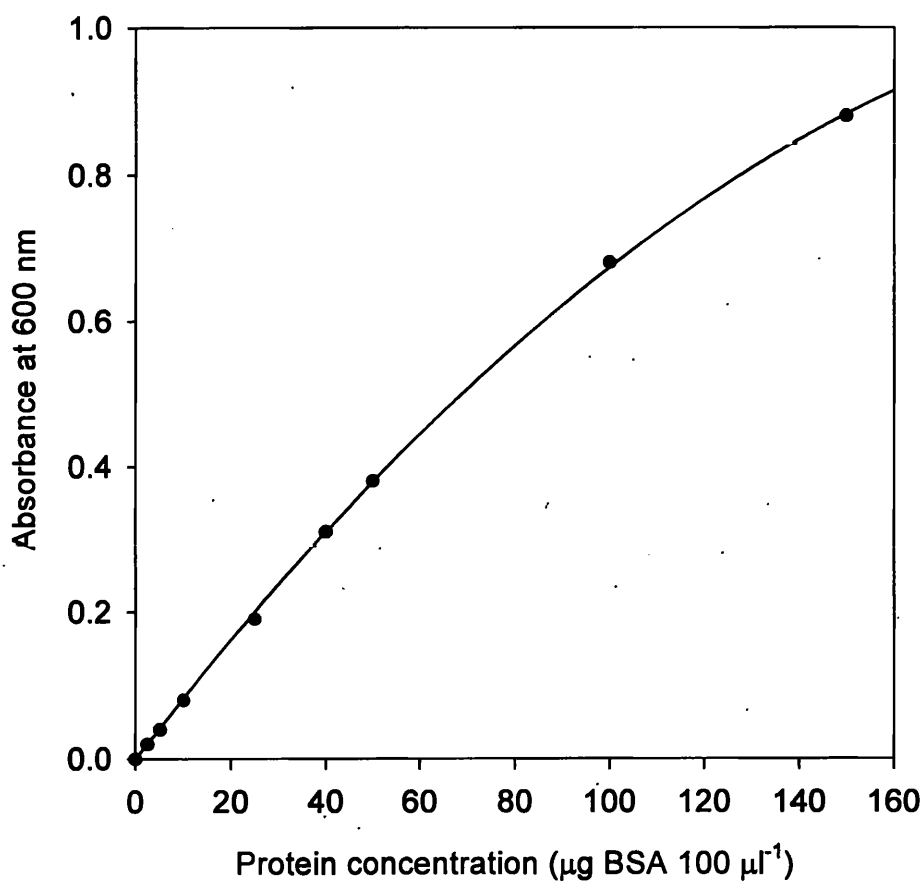
Figure 2.3   A typical standard curve of BSA for the determination of protein

concentration

2.4.8.2   Concentration of protein in dilute solutions

Protein was concentrated from very dilute solutions as described by Wessel and Flugge (1984). In a 2 ml tube, one volume (0.2 ml) of dilute protein solution and 4 volumes (0.8 ml) of methanol were vortexed and centrifuged at 12 000xg in a microfuge for 10 s to collect the whole sample. One volume (0.2 ml) of chloroform was added and the sample was vortexed and centrifuged as before. Three volumes (0.6 ml) of water were added and the sample was vortexed vigorously and centrifuged for 1 min to separate the phases. The upper phase was discarded and a further 3 volumes (0.6 ml) of methanol were added to the lower phase and interphase containing the protein. After vortexing and centrifugation for 2 min the protein pellet was allowed to dry in air. For analysis by SDS-PAGE the pellet was redissolved in 1 x Laemmli sample buffer.

2.4.8.3   Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE was conducted using the discontinuous buffer method of Laemmli (1970). The Bio-Rad Protean II mini-gel system was used to pour and run 0.5 mm thick vertical gels. The composition of the gels is described in Appendix A. Resolving gels (10% (w/v) acrylamide) were poured between ethanol-cleaned glass plates and overlaid with water until polymerized. The water was replaced with resolving gel buffer stock diluted 1:4 and the gel allowed to polymerize fully overnight. The overlay was removed and the stacking gel (3.75% (w/v) acrylamide) poured with a comb in place to form the wells. Once the gel had polymerized, the comb was removed and the wells were rinsed with distilled water to remove any traces of acrylamide. The gel was then set up ready for

electrophoresis in 1 x SDS-PAGE running buffer. Samples and SDS-PAGE molecular weight protein markers were made up in 1 x Laemmli sample buffer, heated to 100°C for 5 min then cooled on ice before loading into the wells. The gel was run at 100 V (40 mA) through the stacking gel and at 200 V (80 mA) through the resolving gel until the bromophenol blue dye front was at the bottom of the gel. The proteins were visualized by staining with Coomassie Blue as described in section 2.4.8.4.1.

2.4.8.4   *Staining for proteins*

2.4.8.4.1   Coomassie Blue staining

Following electrophoresis, proteins were visualized by staining with Coomassie Blue. Gels were incubated for 1 h in 0.05% (w/v) Coomassie Brilliant Blue R-250 in 25% (v/v) methanol and 8% (v/v) acetic acid until the whole gel was a deep blue. Gels were then destained in 25% (v/v) methanol and 8% (v/v) acetic acid with shaking until protein bands were visible on a clear background.

2.4.8.4.2   Silver staining of proteins

Coomassie Blue staining is not sensitive enough to detect very low levels of protein and in these cases the more sensitive method of silver staining was used.

All steps were carried out at RT with shaking. Following electrophoresis, the gel was fixed in Fix/Stop solution for 15 min, washed in 10 gel volumes of distilled water for 3 x 2 min and stained in 5 volumes Stain solution for 10 min. During this time 5 volumes

of developer was freshly prepared on ice. After staining the gel was rinsed in distilled water for 10 s before the ice-cold developer was added. Bands appeared within approximately 3 min and these were fixed in Fix/Stop solution for 2-3 min. The gel was rinsed in distilled water for 2 x 2 min, 2% (w/v) NaOH for 2-3 min and finally in 30% (v/v) Fix/Stop solution for 3 min without shaking.

2.4.8.5   *Electroblotting*

Proteins were transferred from gel to membrane by electroblotting using a BIO-RAD Mini Trans-Blot electrophoretic transfer cell according to the manufacturer's protocol. Prior to blotting the gel and the membrane were equilibrated in the transfer buffer to be used. Electroblotting was carried out at 90 V for 80 min at 4°C.

2.4.8.6   *Protein sequencing*

The eluate containing the purified EGase protein was first concentrated before 20 μg was resolved by SDS-PAGE. The protein was electroblotted from the gel onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P^{SQ}, Millipore) in transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propane-sulphonic acid (CAPS), pH 11, 10% (v/v) methanol. The PVDF membrane was wetted in 100% methanol for 15 s and washed with distilled water prior to use. Both the gel and the membrane were equilibrated in transfer buffer for 15 min prior to blotting. After blotting, the membrane was allowed to air dry to improve protein binding. The membrane was then re-wetted in 100% methanol and stained with 0.1% Coomassie Blue R-250 in 1% (v/v) acetic acid

and 40% (v/v) methanol for 1 min. De-staining was carried out in 50% methanol and the membrane was allowed to dry. The protein band was cut out and sequenced from the N-terminus using a Gas Phase protein sequencer (Pat Barker, The Babraham Institute, Cambridge, UK).

2.4.8.7 Determination of physico-chemical properties

2.4.8.7.1 pH activity profile

The pH optimum of the purified EGase enzyme was determined by viscometric assay over the range pH 3.0 to 9.0, with reaction mixtures containing approximately 7.5 µg purified EGase enzyme. The buffer used was a combination of sodium citrate, sodium phosphate and Tris (CPT) at 50 mM to allow continuity of pH without changing buffer. Control reactions containing no enzyme were carried out at each pH to take account of any effect of pH on the viscosity of the substrate.

2.4.8.7.2 Effect of substrate concentration

The activity of the purified EGase was measured at the following substrate concentrations, 0, 0.15, 0.3, 0.6, 0.9, 1.5 and 2.25% CMC, by reducing sugar assay. The reaction mixture contained the CMC substrate and approximately 1.5 µg purified EGase enzyme in Buffer B (Appendix A2) in a total volume of 100 µl. The reactions were incubated at 30°C and stopped by adding assay reagent after 30 min. To obtain zero-time values assay reagent was added to the reaction mixture before the enzyme. EGase

activity was determined by measuring the amount of reducing sugars released (section 2.4.3) in the first 30 min of the reaction.

2.4.8.8 Determination of substrate specificity

Activity of the purified EGase against various substrates was determined by reducing sugar assay and compared with its activity against CMC taken as a standard. The substrates examined were insoluble CF11 cellulose (medium fibrous, Whatman), starch (soluble potato), laminarin (from *Laminaria digitata*), lichenan (from *Cetraria islandica*), xylan (birchwood, >90% xylose), pectin (*Citrus*, partially methoxylated polygalacturonic acid) (Sigma), xyloglucan (tamarind, amyloid, Ara:Gal:Xyl:Glc 3:16:36:45) and galactan (lupin, Gal:Ara:Rha:Xyl:GalUA 91:2:1.8:0.2:5) (Megazyme). Reactions contained 1% substrate and approximately 0.5 µg purified EGase in Buffer B in a total volume of 100 µl for the soluble substrates or increased to 0.75 µg purified EGase in 150 µl for the insoluble substrates (cellulose, lichenan). The reactions were incubated at 30°C and stopped by adding assay reagent after 60 min. To obtain zero-time values assay reagent was added to the reaction mixture before the enzyme. EGase activity was determined by the release of reducing sugars as described in section 2.4.3. Reactions with insoluble substrates were centrifuged briefly and 100 µl supernatant were taken to determine the release of soluble reducing sugars. To assay reducing groups on the residual insoluble fraction the substrate was washed with 2 x 1 ml sterile water and resuspended in 150 µl water before 37.5 µl ferricyanide reagent was added.

2.5   GENERATION AND ANALYSIS OF TRANSGENIC STRAWBERRY PLANTS

2.5.1   Construction of transformation vectors

2.5.1.1   *Construction of antisense and sense expression cassettes in the intermediate vector pJR1Ri*

The 1779 bp *cell* cDNA insert was excised from the pBK-CMV vector by digestion with the restriction enzyme *Eco*R I, separated on an agarose gel and purified. The sticky-ends were then polished using cloned *Pfu* DNA polymerase (Stratagene Ltd.) to produce blunt-ends. The purified cDNA insert was incubated with 2 μl 10 mM dNTP mix, 2 μl 10 x cloned *Pfu* reaction buffer (200 mM Tris-HCl, pH 8.75, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 20 mM $MgSO_4$, 1% Triton X-100, 1 mg ml^{-1} BSA) and 4 μl cloned *Pfu* DNA polymerase (10 U) in a total volume of 28 μl at 72°C for 30 min. The DNA was precipitated and redissolved in TE, pH 8.0 ready for ligation into pJR1Ri.

The pJR1Ri vector was linearized with the restriction enzyme *Sma* I to leave blunt-ends. These were dephosphorylated to prevent the vector recircularizing during the ligation.

The blunt-end ligation of the *cell* cDNA into pJR1Ri in either orientation was performed using the pCR-Script™ SK(+) cloning kit reagents (Stratagene Ltd.). For a blunt-end ligation the molar ratio of insert-to-vector DNA used was 100:1. The polished *cell* cDNA and dephosphorylated pJR1Ri vector in a total volume of 15 μl were

incubated with 2 µl 10 x pCR-Script reaction buffer (250 mM Tris-HCl, pH 7.5, 100 mM MgCl$_2$, 100 mM DTT, 200 µg ml^{-1} BSA), 1 µl 10 mM rATP and 2 µl T4 DNA ligase (8 U) at 16°C overnight.

The ligation mixture was diluted 5-fold in TE, pH 7.5 and 5 µl was transformed into 100 µl DH5α™ competent cells (Life Technologies Ltd.) using the protocol supplied. This was essentially the same as the method described in section *2.3.9.3* for supercompetant *E. coli* XLi-Blue MRF' cells but without the addition of β-mercaptoethanol. The transformation mixture was spread on LB plates containing 50 µg ml^{-1} kanamycin. A control transformation was set up using 5 µl pUC19 control plasmid supplied with the cells, diluted 1:10 and spread on an LB plate containing 50 µg ml^{-1} ampicillin. Transformed cells grew into white colonies and these were picked off individually into 50 µl TB ready for PCR analysis to confirm a) the presence of pJR1Ri containing the *cell* cDNA and b) the orientation of the cDNA between the promoter and terminator.

PCR was carried out using 2 µl of the resuspended colonies as the template DNA. Primers were designed to the CaMV 35S promoter (5' ACTATCCTTCGCAAGA CCCTTCCT 3'), the nos 3' terminator (5' ATCATCGCAAGACCGGCAACAGGA 3') and a 5' region of the *cell* cDNA (5' TGAAGGCCACGGCGGTTCCTGGCG 3'). Each of the three combinations of primers were used for each template DNA. Reaction conditions and cycles were as described previously (section *2.3.8.5*) but at an annealing temperature of 70°C. The PCR products were separated on an agarose gel to ascertain the presence of the *cell* cDNA and its orientation. This was verified by DNA sequencing using the CaMV 35S primer. The confirmed antisense and sense expression cassettes were then cloned into pBINPLUS.

2.5.1.2 Cloning of antisense, sense and control expression cassettes into the binary vector pBINPLUS

The antisense and sense expression cassettes from the transformants and the control cassette (promoter and terminator only) were excised from pJR1Ri by digestion with the restriction enzymes *Eco*R I and *Hin*d III, purified and dephosphorylated. The vector pBINPLUS was cut with the same enzymes to allow directional cloning of the cassettes into the vector. The ligation was carried out with an insert-to-vector molar ratio of 10:1 and transformed into DH5α™ competent cells as described before. Colonies were analyzed by PCR as in section *2.5.1.1*) to confirm transformants. Restriction analysis was also performed using *Pac* I and *Asc* I as final confirmation of the presence of each of the expression cassettes in pBINPLUS.

2.5.1.3 Transformation of vectors into Agrobacterium

The vectors were transformed into the hypervirulent *Agrobacterium tumefaciens* strain EHA105 (Hood *et al.*, 1993) by electroporation. This strain is streptomycin resistant (Sm^R) but kanamycin sensitive (Km^S) allowing selection of the transformation vectors which contain the kanamycin resistance gene (*nptIII*).

A culture of EHA105 was grown in 100 ml LB medium containing 0.1% (w/v) glucose and 200 μg ml^{-1} streptomycin at 29°C overnight with shaking until an OD$_{660}$ of 1-1.5 was attained. To prepare electrocompetent cells the culture was chilled on ice for 15 min and centrifuged at 2 300xg for 20 min at 4°C to pellet the cells. The pellet was washed

in 3 x 10 ml ice-cold 1 mM HEPES, pH 7.0 and 1 x 10 ml 10% (w/v) glycerol in 1 mM HEPES, pH 7.0. The pellet was finally resuspended in 500 µl 10% (w/v) glycerol in 1 mM HEPES, pH 7.0, distributed into 45 µl aliquots, frozen in liquid nitrogen and stored at -70°C.

For electroporation, 500 ng of each of the three transformation vectors was used. Aliquots of electrocompetent cells were thawed on ice, the vector DNA added and the mixture transferred to a pre-chilled electroporation cuvette (0.1 cm electrode gap). The load resistance was set to 100 Ω, the capacitance to 1.5 kV and the electric pulse applied. The cells were diluted immediately in 1 ml SOC medium at RT and incubated at 29°C for 1-1.5 h with shaking. The cells were plated on LB plates containing 50 µg ml^{-1} kanamycin and incubated at 29°C for 2 d.

A transformed colony, confirmed by PCR as before (section *2.5.1.1*), for each of the three transformation vectors was chosen and the remaining resuspended cells were used to grow a culture in YEP medium containing 50 µg ml^{-1} kanamycin at 29°C for 2 d. A glycerol stock was prepared for each by mixing 0.5 ml culture with 0.5 ml glycerol, freezing in liquid nitrogen and stored at -70°C. The stocks were used to prepare the *Agrobacterium* containing the antisense, sense and control vectors for transforming strawberry.

2.5.2 Transformation of strawberry

Strawberry plants were transformed with *Agrobacterium tumefaciens* EHA105, containing either the antisense, sense or control transformation vector, using the methods described by James *et al.* (1990) with modifications.

The stages of the transformation of strawberry (*Fragaria x ananassa* Duch. cv. Calypso) are described below.

2.5.2.1 *Preparation of Agrobacterium containing the transformation vectors*

Day 1

A scrape from each of the three transformed *Agrobacterium tumefaciens* EHA105 glycerol stocks was streaked onto an LB plate containing 50 $\mu g\ ml^{-1}$ kanamycin and incubated at 29°C for 2 d.

Day 3

am A single colony of each of the three transformed *Agrobacterium* was inoculated into 5 ml YEP medium and incubated at 29°C with shaking at 200 rpm.

pm Kanamycin was added to a concentration of 50 $\mu g\ ml^{-1}$ and the cultures incubated as before overnight.

For each culture 1 ml was inoculated into 9 ml fresh YEP medium containing 50 μg ml^{-1} kanamycin and incubated at 29°C with shaking overnight.

2.5.2.2   Preparation of strawberry explants and infection with Agrobacterium

Day 5

The OD_{420} of the *Agrobacterium* cultures were determined and the cells were pelleted by centrifugation at 3 000xg for 15 min. Each pellet was redissolved in a volume of MS20 solution equal in ml to the OD_{420} x 200, separated into 10 ml aliquots and incubated at 20°C with shaking at 200 rpm for 5 h.

The strawberry explants were prepared from aseptically micropropagated plants at approximately 8 weeks old. Leaf discs were cut from the leaves using a sterile no.2 cork borer and placed onto 100 mm^2 ZN102 plates ensuring good contact with the agar. For each plate 25 discs were placed in a 5 x 5 grid. For each *Agrobacterium* transformation 100 explants were infected (4 plates). In addition to the transformations, two controls were set up; no infection/no selection and no infection/with selection using 50 explants (2 plates) for each.

The explants were innoculated with the relevant *Agrobacterium* by adding a 10 ml aliquot of bacteria per plate and incubated for 20 min, occasionally swirling the plates. The leaf discs were blotted on sterile filter paper and then transferred to fresh ZN102 plates with 2 sterile filter papers dampened with 0.5 ml MS20 on top. As before, they

were arranged in a 5 x 5 grid. The plates were sealed with parafilm and incubated at

22°C in the dark for 2 d to allow infection of the leaf discs with *Agrobacterium*.

2.5.2.3   *Washing and regeneration of infected explants*

Day 8

The leaf discs from each plate were transferred into 10 ml wash solution in a 30 ml

sterilin tube. The tubes were shaken sideways at 50 rpm for 5 h at RT. After washing,

the discs were blotted on sterile filter paper and transferred to the relevant plates.

Explants infected with *Agrobacterium* and the no infection/with selection controls were

placed on selection repli plates (ZN102 containing 100 μg ml^{-1} kanamycin and 200 μg

ml^{-1} cefotaxime to kill any remaining *Agrobacterium* cells). The no infection/no

selection controls were placed on plain ZN102 repli plates. The plates were placed in

controlled environmental conditions at 20-22°C, 16 h day and at a light intensity of 70

μmol m^{-2} s^{-1} (Phillips 70 W Type 84 fluorescent tubes sited 25 cm above the shelf) for 3

weeks.

3 weeks onwards

After 3 weeks, explants were transferred onto regeneration repli plates with no selection

(ZN102 containing 200 μg ml^{-1} cefotaxime only) and the first shoots started to appear at

4-6 weeks. Shoots were removed and placed on S5 proliferation medium in Coulter pots

for 2 weeks (weeks 6-8) to increase in size. Shoots were then screened again on S5

medium containing 50 μg ml^{-1} kanamycin to select for transformed shoots and those

surviving were considered putative transformants and rooted. To root, shoots were

placed on R13 rooting medium in Coulter pots for 4 d and then transferred to R37

rooting medium in honey jars. After 4-8 weeks the roots were well established and the

plants were at least 5 cm tall. At this stage the plants were removed from aseptic media

and transferred to Levingtons F2 compost (medium nutrient/standard pH/fine structure)

in 6.5 cm pots. The compost was heat sterilized by autoclaving to reduce the risk of

fungal or bacterial contamination during establishment. The plants were placed in a

propagator with the vents closed and grown at 20-22°C, in a 16 h day, and at a light

intensity of 70 μmol m^{-2} s^{-1}. After 1 week the vents were gradually opened and after 2

weeks the lid was removed and the plants were hardened off. The plants were then

transferred to 9 cm pots in a compost mix consisting of Richmoor Mix 1, Osmocote

Plus and Suscon Green (900 litres : 4 kg : 550 g) and placed in the glasshouse.

2.5.3   PCR analysis of putative transformants

Putative transformants that had survived the kanamycin selection were analyzed for the

presence of the transformation vector by PCR using genomic DNA extracted from

young leaf tissue in a small scale method (section 2.3.15) as the template. Genomic

DNA from a wild-type plant was used as a negative control. The primers used were

designed to the *nptII* gene:

NPTII 156   5' CCTGTCCGGTGCCCTGAATGAAC 3'

NPTII 631   5' GGCCACAGTCGATGAATCCAGAAAAG 3'.

Each reaction contained 100 ng template DNA, 10 pmoles of each NPTII primer and a

Ready-To-Go® PCR Bead (Amersham Pharmacia Biotech), containing 200 μM of each

dNTP, ~1.5 units of *Taq*, 10 mM Tris-HCl, pH 9.0, 50 mM KCl and 1.5 mM MgCl$_2$, in

a total volume of 25 μl. The PCR was carried out in a thermal cycler (Omn-E, Hybaid Ltd.) with the following cycle program :

95°C for 5 min		1 cycle
95°C for 0.8 min	]	
65°C for 1 min	]	35 cycles
73°c for 1.5 min	]	
73°C for 6 min		1 cycle

The PCR products were analyzed by electrophoresis for the presence of a band of the expected size of 475 bp to confirm that they were transformed. Confirmed primary transformants were grown to maturity in the glasshouse. In addition, transformed plants that tested negative for the transformation vector (non-transformed) and wild-type (untransformed) plants were grown as controls.

2.5.4   Southern analysis of the primary transformants

Southern analysis was used to confirm the presence of the antisense or sense transgene in the genomes of the *cel1*-transformed lines. Genomic DNA was extracted from young leaf tissue in a small scale method, digested with the restriction enzyme *Hin*c II and blotted onto membrane. The 542 bp CaMV 35S promoter fragment, isolated from pJR1Ri by restriction with *Eco*R I and *Kpn* I, was used as the probe in Southern blot hybridizations as previously described (sections 2.3.15 and 2.3.16). Hybridizing bands of 1466 bp and 1169 bp should be present in antisense and sense *cel1*-transformed plants respectively.

2.5.5   Northern analysis of the primary transformants

The expression of both *cel1* and *cel2* in ripe fruit of all the primary transformants was determined by northern analysis. Ripe receptacle tissue from each transformed line, non-transformed line and a wild-type untransformed control was frozen in liquid nitrogen, ground to a fine powder in a pestle and mortar and stored at -70°C until extraction. RNA was extracted in a scaled-down version of the method described previously (section 2.3.13). For each sample, a 1 g aliquot of frozen, powdered tissue was weighed into 2.5 ml of RNA extraction buffer at RT in a pestle and mortar and ground thoroughly. Two 0.9 ml aliquots were transferred to two 2 ml microfuge tubes on ice. An equal volume (0.9 ml) of phenol:chloroform (1:1) was added and mixed by shaking. The phases were separated by centrifugation at 12 000xg for 5 min at RT in a microfuge and the upper aqueous phase was transferred to a 10 ml tube. The upper phase was made up to a volume of 4.32 ml with sterile distilled water, 0.18 ml 1 M sodium acetate/acetic acid buffer, pH 4.5 and 1.8 ml 2-BE were added and mixed by shaking. After incubation on ice for 30 min and centrifugation at 20 000xg for 10 min at 4°C, the supernatant was transferred to a clean tube. A further 2.7 ml 2-BE was added, mixed and incubated on ice for 30 min. The precipitated nucleic acids were pelleted by centrifugation at 20 000xg for 10 min at 4°C and the supernatant was discarded. The pellet was washed sequentially with 5 ml 40 mM sodium acetate, pH 4.5:2-BE (1:1 (v/v)), cold 70% (v/v) ethanol and 100% ethanol before being dried under vacuum. The pellet was redissolved in 0.3 ml TE, pH 8.0 on ice for 1 h and transferred to a 1.5 ml microfuge tube. To precipitate the RNA, 0.1 ml 12 M LiCl was added and incubated on ice for at least 1 h. The RNA pellet was collected by centrifugation at 12.000xg for 10 min at RT, washed

sequentially with 0.5 ml 3 M LiCl, cold 70% (v/v) ethanol and 100% ethanol and dried under vacuum. The RNA was redissolved in 40 µl sterile distilled water on ice for at least 1 h. An 8 µl aliquot was taken for determination of RNA concentration by spectrophotometry (section 2.3.2). The remaining 32 µl of RNA solution was re-precipitated for storage by adding 1.3 µl 4 M sodium acetate/acetic acid buffer, pH 6.0 and 2.5 volumes 100% ethanol in a total volume of 125 µl, incubated at -70°C for 15 min and centrifuged at 12 000xg for 15 min at RT. The pellet was washed with 0.5 ml cold 70% (v/v) and 100% ethanol and stored at -70°C under 100% ethanol until required.

The RNA pellets were dissolved in sterile distilled water on ice to give a concentration of 3 µg µl^{-1}. For each sample, two 15 µg aliquots of RNA were made up in RNA loading buffer, run on duplicate RNA denaturing gels and blotted. The duplicate blots were hybridized first with the *cel1* cDNA probe and then with the *cel2* cDNA fragment probe (after removal of *cel1* probe) as previously described (section 2.3.14).

2.5.6   Assay of EGase activity in the primary transformants

Endoglucanase activity was determined in ripe fruit of all the primary transformants. For each sample, proteins were extracted from a 5 g aliquot of the frozen, powdered ripe receptacle tissue prepared for RNA extraction (section 2.5.5) as previously described (section 2.4.1). The dried protein pellet was dissolved at a concentration equivalent to 2 g FW ml^{-1} and 1 ml was used per viscometric assay (section *2.4.2.3*). EGase activity was determined from the mean value of two replicate assays for each sample. Similarly, two

replicate protein assays were carried out on the extract from each sample (section *2.4.8.1*) and the mean EGase specific activity was calculated for each primary transformant.

2.5.7   Measurement of fruit firmness of the primary transformants

The firmness of ripe fruit from all the primary transformants was determined by penetrometry as previously described (section 2.4.5). The measurements were necessarily conducted on separate fruit to those used for the RNA and protein extractions due to the destructive nature of the firmness assay.

CHAPTER 3.   ISOLATION AND CHARACTERIZATION OF A

STRAWBERRY ENDO-β-1,4-GLUCANASE cDNA

3.1   INTRODUCTION

In an effort to understand the role of cell wall hydrolases in modifying cell wall structure

and hence texture during fruit ripening, much research has focused on the isolation of

ripening-related cDNAs encoding hydrolytic enzymes in order to allow characterization

of the corresponding genes. Endo-β-1,4-glucanase (EGase) in particular is considered to

play an important role in the softening of many fruits and ripening-related cDNAs

encoding EGases have been isolated from several fruits including tomato, avocado and

pepper. In tomato, two different cDNAs have been identified in the fruit indicating the

expression of multiple EGase genes (*cel1* and *cel2*) during ripening. The individual

family members are differentially expressed suggesting that multiple activities are

required for the cooperative disassembly of the cell wall during ripening and that each

may have a distinct role to play (Lashbrook *et al.*, 1994). In contrast, all cDNAs isolated

from ripe fruit of avocado are derived from a single gene, *cel1* (Cass *et al.*, 1990). Of

three cDNAs isolated from pepper, one encodes Cel1, the ripening-related EGase

present in ripe fruit (Harpster *et al.*, 1997).

Isolation of a cDNA provides the potential for genetic manipulation of the level

of the corresponding enzyme in transgenic plants. The expression of both *cel1* and *cel2*

genes has been suppressed in transgenic tomato plants to study their roles in fruit

softening (Lashbrook *et al.*, 1998; Brummell *et al.*, 1999a). In order to produce

transgenic plants for elucidating the *in vivo* role of EGase in ripening strawberry fruit it

was necessary to isolate a ripening-related EGase cDNA clone. Previous work has identified a partial-length EGase cDNA clone (FAN R97) from a ripe strawberry receptacle cDNA library (Manning, 1998a) by differential screening. This cDNA was used as a probe to re-screen the ripe library and isolate the full-length homologue which could then be characterized.

3.2   RESULTS

3.2.1   Isolation of a full-length EGase cDNA from strawberry

To isolate a full-length ripening-related EGase cDNA clone the amplified cDNA library prepared from ripe receptacle tissue of strawberry (cv Brighton) was screened with the partial-length EGase cDNA (FAN R97, 1.5 kb) as a homologous probe. In the primary round of screening, 60 000 pfu were plated. From these, 12 of the strongest hybridizing plaques were isolated and taken through a higher stringency secondary screen. After the second round of screening, 7 out of the original 12 clones were positive. Representative hybridizing plaques from the primary and secondary rounds of screening are shown in Figure 3.1. A few well-isolated plaques from each positive clone were taken to estimate the size of the cDNA inserts by PCR analysis. The sizes ranged from 1.4 kb to 2.7 kb (Table 3.1). The cDNA inserts of four of the longest clones (1.1, 1.2, 3.2 and 4.2) were released from the λgt10 vector by restriction with EcoR I to allow sub-cloning into the vector pBK-CMV. However, the pattern of bands observed after restriction did not agree with the PCR results in all cases. One clone (3.2) did not contain an insert at all. Two clones (1.1 and 4.2) produced multiple bands on restriction with EcoR I. In each

case the size of the largest fragment was the sum of the sizes of the two smaller

fragments indicating that the cDNAs contained internal *Eco*R I sites which had been

partially restricted. It is unlikely that the multiple fragments represented different

cDNAs cloned into λgt10 as the apparent insert size from PCR analysis would have

been much larger.

Table 3.1   Putative strawberry EGase cDNAs isolated by screening a cDNA

library prepared from ripe fruit with the partial-length EGase cDNA,

FAN R97, as a homologous probe

Positive clone from 1° screen	Clone positive after 2° screen	Approx. insert size from PCR analysis (kb)	Approx. insert size from restriction analysis (kb)
1.1	+	2.5	2.7, 2.0 and 0.7
1.2	+	1.8	1.9
2.1	-	-	-
2.2	-	-	-
3.1	+	1.4	-
3.2	+	1.8	no insert
4.1	-	-	-
4.2	+	2.7	2.8, 1.9 and 0.9
5.1	-	-	-
5.2	+	1.8	-
6.1	+	1.7	-
6.2	-	-	-

A.

B.

Figure 3.1   Isolation of positive plaques from the first (A) and second (B) round

screens of the ripe fruit cDNA library from strawberry hybridizing with

the partial-length EGase cDNA probe, FAN R97

To facilitate subsequent sub-cloning and manipulation, the clone with an insert of 1.8-1.9 kb and lacking an internal *Eco*R I site (clone 1.2), was selected for further analysis. The cDNA insert from this clone was sub-cloned into the vector pBK-CMV and partially sequenced in both directions using M13 forward and reverse primers. Sequences were obtained from each end of the clone and analyzed using the University of Wisconsin GCG software package. Comparison of the sequence with the partial-length EGase cDNA, FAN R97, used as the probe and sequences in the nucleic acid databases confirmed that the cDNA encoded a EGase. Alignment of the 5' end of the nucleic acid sequence of this clone, designated *cel1*, with that of FAN R97 is shown in Figure 3.2. The two mismatches are likely to be due to errors in the sequence of FAN R97 which was generated in a single pass for the purpose of database homology searching only and was not verified by sequencing the opposite strand.

```
  1 GGGACGGAGCAGAGGAACGCGGTCAAGGCGTTACGGTGGGGGACAGACTA 50
    ||||||||||||||||||||||||||||||||||||||||||||||||||
366 gggacggagcagaggaacgcggtcaaggcgttacggtggggggacagacta 415

 51 CCTCCTGAAGGCCACGGCGGTTCCTGGCGTCGTCTTCGTCCAAGTCGGCG 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
416 ·cctcctgaaggccacggcggttcctggcgtcgtcttcgtccaagtcggcg 465

101 ACCCATACTCCGATCACAACTGCTGGGAGAGGCCGGAAGACATGGACACA 150
    |||||||||||||||||||||||||||||||| |||||||||||||||||
466 acccatactccgatcacaactgctgggagaagccggaagacatggacaca 515

151 CGCCGCACGGTGTACAAAATCGACCACAACAACCCGGGATCCGACGTGGC 200
    ||||||||||||||||||||||||||||||||||||||||||||||||||
516 cgccgcacggtgtacaaaatcgaccacaacaacccgggatccgacgtggc 565

201 AGGCGAAACCGCAGCCGCGCTCGCCGCCGCCTCTATCGTTTTCAGGTCAC 250
    |||||||||||||||||||||||||||||||||| |||||||||||||||
566 aggcgaaaccgcagccgcgctcgccgccgcctccatcgttttcaggtcac 615

251 GTGACCCCGCTTACTCGAGACTGCTTCTCAATCGAGCCGTTAAGGTTTTC 300
    ||||||||||||||||||||||||||||||||||||||||||||||||||
616 gtgaccccgcttactcgagactgcttctcaatcgagccgttaaggttttc 665·

301 GAGTTCGCTGATACCCACCGCGGCGCGTACAGCTCCAGCCTCAAAAACGC 350
    ||||||||||||||||||||||||||||||||||||||||||||||||||
666 gagttcgctgatacccaccgcggcgcgtacagctccagcctcaaaaacgc 715

351 CGTGTGCCCTTTTTACTGCGACGTCAACGG                     380
    ||||||||||||||||||||||||||||||
716 cgtgtgccctttttactgcgacgtcaacgg                     745
```

Figure 3.2   Alignment of the nucleic acid sequence of the 5' end of the partial-length

cDNA FAN R97 (uppercase) with that of the isolated full-length cDNA

cell (lowercase)

3.2.2 Characterization of the EGase cDNA *cell* from strawberry

The EGase cDNA *cell* was fully sequenced on both strands by designing primers to walk along the sequence from the 3' and 5' ends. The complete nucleotide sequence and deduced amino acid sequence is shown in Figure 3.3. This clone had an insert size of 1779 bp with an open reading frame from nucleotides 24 to 1511. Analysis of the cDNA identified 23 bp of 5' untranslated sequence upstream of the putative ATG initiation codon and 268 bp of 3' untranslated sequence, containing a potential polyadenylation signal (AATAAA) approximately 30 nucleotides upstream of the poly(A) tail. The open reading frame encodes a polypeptide of 496 amino acids. The polypeptide contains a putative signal sequence with a predicted cleavage site (von Heijne, 1986) at or close to the Ala residue at position 32. The mature protein with the signal peptide removed has a predicted molecular mass of 53 kDa. The mature protein is a basic protein with a calculated pI of 9.18 and contains one potential glycosylation site (Asn-X-Ser/Thr). Since this work was completed, further EGase cDNAs have been isolated from different strawberry cultivars, namely cvs Chandler (database accession numbers AJ006348, Trainotti *et al.*, 1999b; AF074923, Harpster *et al.*, 1998) and Selva (database accession number AF051346, Llop-Tous *et al.*, 1999). A comparison of the deduced amino acid sequences of these with that of the *cell* cDNA is shown in Figure 3.4. Alignment of the isolated *cell* cDNA (from cv Brighton) with the cDNAs from the other cultivars revealed a high level of similarity, but not identity, between the sequences. The deduced Cell protein showed 11 amino acid differences to the deduced amino acid sequence of the *cell* cDNA from cv Selva and 3 and 9 amino acid differences to those of two independant *cell* cDNAs from cv Chandler. Only one of these differences was common

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to all and it was a conserved amino acid substitution from Arg to Lys at position 158 (double underlined in Figure 3.4) within one of the highly conserved domains characteristic of plant EGases. It is interesting to note that amino acid differences exist between the reported sequences of the two independently isolated *cel1* cDNAs from cv Chandler. The database comparisons highlighted the presence of a second EGase gene in strawberry, *cel2*. Two cDNAs encoding *cel2* have been isolated, one from cv Chandler (database accession number AJ006349, Trainotti *et al.*, 1999b) and the other from cv Selva (database accession number AF054615, Llop-Tous *et al.*, 1999). The deduced amino acid sequences of both are considerably less homologous to the deduced Cel1 sequence, with only 48% identity at the amino acid level (Figure 3.4). This is primarily due to the presence of an unusually long C-terminus peptide in Cel2 which is absent from both strawberry Cel1 and EGases from other plants. Comparison of the deduced amino acid sequence of the *cel1* cDNA with those of other plant EGases indicates that strawberry Cel1 has highest homology with *Arabidopsis* Cel1 (Shani *et al.*, 1997), pepper Cel3 (Trainotti *et al.*, 1998b) and tomato Cel2 (Lashbrook *et al.*, 1994) showing 82%, 81% and 80% identity at the amino acid level respectively (Figure 3.5).

```
GCAAAAACGAGAGAGAGAAAAAAAAAATGGCGCGAAATGGCCTTTGCTTACCGGGAAATGCTCCCGCATTTCG  70
   k  n  e  r  e  k  k  m  a  r  n  g  l  c  l  p  g  n  a  p  a  f  r
CGCAACACTCGTCCTCTCGCTGCTCCTGCTTCTCCAGCCAATCCGCGCCGGCCACGACTACCACGACGCC 140
   a  t  l  v  l  s  l  l  l  l  q  p  i  r  a »G  H  D  Y  H  D  A
CTCCGCAAGAGCATCCTCTTCTTCGAAGGCCAGCGCTCCGGCAAGCTCCCGCCCGATCAACGCCTCAAAT 210
 L  R  K  S  I  L  F  F  E  G  Q  R  S  G  K  L  P  P  D  Q  R  L  K  W
GGCGCCGCGACTCCGCATTGCACGACGGCTCCACCGCCGGCGTAGACTTAACCGGCGGCTACTACGACGC 280
   R  R  D  S  A  L  H  D  G  S  T  A  G  V  D  L  T  G  G  Y  Y  D  A
CGGCGACAACGTGAAGTTCGGCTTTCCGATGGCGGTTCACGACCACTCTGCTGGCCGTGGAGCATTATAGAC 350
 G  D  N  V  K  F  G  F  P  M  A  F  T  T  T  L  L  A  W  S  I  I  D
TTCGGGAGGGTCATGGGGACGGAGCAGAGGAACGCGGTCAAGGCGTTACGGTGGGGGACAGACTACCTCC 420
 F  G  R  V  M  G  T  E  Q  R  N  A  V  K  A  L  R  W  G  T  D  Y  L  L
TGAAGGCCACGGCGGTTCCTGGCGTCGTCTTCGTCCAAGTCGGCGACCCATACTCCGATCACAACTGCTG 490
   K  A  T  A  V  P  G  V  V  F  V  Q  V  G  D  P  Y  S  D  H  N  C  W
GGAGAAGCCGGAAGACATGGACACACGCCGCACGGTGTACAAAATCGACCACAACAACCCGGGATCCGAC 560
   E  K  P  E  D  M  D  T  R  R  T  V  Y  K  I  D  H  N  N  P  G  S  D
GTGGCAGGCGAAACCGCAGCCGCGCTCGCCGCCGCCTCCATCGTTTTCAGGTCACGTGACCCCGCTTACT 630
 V  A  G  E  T  A  A  A  L  A  A  A  S  I  V  F  R  S  R  D  P  A  Y  S
CGAGACTGCTTCTCAATCGAGCCGTTAAGGTTTTCGAGTTCGCTGATACCCACCGCGGCGCGTACAGCTC 700
   R  L  L  N  R  A  V  K  V  F  E  F  A  D  T  H  R  G  A  Y  S  S
CAGCCTCAAAAACGCCGTGTGCCCTTTTTACTGCGACGTCAACGGCTTCCAGGATGAGTTACTGTGGGGA 770
   S  L  K  N  A  V  C  P  F  Y  C  D  V  N  G  F  Q  D  E  L  L  W  G
GCAGCGTGGTTGCACAAGGCGTCGAGAAGGCGGCAGTACAGAGAATACATAGTGAGAAACGAGGTCATTT 840
 A  A  W  L  H  K  A  S  R  R  R  Q  Y  R  E  Y  I  V  R  N  E  V  I  L
TGAGAGCTGGAGATACCATTAACGAGTTTGGTTGGGATAACAAGCATGCTGGGATTAATATTCTCATTTC 910
   R  A  G  D  T  I  N  E  F  G  W  D  N  K  H  A  G  I  N  I  L  I  S
TAAGGAAGTGCTTATGGGAAAAGCAGATTATTTCGAATCTTTCAAGCAAAATGCAGATGGATTTATATGC 980
   K  E  V  L  M  G  K  A  D  Y  F  E  S  F  K  Q  N  A  D  G  F  I  C
TCTGTTTTGCCTGGACTTGCCCATACCCAAGTCCAATATTCTCCAGGTGGTTTGATCTTCAAGCCTGGAG 1050
 S  V  L  P  G  L  A  H  T  Q  V  Q  Y  S  P  G  G  L  I  F  K  P  G  G
GGAGTAACATGCAGCATGTAACTTCGCTATCGTTCCTGCTTTTGACTTATTCCAACTATCTAAGCCACGC 1120
   S  N  M  Q  H  V  T  S  L  S  F  L  L  L  T  Y  S  N  Y  L  S  H  A
CAATAAGAACGTGCCGTGTGGCATGACCTCCGCCTCCCCGGCCTTCCTCAAACAATTGGCTAAACGCCAG 1190
 N  K  N  V  P  C  G  M  T  S  A  S  P  A  F  L  K  Q  L  A  K  R  Q
GTGGATTACATTTTGGGTGACAATCCATTAAGAATGTCTTACATGGTTGGATATGGGCCGCGTTACCCGC 1260
 V  D  Y  I  L  G  D  N  P  L  R  M  S  Y  M  V  G  Y  G  P  R  Y  P  Q
AGAGGATTCACCACCGGGGCAGCTCACTTCCATCCGTGCAGGCCCATCCGGCCCGTATCGGATGCAAAGC 1330
   R  I  H  H  R  G  S  S  L  P  S  V  Q  A  H  P  A  R  I  G  C  K  A
CGGTTCTCATTATTTTCTGAGTCCGAATCCAAACCCGAATAAATTAGTCGGGGCTGTTGTGGGCGGACCC 1400
 G  S  H  Y  F  L  S  P  N  P  N  P  N  K  L  V  G  A  V  V  G  G  P
AATAGCTCGGATGCATTTCCGGACTCGAGGCCTTACTTTCAAGAGTCTGAGCCCACGACGTACATAAATG 1470
 N  S  S  D  A  F  P  D  S  R  P  Y  F  Q  E  S  E  P  T  T  Y  I  N  A
CGCCTCTTGTGGGCCTACTTTCGTATTTTGCAGCCCATTACTAATTCTCGAAGTGTAAACAGTGATTGAG 1540
   P  L  V  G  L  L  S  Y  F  A  A  H  Y  *
AATTTGTTGTGGTGCGCCAATACTCACCCACCAATCCCCCACACTACCAATTGTTGTTACTTTTGGAAAG 1610
TTCTAAATTTAAGAAATTGTTAAGAAAGAAAATGGCCCAAGCTTAGTTATGGAATTTAGTCTCAAAAGCC 1680
CTACTGTTGTGCTTTTGAAATGTTCTAGCTGTAACATAATTTCTATCAATGAATAAAGAAAATGGGCCAA 1750
GCCTAAATGTGGAAAAAAAAAAAAAAAAAA                                       1779
```

Figure 3.3   Nucleotide sequence and deduced amino acid sequence of the *cell* cDNA. The translation initiation codon (position 24) and corresponding methionine residue are shown in bold. The putative signal sequence is shown in lowercase. The predicted cleavage site is indicated by ». A potential glycosylation site is shown in bold italics and a potential polyadenylation signal is shown in bold underlined

```
AF041405   MARNGLCLPGNAPAFRATLVLS..LLLLLQPIR.AGHDYHDALRKSILFFEGQRSGKLPPDQRLKWRRDSALHDGSTAGV   77
AF074923   MARNGLCLPGNVPAFRATLVLS..LLLLLQPIS.AGHDYHDALRKSILFFEGQRSGKLPPDQRLKWRRDSALHDGSTAGV   77
AF051346   MARNGLCLPGNAPAFRATLVLS..LLLLLQPIR.AGHDYHDALRKSILFFEGQRSGKLPHDQRLKWRGDSALHDGSTAGV   77
AJ006348   MARNGLCLPGNAPAFRATLVLS..LLLLLQPIR.AGHDYHDALRKSILFFEGQRSGKLPPDQRLKWRRDSALHDGSTAGV   77
AF054615   M..........EKFVRLVSLAPLFLLLCFPLALAGHDYGARLSKSILFFEAQRSGVLPHNQRVTWRSHSGLYDGKASGV   69
AJ006349   M..........EKFVRLVSLAPLFLLLCFPLALAGHDYGAALSKSILFFEAQRSGVLPHNQRVTWRSHSGLYDGKASGV   69
                  *        * .*. .*** * . ***** * *******.**** ** .**. ** .*.* ** ..**

AF041405   DLTGGYYDAGDNVKFGFPMAFTTTLLAWSIIDFGRVMGT..EQRNAVKALRWGTDYLLKATAVPGVVFVQVGDPYSDHNC   155
AF074923   DLTGGYYDAGDNVKFGFPMAFTTTLLAWSIIDFGRVMGT..EQRNAVKALRWGTDYLLKATAVPGVVFVQVGDPYSDHNC   155
AF051346   DLTGGYYDAGDNVKFGFPMAFTTTLLAWSIIDFGRVMGT..EQRNAVKALRWGTDYLLKATAVPGVVFVQVGDPYSDHNC   155
AJ006348   DLTGGYYDAGDNVKFGFPMAFTTTLLAWSIIDFGRVMGT..EQRNAVKALRWGTDYLLKATAVPGVVFVQVGDPYSDHNC   155
AF054615   NLVGGYYDAGDNVKFGLPMAFTVTMMSWSNIEYGKQMASSGELGHALDAVKWGTDYFIKAHPEPNVLYGEVGDGNTDHYC   155
AJ006349   NLVGGYYDAGDNVKFGLPMAFTVTMMSWSIIEYGKQMASSGELGHALDAVKWGTDYFIKAHPEPNVLYGEVGDGNTDHYC   149
             *.*************.***** *...** *..*. *.. * .*. *.,***** .** . * * .. *** .** *

AF041405   WEKPEDMDTRRTVYKIDHNNPGSDVAGETAAALAAASIVFRSRDPAYSRLLLNRAVKVFEFADTHRGAYSSSLKNAVCPF   235
AF074923   WERPEDMDTRRTVYKIDHNNPGSDVAGETAAALAAASIVFRSHDPAYSRLLLNRAVRVFEFADTHRGGYSSSLKNAVCPF   235
AF051346   WERPEDMDTRRTVYKIDHNNPGSDVAGETAAALAAASIVFRSRDPAYSRLLLNRAVKVFEFADTHRGAYSSSLKNAVCPF   235
AJ006348   WERPEDMDTRRTVYKIDHNNPGSDVAGETAAALAAASIVFRSRDPAYSRLLLNRAVKVFEFADTHRGAYSSSLKNAVCPF   235
AF054615   WQRPEDMTTDRRAYKSSPSNPGSDLAGETAAAMAAASIVFRRTNPAYSRELLQHAYLLFDFADKYRGKYDSSITVAQ.KY   235
AJ006349   WQRPEDMTTDRRAYKISPSNPGSDLAGETAAAMAAASIVFRRTNPAYSRELLQHAYQLFDFADKYRGKYDSSITVAQ.KY   228
             *..**** * * ** .*****.******* .******** .***** **. * .*.*** ** * **. *

AF041405   YCDVNGFQDELLWGAAWLHKASRRRQYREYI.VRNEVILRAGDTINEFGWDNKHAGINILISKEVLMGKAD....YFESF   310
AF074923   YCDVNGFQDELLWGAAWLHKASRRRQYREYI.VRNEVVLRAGDTINEFGWDNKHAGINILISKEVLMGKAD....YFESF   310
AF051346   YCDVNGFQDELLWGAAWLHKASRRRQYREYI.VRNEVILRAGDTINEFGWDNKHAGINILISKEVLMGKSD....YFESF   310
AJ006348   YCDVNGFQDELLWGAAWLHKASRRRQYREYI.VRNEVILRAGDTINEFGWDNKHAGINILISKEVLMGKAD....YFESF   310
AF054615   YRSISGYNDELLWAAAWLYQASNNEYYLNYLAVNGDSMGGTGWGITEFGWDVKYSGVQTLVAKFLMQGKAGKHAAVFQKY   310
AJ006349   YRSISGYNDELLWAAAWLYQASNNEYYLNYLAVNGDSMGGTGWGMTEFGWDVKYSGVQTLVAKFLMQGKAGKHAAVFQKY   308
             * ..*..***** .**** .** * .*. * .* .. ..* .***** *.*. * ** ..* * ..

AF041405   KQNADGFICSVLPGLAHTQVQYSPGGLIFKPGGSNMQHVTSLSFLLLTYSNYLSHANKNVPCGMTSASPAFLKQLAKRQV   390
AF074923   KQNADGFICSVLPGLAHTQVQYSPGGLIFKPGGSNMQHVTSLSFLLLTYSNYLSHANKNVPCGMTSASPAFLKQLAKRQV   390
AF051346   KQNADGFICSVLPGLAHTQVQYSPGGLIFKPGGSNMQHVTSLSFLLLTYSNYLSHANKNVPCGMTSASRPSSNKLAKRQV   390
AJ006348   KQNADGFICSVLPGLAHTQAQYSPGGLIFKPGGSNMQHVTSLSFLLLTYSNYLSHANKNVPCGMTSASPAFLKQLAKRQV   390
AF054615   TAKAEYFMCSCL.GKGSRNAQKTPGGLLFRQRWNNMQFVTSASFLATVYSDYLTSSRRTLKCASGNVAPSELLSFAKSQV   390
AJ006349   TAKAEYFMCSCL.GKGSRNAQKTPGGLLFRQRWNNMQFVTSASFLATVYPDYLTSSRRTLKCASGNVAPSELLSFAKSQV   387
             .*. *.** * *. * .**** .* .*** *** *** *..**.... * ** **

AF041405   DYILGDNPLRMSYMVGYGPRYPQRIHHRGSSLPSVQAHPARIGCKAG.SHYFLSPNPNPNKLVGAVVGGPNSSDAFPDSR   469
AF074923   DYILGDNPLRMSYMVGYGPRYPQRIHHRGSSLPSVQAHPARIGCKAG.SRYFMSPNPNPNKLVGAVVGGPNSSDAFPDSR   469
AF051346   DYILGDNPLRMSYMVGYGPRYPQRIHHRGSSLPSVQAHPARIGCKAG.SRYFLSPNPNPNKLVGAVVGGPNSSDAFPDSR   469
AJ006348   DYILGDNPLRMSYMVGYGPRYPQRIHHRGSSLPSVQAHPARIGCKAG.SRYFLSPNPNPNKLVGAVVGGPNSSDAFPDSR   469
AF054615   DYILGDNPRATSYMVGYGNNYPQQVHHRGSSIVSIKKDSSFVSCRGGYATWFSRKASDFNDNFADQR   469
AJ006349   DYILGDNPRATSYMVGYGNNYPQQVHHRGSSIVSIKKDSSFVSCRGGYATWFSRKASDPNLLTGAIVGGPDAYDNFADQR   467
             ******* ***********..****. .*... .. .*..*. .* .** ** ***** * *.* *

AF041405   PYFQESEPTTYINAPLVGLLSYFAAHY*.................................................   496
AF074923   PYFQESEPTTYINAPLVGLLSYFAAHY*.................................................   496
AF051346   PYFQESEPTTYINAPLVGLLSYFAAHY*.................................................   496
AJ006348   PYFQESEPTTYINAPLVGLLSYFAAHY*.................................................   496
AF054615   DNYEQTEPATYNNAPLIGILARLGGGQSSYNQLLPVVTSQPKQTPVPKLTPAAPASTSGPIAIAQKVTSSWGFQGSNLLQ   496
AJ006349   DNYEQTEPATYNNAPLIGILARLGGGQSSYNQLLPVVTSQPKQTPVPKLTPAAPASTSGPIAIAQKVTSSWVSKGVTYYR   547
             ....**.** ****.*.*. ...

AF041405   ..........................................................................
AF074923   ..........................................................................   496
AF051346   ..........................................................................   496
AJ006348   ..........................................................................   496
AF054615   ...IFHNCDQQVWEDTQ*.........................................................   496
AJ006349   YSTTVTNKSGKTLNNLKLTISKLYGPLWGLTKTGDSYVFPSWLNSLPAGKSLEFVYIHAASAANVLVSSYSLA*   561
                                                                                        620
```

Figure 3.4   Alignment of the deduced amino acid sequence of the isolated

strawberry cDNA *cel1* (database accession number AF041405) with

those of EGase cDNAs isolated from other strawberry cultivars

(database accession numbers AF074923 to AJ006349). Identical amino

acids are represented by asterisks and conservative substitutions by dots

```
AF041405    MARNGLCLPGNAPAFRATLVLSLLLLL.QPI.RAGHDYHDALRKSILFFEGQRSGKLPPDQRLKWRRDSALHDGSTAGVD    78
X98544      MARKSLIFP........VILHAVLLFS.PPIYSAGHDYRDALRKSILFFEGQRSGKLPPDQRLKWRRDSALRDGSSAGVD    71
X97189      MAPK.........H...IFLLLLLISIRHSSGGGHDYHDALRKSILFYEGQRSGKLPPDQRIKWRRDSALHDGASAGVD    67
U13055      MAPK.........YTSIIFLFLLFNSFSCSFGGGHDYHDALRKSILFYEGQRSGKLPPDQRIKWRRDSALHDGASAGVD    70
            **  .            .  .*.     .**** ********.************.******** **..****


AF041405    LTGGYYDAGDNVKFGFPMAFTTTLLAWSIIDFGRVMGTEQRNAVKALRWGTDYLLKATAVPGVVFVQVGDPYSDHNCWEK    158
X98544      LSGGYYDAGDNIKFGFPMAFTTTMLSWSIIDFGKTMGPELRNAVKAVKWGTDYLLKATAIPGVVFVQVGDAYSDHNCWER    151
X97189      LAGGYYDAGDNVKFVFPMAFTATLLSWSIIDFKRNMGSELGNAVKAVKWATDFLLKATAKEGVVYVQVGDPFSDHSCWER    147
U13055      LTGGYYDAGDNVKFVFPMAFTTTLLSWSIIDFKRNIGNELGNAVKAVKWGTDFLLKATARDGVIYVQVGDAFSDHSCWER    150
            *.*********.** ****** .*.*.****** . .* * ****..*.**.****** **..*****.***.***.


AF041405    PEDMDTRRTVYKIDHNNPGSDVAGETAAALAAASIVFRSRDPAYSRLLLNRAVKVFEFADTHRGAYSSSLKNAVCPFYCD    238
X98544      PEDMDTLRTVYKIDRAHPGSDVAGETAAALAAASIVFRKRDPAYSRLLLDRATRVFAFANRYRGAYSNSLYHADCPFYCD    231
X97189      PEDMDTLRTVYKIDQNHPGSDVAGEIAAALAAASIVFRSLDASYSNLLLDRAVKVFEFANRHRGAYSSSLHSAVCPFYCD    227
U13055      PEDMDTLRTVYKIDANNPGSDVAGEIAAALAAASIVFRSLDSSYSNLLLDRAVKVFDFANRHRGAYSSSLHSAVCPFYCD    230
            ****** ******* .******** ************  *..** ***.** .** **. *****.** * ******


AF041405    VNGFQDELLWGAAWLHKASRRRQYREYIVRNEVILRAGDTINEFGWDNKHAGINILISKEVLMGKADYFESFKQNADGFI    318
X98544      FNGYQDELLWGAAWLHKASRKRAYREFIVKNEVILKAGDTINEFGWDNKHAGINVLISKEVLMGKAEYFESFKQNADGFI    311
X97189      FNGYQDELLWGAAWLHKATRRRQYREYIVKNEVILRAGDTINEFGWDNKHAGINVLISKEVLMGRAPDLKSFQVNADAFI    307
U13055      FNGYQDELLWGAAWLHKATRRRQYREYIVKNEVILRAGDTINEFGWDNKHAGINVLISKEVLMGKAPDLKSFQVNADAFI    310
            **.************** .*.* ***.**.***** .****************.********.*   .**. ***.**


AF041405    CSVLPGLAHTQVQYSPGGLIFKPGGSNMQHVTSLSFLLLTYSNYLSHANKNVPCGMTSASPAFLKQLAKRQVDYILGDNP    398
X98544      CSILPGISHPQVQYSRGGLLVKTGGSNMQHVTSLSFLLLAYSNYLSHAKKVVPCGELTASPSLLRQIAKRQVDYILGDNP    391
X97189      CSILPGIAHPQVQYSPGGLIVKPGVCNMQHVTSLSFLFLAYSNYLSHANHVVPCGSMSATPALLKHIAKRQVDYILGDNP    387
U13055      CSILPGISHPQVQYSPGGLIVKPGVCNMQHVTSLSFLLLTYSNYLSHANHVVPCGSMTATPALLKHIAKRQVDYILGDNP    390
            **.***..* ***** ***. * *  **********.*.********.  ****  .*.*..*...****.********


AF041405    LRMSYMVGYGPRYPQRIHHRGSSLPSVQAHPARIGCKAGSHYFLSPNPNPNKLVGAVVGGPNSSDAFPDSRPYFQESEPT    478
X98544      MGLSYMVGYGQKFPRRIHHRGSSVPSVSAHPSHIGCKEGSRYFLSPNPNPNLLVGAVVGGPNVTDAFPDSRPYFQQSEPT    471
X97189      QRMSYMVGYGPHYPLRIHHRGSSLPSMAAHSARIGCKEGSRYFFSPNPNPNRLIGAVVGGPNLTDSFPDARSFFQESEPT    467
U13055      QRMSYMVGYGPHYPQRIHHRGSSVPSVATHSARIGCKEGSRYFFSPNPNPNRLIGAVVGGPNLTDSFPDARPYFQESEPT    470
             .******* .* * ********.**. .*.. **** ** **.******* *.******** .*.*** .*. .** ****


AF041405    TYINAPLVGLLSYFAAHY*...    496
X98544      TYINAPLVGLLGYFSAHSTWR*    492
X97189      TYVNAPLVGLLAYFSAHS*...    485
U13055      TYVNAPLVGLLAYFAAHSN*..    489
            **.********.**.**
```

Figure 3.5   Alignment of the deduced amino acid sequence of the isolated strawberry

cDNA *cel1* (database accession number AF041405) with those of

EGase cDNAs isolated from *Arabidopsis* (X98544), pepper (X97189)

and tomato (U13055). Identical amino acids are represented as asterisks

and conservative substitutions by dots

3.2.3 Isolation of a *cel2* cDNA fragment from strawberry

RT-PCR with degenerate primers was used to amplify the 3' end of a second EGase cDNA, *cel2*, from strawberry (cv Calypso). A product of the expected size of 262 bp was obtained and sequenced. The deduced amino acid sequence of the C-terminus was compared with those of the two full-length *cel2* cDNAs that have been isolated from strawberry cv Selva (database accession number AF054615, Llop-Tous *et al.*, 1999) and cv Chandler (database accession number AJ006349, Trainotti *et al.*, 1999b). The translated sequence of the *cel2* cDNA fragment from cv Calypso was identical to that of the corresponding region of the *cel2* cDNA from cv Chandler except for 2 amino acid differences at positions 552 and 553 (Figure 3.6A). Thus sequence differences between cultivars of octoploid strawberry are a feature of *cel1* and *cel2*. However, the comparison between the translated *cel2* cDNA fragment and that of the corresponding region of the *cel2* cDNA from cv Selva revealed 15 mostly consecutive amino acid differences (Figure 3.6B). This is considerably higher variation than that seen between any of the *cel1* homologues from different cultivars. In addition, if the translated sequences of the two full-length *cel2* cDNAs are compared they show no homology from position 539 onwards and only the sequence from cv Chandler indicates the presence of a potential glycosylation site (Figure 3.7). The reason for this becomes clear from a comparison of the nucleotide sequences of the two cDNAs (Figure 3.8). The sequence of the *cel2* cDNA from cv Selva contains an additional base (G) at nucleotide position 1733 which is absent in the sequence from cv Chandler. This base is also absent from the *cel2* cDNA fragment from cv Calypso which accounts for its high homology with the cDNA from cv Chandler and not with that from cv Selva.

```
A.
   1 DNYEQTEPATYNNAPLIGILARLGGGQSSYNQLLPVVTSQPKQTPVPKLT 50
     |||||||||||||||||||||||||||||||||||||||||||||||||
 468 dnyeqtepatynnapligilarlgggqssynqllpvvtsqpkqtpvpklt 517

  51 PAAPASTSGPIAIAQKVTSSWVSKGVTYYRYSTTAIN                87
     |||||||||||||||||||||||||||||||||||  |
 518 paapastsqpiaiaqkvtsswvskgvtyyrysttvtn               554

B.
   1 DNYEQTEPATYNNAPLIGILARLGGGQSSYNQLLPVVTSQPKQTPVPKLT 50
     |||||||||||||||||||||||||||||||||||||||||||||||||
 468 dnyeqtepatynnapligilarlgggqssynqllpvvtsqpkqtpvpklt 517

  51 PAAPASTSGPIAIAQKVTSSWVSKGVTYYRYSTTAIN                87
     ||||||||||||||||||||||   .|
 518 paapastsgpiaiaqkvtsswgfqgsnllqifhncdq               554
```

Figure 3.6   Alignment of the deduced amino acid sequence of the *cel2* cDNA

fragment from strawberry cv Calypso (uppercase) with that of the

corresponding region of the *cel2* cDNAs from cv Chandler (A) and cv

Selva (B) (lowercase)

```
 468 DNYEQTEPATYNNAPLIGILARLGGGQSSYNQLLPVVTSQPKQTPVPKLT 517
     |||||||||.|||||||||||||||||||||||||||||||||||||||
 468 dnyeqtepatynnapligilarlgggqssynqllpvvtsqpkqtpvpklt 517

 518 PAAPASTSGPIAIAQKVTSSWVSKGVTYYRYSTTVTNKSGKTLNNLKLTI 567
     |||||||||||||||||||||   .|    .      |   .   . .
 518 paapastsgpiaiaqkvtsswgfqgsnllq...ifhncdqqvwedtq*.. 561

 568 SKLYGPLWGLTKTGDSYVFPSWLNSLPAGKSLEFVYIHAASAANVLVSSY 617
 618 SLA*                                               620
```

Figure 3.7   Alignment of the C-terminus of the deduced amino acid sequence of the

two full-length *cel2* cDNAs from cv Chandler (uppercase) and cv Selva

(lowercase). The potential glycosylation site in the sequence from cv

Chandler is shown in bold

```
1524 GACAACTATGAACAGACTGAACCTGCTACCTACAACAATGCTCCTCTTAT 1573
     |||||||||||| |||||||||||||||||||||||||||||||||||||
1522 gacaactatgagcagactgaacctgctacctacaacaatgctcctcttat 1571

1574 CGGTATATTGGCTCGTCTGGGAGGTGGTCAGAGCAGCTATAACCAGCTTC 1623
     |||||||||||||||||| ||||||||||||||||||||||||||||||||
1572 tggtatattggctcgtctaggaggtggtcagagcagctataaccagcttc 1621

1624 TTCCAGTTGTTACATCCCAGCCAAAACAAACCCCAGTACCTAAGCTTACT 1673
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1622 ttccagttgttacatcccagccaaaacaaaccccagtacctaagcttact 1671

1674 CCAGCTGCCCCAGCTTCAACTTCTGGCCCAATTGCAATAGCACAGAAGGT 1723
     |||||||||||||||||||||||||||||||||||||||||||||||||||
1672 ccagctgccccagcttcaacttctggcccaattgcaatagcacagaaggt 1721

1724 GACATCTTCAT.GGGTTTCCAAGGGAGTAACTTACTACAGATATTCCACA 1772
     |||||||||||| ||||||||||||||||||||||||||||||||||||||
1722 gacatcttcatggggtttccaagggagtaacttactacagatattccaca 1771

1773 ACTGTGACCAACA                                       1785
     |||||||||||||
1772 actgtgaccaaca                                       1784
```

Figure 3.8   Alignment of the nucleotide sequences of the two full-length *cel2* cDNAs

from cv Chandler (uppercase) and cv Selva (lowercase) over the region

corresponding to the *cel2* cDNA fragment from cv Calypso. The

additional base (**g**) present in the sequence from cv Selva is shown in

bold

145

3.2.4 Expression analysis of *cel1* and *cel2*

Northern analysis of total RNA was used to follow *cel1* and *cel2* expression throughout fruit development. Duplicate northern blots were hybridized with *cel1* or *cel2* cDNA probes and exposed to X-ray film for the same length of time. Fruit were studied at the following stages as defined by their receptacle colour and size: small green, white with green achenes, white with yellow achenes, turning, orange, red ripe and over-ripe (Figure 3.9). Differences in the temporal patterns of transcript accumulation in fruit were observed for the two EGase genes and is taken as evidence that the probes did not cross-hybridize. There was no detectable *cel1* expression in unripe fruit. *Cel1* transcripts were detected when fruit showed the first development of red colour at the turning stage. Thereafter *Cel1* mRNA accumulated significantly during ripening to reach a maximum in red ripe fruit. The level of message then declined slightly in over-ripe fruit (Figure 3.10). The size of the transcript was estimated at 1.9 kb. In contrast, *Cel2* transcripts were first detected in small green fruit at the earliest stage of fruit development. Levels of *Cel2* mRNA then increased as fruit progressed from green to white. Expression increased significantly as ripening proceeded and, as observed for *cel1*, reached a maximum in red ripe fruit (Figure 3.11). The size of the *Cel2* transcript was estimated at 2.6 kb. Expression of the two EGase genes was also studied in other tissues of strawberry. *Cel1* transcripts were not detected in fully expanded leaf, petiole or root tissue indicating that *cel1* expression is specific to ripening fruit (Figure 3.10). However *cel2* expression was not restricted to fruits, although transcript levels in other tissues were low in comparison with fruit. *Cel2* mRNA was detected in petiole tissue, to a lesser extent in root tissue and was barely detectable in leaf tissue (Figure 3.11).
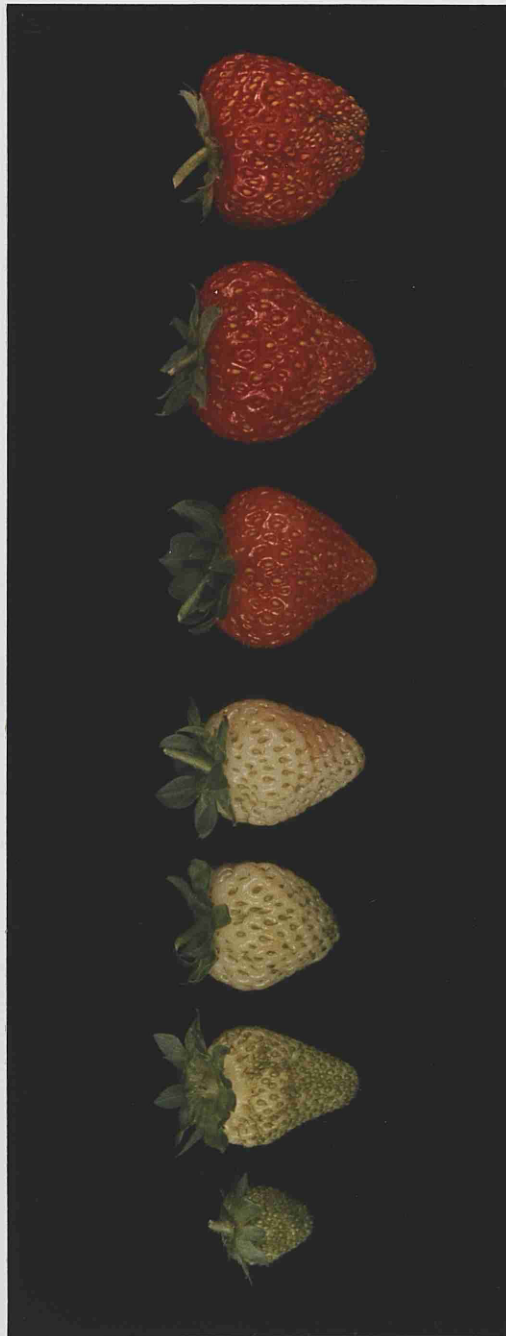
Figure 3.9   Representative examples of strawberry fruit sampled at various stages throughout fruit development. (Left to right: SG, small green; W+G, white with green achenes; W+Y, white with yellow achenes; T, turning; O, orange; R, red ripe; OR, over-ripe)

A.

B.
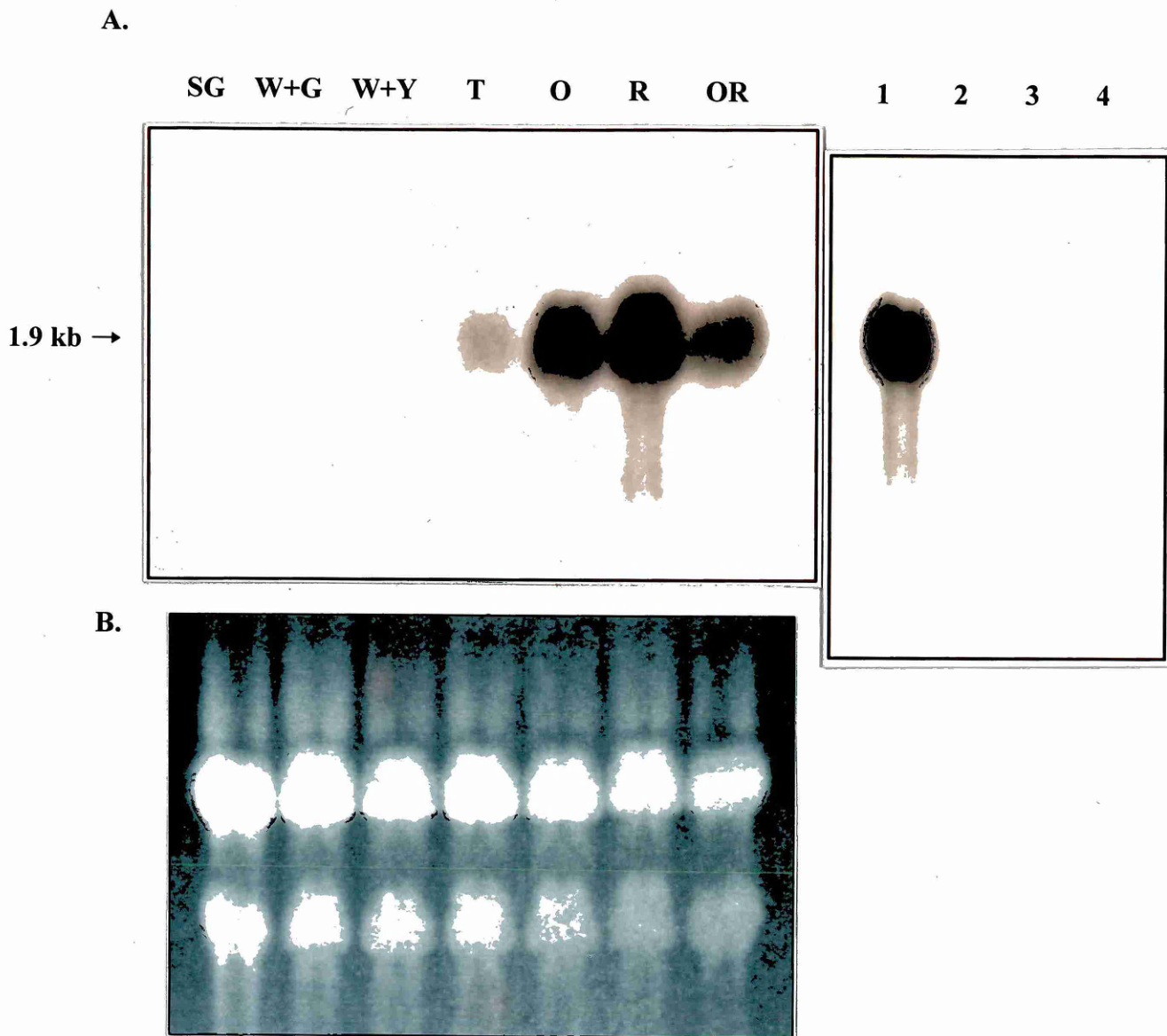
Figure 3.10   Northern analysis of *cel1* expression in developing fruit (SG, small

green; W+G, white with green achenes; W+Y, white with yellow

achenes; T, turning; O, orange; R, red ripe; OR, over-ripe) and other

tissues (1, red ripe fruit; 2, leaf; 3, petiole; 4, root) of strawberry cv

Calypso plants (A). The gel was stained with ethidium bromide and

photographed under UV light to verify equal loading of RNA samples

(B)

2.6 kb $\longrightarrow$
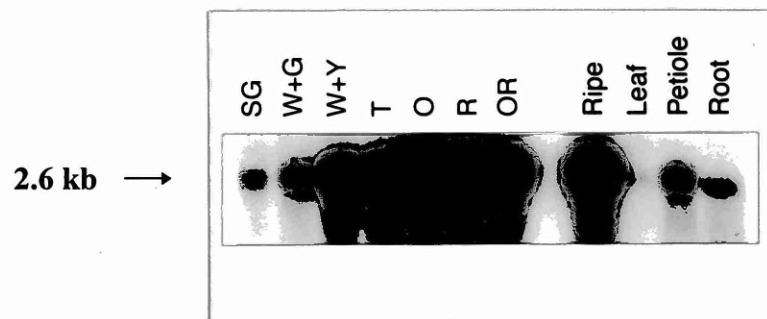
Figure 3.11   Northern analysis of *cel2* expression in developing fruit (SG, small

green; W+G, white with green achenes; W+Y, white with yellow

achenes; T, turning; O, orange; R, red ripe; OR, over-ripe) and other

tissues of strawberry cv Calypso plants

149

3.2.5   Southern analysis of *cel1* and *cel2*

Southern blot analysis of genomic DNA was carried out at high stringency using both the *cel1* cDNA and *cel2* cDNA fragments as probes hybridized to duplicate Southern blots to identify any related sequences present in the strawberry genome. Genomic DNA was digested to completion with the restriction enzymes *Bcl* I, *Sal* I, *Spe* I and *Xba* I, none of which cut within the *cel1* cDNA or the published full-length sequence for the *cel2* cDNA. Genomic DNA from the diploid *Fragaria vesca* was analyzed alongside that from *F. x ananassa* Duch. cv Calypso. The octoploid nature of the *F. x ananassa* genome produces complex hybridization patterns (Medina-Escobar *et al.*, 1997b) which can confuse interpretations of Southern analysis. The inclusion of the diploid strawberry genome in the analysis allows a more accurate assessment to be made of the copy number of EGase genes in strawberry. Comparison of the Southern blots showed that each probe hybridized to a distinct set of fragments indicating that the probes for *cel1* and *cel2* did not cross-hybridize. The patterns of bands observed in the *F. vesca* blots were a subset of those seen in the *F. x ananassa* blots. Both probes showed strong hybridization to single bands in each diploid digest which suggests the presence of a single gene per diploid genome for each of *cel1* and *cel2*. Other fragments hybridized more weakly indicating the presence of related sequences that may represent a small EGase multigene family in strawberry (Figure 3.12).
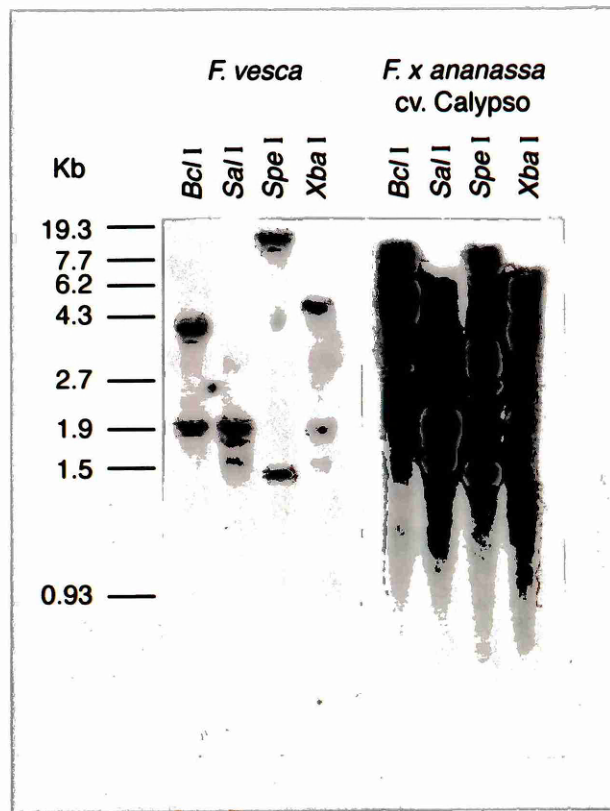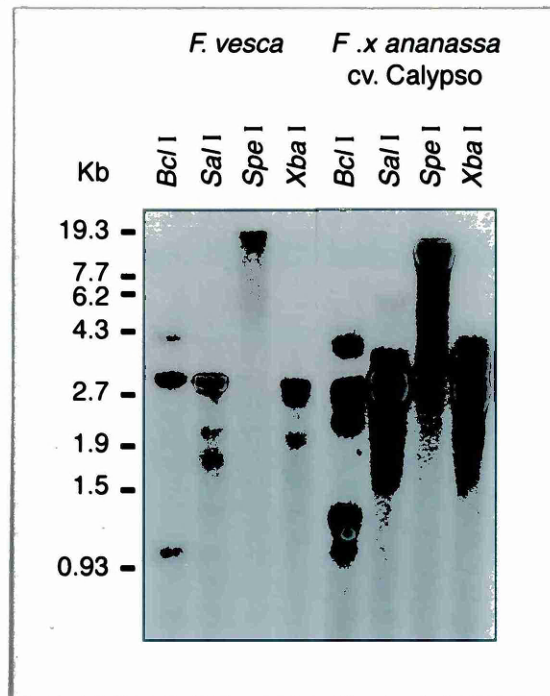
Figure 3.12 Southern analysis of *cel1* (A) and *cel2* (B). Genomic DNA from strawberry *F. vesca* and *F. x ananassa* Duch. cv Calypso was digested with the restriction enzymes shown and hybridized to the *cel1* cDNA or *cel2* cDNA fragment

3.3   SUMMARY

A full-length cDNA clone, *cel1*, encoding a ripening-related EGase from strawberry, was isolated from a cDNA library prepared from ripe fruit and probed with the partial-length cDNA FAN R97. The *cel1* clone had an open reading frame encoding a polypeptide of 496 amino acids. The predicted molecular mass of the mature protein after cleavage of the putative signal sequence was 53 kDa and the predicted pI was 9.18.

Comparison of the deduced amino acid sequence of the *cel1* clone with homologues recently isolated from different strawberry cultivars revealed a high degree of homology, but not identity, between the sequences. The comparison also showed a much lower level of homology with a second strawberry EGase, Cel2. When compared with EGases from other plants, the deduced strawberry Cel1 sequence was most closely related to *Arabidopsis* Cel1, tomato Cel2 and pepper Cel3. RT-PCR was used to amplify the 3' end of a second EGase cDNA, *cel2*, from strawberry. The deduced amino acid sequence of the *cel2* cDNA fragment showed a high degree of homology with the corresponding region of a full-length *cel2* cDNA isolated from strawberry cv Chandler, but not with that from cv Selva.

Northern analysis revealed that expression of strawberry *cel1* is fruit-specific and ripening-enhanced, with maximum expression in ripe fruit. Strawberry *cel2*, however, is not fruit-specific as it was also expressed in petiole, root and leaf tissue although at much lower levels than in fruit. *Cel2* expression was also less tightly linked to ripening as, unlike Cel1, Cel2 transcripts were present in unripe fruit.

Southern analysis of *cel1* and *cel2* revealed the presence of related sequences in the strawberry genome, indicating a small multigene family. This is consistent with the isolation of two different EGase cDNAs from strawberry.

3.4   DISCUSSION

CHARACTERIZATION OF THE ENDO-β-1,4-GLUCANASE cDNA *CEL1* FROM STRAWBERRY

The strawberry *cel1* cDNA encodes a polypeptide with a hydrophobic signal sequence at its N-terminus and a single consensus site (Asn-X-Ser/Thr) for potential N-glycosylation near the C-terminus. The majority of plant EGases identified to date, and indeed many cell wall modifying proteins, possess typical eukaryotic signal sequences which are characteristic of secreted proteins and target them to the endomembrane system for processing and secretion to the cell surface. This allows EGases to be directed to their proposed site of action, the cell wall. The cDNA sequences of many plant EGases also predict the presence of sites for potential N-glycosylation in the mature proteins. Avocado EGase has been shown to be a glycoprotein that is synthesized with a signal peptide at the N-terminus. The signal peptide is removed prior to glycosylation of the protein to a membrane-associated secretory form which undergoes further processing during transport to the cell wall (Bennett and Christoffersen, 1986). Thus, avocado EGase appears to be produced via the typical eukaryotic pathway for secretory glycoproteins (Christoffersen, 1987). The presence of two oligosaccharide side-chains in the mature glycoprotein indicated by partial endoglycosidase H digestion was found to

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be in agreement with the number of potential glycosylation sites predicted from the corresponding cDNA sequence (Tucker *et al.*, 1987). The pepper ripening-related EGase cDNA, *Cel1*, contains three consensus glycosylation sites (Harpster *et al.*, 1997; Trainotti *et al.*, 1998b) and the corresponding purified protein was shown to be glycosylated (Ferrarese *et al.*, 1995). The other two identified pepper EGases, Cel3 and Cel2, contain one and no potential glycosylation sites respectively (Trainotti *et al.*, 1998b). EGase cDNAs isolated from peach (Trainotti *et al.*, 1997), poplar (Nakamura *et al.*, 1995) and elder (Taylor *et al.*, 1994), in addition to tomato *Cel1* and *Cel2* (Lashbrook *et al.*, 1994) and *Arabidopsis cel1* (Shani *et al.*, 1997) cDNAs, also predict glycosylation sites in the proteins. In contrast, the cDNAs of a bean abscission EGase (Tucker and Milligan, 1991), pea EGase (Wu *et al.*, 1996) and the tomato cDNAs *Cel4* (Milligan and Gasser, 1995) and *Cel7* (Catala *et al.*, 1997) indicated the presence of a signal peptide, but not of glycosylation sites. One plant EGase is highly divergent from other plant EGases and does not follow the general structure exhibited by them. The tomato EGase cDNA *Cel3* encodes a polypeptide that lacks the typical cleavable signal peptide, yet possesses seven potential N-glycosylation sites. It has a structure more characteristic of an integral membrane protein which accounts for its localization on Golgi and plasma membranes instead of the cell wall location suggested for plant EGases (Brummell *et al.*, 1997a).

Thus the strawberry *cel1* gene encodes a polypeptide that shares characteristics with the majority of plant EGases. EGases have been grouped into six major families (A to F) identified by homology of their catalytic cores based on hydrophobic cluster analysis (Henrissat *et al.*, 1989; Beguin, 1990). All identified plant EGases belong to the E family and more specifically the E_2 subgroup, which also contains bacterial EGases

but no fungal representatives. Sequence analysis of the catalytic cores of the members of this subgroup reveal several amino acid domains that are conserved. The two most highly conserved motifs are represented by GGYYDAGDN and DELLWGAA in strawberry Cel1. It is likely that these domains contain amino acid residues that are required for catalytic activity, substrate binding or the tertiary structure of the protein (Gilkes *et al.*, 1991; Brummell *et al.*, 1994).

Comparison of the deduced amino acid sequence of the *cel1* cDNA with those of other plant EGases indicates that strawberry Cel1 has highest homology with *Arabidopsis* Cel1 (Shani *et al.*, 1997), pepper Cel3 (Trainotti *et al.*, 1998b) and tomato Cel2 (Lashbrook *et al.*, 1994). Strawberry Cel1 also shares 60-63% amino acid identity with tomato Cel4 (Milligan and Gasser, 1995) and Cel5 (Kalaitzis *et al.*, 1999), pine Cel1 and Cel2 (Loopstra *et al.*, 1998), pepper Cel2 (Trainotti *et al.*, 1998a) and poplar Cel1 (Nakamura *et al.*, 1995). It may be expected that EGases that share high levels of sequence homology also share similar expression patterns and physiological functions. Based on phylogenetic analysis of deduced amino acid sequences, it has been suggested that plant EGases fall into two main groups (Brummell *et al.*, 1994). The first contains members which are found to be expressed in abscission zones and are hence involved in abscission processes, such as the bean abscission EGase. The second group includes members expressed predominantly in ripening fruit or vegetative tissues which are associated with fruit ripening and cell expansion such as avocado Cel1 and pea EGL1 respectively (Wu *et al.*, 1996; Brummell *et al.*, 1997a,b; Catala *et al.*, 1997). Indeed, tomato Cel1 is involved in cell separation events and shows greatest sequence homology to the bean abscission zone EGase (68% amino acid identity). Tomato Cel2, however, is most abundant in ripening fruit and is most similar (57% amino acid identity) to

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avocado fruit EGase (Lashbrook *et al.*, 1994). Strawberry Cel1 is also ripening-related and exhibits 80% amino acid identity with tomato Cel2. However, strawberry Cel1 shows similarly high amino acid identities of 82% and 81% with the elongation-specific *Arabidopsis* Cel1 and the abscission-related pepper Cel3 respectively. In addition, strawberry Cel1 is considerably less similar to the ripening-related EGases avocado Cel1 (Tucker *et al.*, 1987) and pepper Cel1 (Harpster *et al.*, 1997), showing 60% and 54% amino acid identity, respectively. This clearly indicates that despite the general phylogenetic grouping described above, similarity between amino acid sequences does not neccessarily correlate with similarity in expression pattern and protein function. An imperfect correlation between sequence similarity and expression pattern was also revealed from a phylogenetic comparison of tomato Cel5 with other plant EGases. At best, the resulting dendogram could only provide clues as to the exprèssion pattern of related genes (Kalaitzis *et al.*, 1999). Similarly, comparison of pepper Cel1 with other plant EGases indicated that it is not possible to reliably group EGases of specific expression patterns or physiological function on the basis of their amino acid sequence relatedness. Pepper Cel1 exhibits highest sequence homology to tomato Cel1, yet its expression profile is different and is instead more like that of the predominantly fruit expressed tomato Cel2. Likewise, pepper Cel1 is ripening-related but is more homologous to the bean abscission EGase than to the ripening-related avocado EGase (Harpster *et al.*, 1997). The opposite situation is seen for pepper Cel2 which is expressed in abscission zones and has a higher similarity to the avocado ripening EGase than the bean abscission EGase (Trainotti *et al.*, 1998a). This is also the case for the peach abscission EGase, ppEG1, and again questions the usefulness of grouping EGases with similar sequences on the basis of physiological function (Trainotti *et al.*, 1997).

Since this work was completed, further EGase cDNAs have been isolated from different strawberry cultivars. Alignment of the strawberry *cel1* cDNA isolated here (from cv Brighton) with the cDNAs from the different cultivars has revealed a high degree of similarity, but not identity, between the sequences. The deduced Cel1 protein exhibited 98% amino acid identity with the deduced amino acid sequence of the *cel1* cDNA from cv Selva (Llop-Tous *et al.*, 1999) in addition to 99% and 98% amino acid identity with those of two independent *cel1* cDNAs from cv Chandler (Trainotti *et al.*, 1999b and Harpster *et al.*, 1998 respectively). The subtle differences revealed included only one that was common between all cultivars. The deduced Cel1 protein from cv Brighton has a conserved amino acid substitution from Arg to Lys at position 158 within one of the highly conserved domains characteristic of all plant EGases. The high level of amino acid identity shared between the individual sequences suggests that the cDNAs represent homologues of the *cel1* gene in the different cultivars and that the minor sequence differences may be because of cultivar variability. Different cultivars have widely different parentage as they originate from different continents, so some variation is not surprising. It is not known if any of the cultivar-specific sequence variations affect the functional properties of the enzymes. It is possible that the differences may subtly affect the catalytic activity, substrate-specificity or tertiary structure of the enzymes. This could account for the variations observed in the texture characteristics of the fruit from different cultivars. Cultivar-specific variations in sequence were also observed for tomato Cel5. A cDNA (TAC1) was isolated from tomato cv Rutgers (Kalaitzis *et al.*, 1999) and the deduced protein was found to have 99% amino acid identity with that of the *cel5* cDNA independently isolated from tomato cv Castlemart (del Campillo and Bennett, 1996). It was suggested that *cel5* and TAC1 were likely to be allelic, encoding

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the same gene in the two different cultivars (Kalaitzis *et al.*, 1999). Similarly, minor sequence variation observed between two cDNAs encoding pepper Cel1 that were independently isolated from different cultivars was attributed to cultivar variability (Trainotti *et al.*, 1998b).

Comparison of the *cel1* homologues from different cultivars has also revealed sequence variation within a cultivar. The two independently isolated *cel1* cDNAs from cv Chandler (Harpster *et al.*, 1998 and Trainotti *et al.*, 1999b) showed 98% identity at the amino acid level. An explanation for this may lie in the fact that strawberry (*Fragaria x ananassa* Duch.) is a polyploid species and more specifically octoploid. This polyploidization resulted from the merging of fully differentiated genomes, known as allopolyploidy (Wendel, 2000), to give the genomic constitution AAA'A'BBBB, where the AA genomes come from *Fragaria vesca* and *Fragaria viridis* (Senanayake and Bringshurst, 1967). Thus, strawberry contains multiple copies, or homoeologues, of all its genes. As the homoeologues are originally derived from different species, slight variations in sequence at the same locus may be expected. Hence, the different *cel1* cDNAs isolated from cv Chandler most likely represent different *cel1* homoeologues, that is the *cel1* locus from the different genomes present within octoploid strawberry. It is possible that sequence variation due to polyploidy also accounts for the cultivar variations observed in that different individual homoeologues were isolated from each cultivar. However, it is still probable that the generation of different cultivars has introduced further sequence variations. Genes present in multiple copies due to polyploidy may retain their original or similar function, undergo diversification in protein function or regulation, or become silenced (Wendel, 2000). What happens to

each of the *cel1* homoeologues may vary between cultivars and may result in subtle physiological differences between cultivars.

The database comparison of strawberry Cel1 highlighted the presence of a second, distinct EGase gene in strawberry, *cel2*. Two cDNAs encoding the divergent Cel2 have been independently isolated (Trainotti *et al.*, 1999b; Llop-Tous *et al.*, 1999). The deduced Cel1 sequence shares only 48% amino acid identity with that of Cel2. This is mainly due to the presence of an unusually long C-terminus peptide in Cel2 which is absent from Cel1 and EGases from other plants. The C-terminal extension of about 130 amino acids has not been found in any other plant EGase and has some similarity to microbial EGases. In fact it contains a sequence which has the characteristics of a putative microbial cellulose binding domain (Trainotti *et al.*, 1999b). Cellulose binding domains (CBDs) are non-catalytic elements coupled to the catalytic core of microbial cellulolytic enzymes by a linker sequence. Some CBDs have been shown to be capable of binding the cellulose substrate (Beguin and Aubert, 1994). This feature, along with the ability of individual cellulolytic enzymes to assemble into a structure known as a cellulosome and act in a synergistic manner, is believed to account for the ability of microbes to efficiently degrade crystalline cellulose (Bayer *et al.*, 1998). Endogenous EGases of two plant-parasitic cyst nematodes have also been found to contain bacterial-like CBDs and it was suggested that these allow the enzymes to partially degrade the cell wall to allow the nematode entry into the plant (Smant *et al.*, 1998). Thus the potential presence of a CBD in a plant EGase raises questions about the presence of novel biochemical properties of the enzyme and may indicate a mode of action or substrate specificity not yet observed for plant EGases.

The characterization of a second EGase gene in strawberry confirms the results of Southern analysis which indicated the presence of an EGase multigene family in strawberry. Multigene families for EGase have been observed in many plants, as they have for many other cell wall modifying proteins. The presence of multigene families whose members have different or overlapping patterns of temporal and spatial expression suggests that they each have a particular defined role and can work in a synergistic and coordinated manner to effect the various physiological processes that require modification of the cell wall. Strawberry fruit exhibit overlapping temporal expression of the two genes *cel1* and *cel2*. The expression of strawberry *cel1* is fruit-specific and ripening-enhanced, with maximum expression in ripe fruit. Strawberry *cel2* expression also reaches a maximum in ripe fruit. However, it appears to be less tightly linked to the ripening process than *cel1* since it is initially expressed in the fruit well before the colour and texture changes associated with ripening. The increase in EGase activity throughout fruit development (Barnes and Patchett, 1976; Abeles and Takeda, 1990; Chapter 3) parallels increases in *Cel1* and *Cel2* transcripts. Taken together, the expression of *cel1* and *cel2* could account for the observed pattern of EGase activity in the fruit. The coincidence of maximum expression of *cel1* and *cel2* just prior to the time of greatest EGase activity and ripening-associated loss of firmness strongly suggests that these genes have roles in fruit softening and may act cooperatively to alter texture. The additional presence of *Cel2* mRNA in unripe fruit suggests that Cel2 may also be involved in the early modification of the cell wall prior to the ripening-associated textural changes. Ultrastructural studies in strawberry have shown a progressive

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disorganization of cellulose microfibrils first apparent in white fruit (Trainotti *et al.*, 1999a) and some hemicellulose degradation is already evident in fruit before the white stage (Huber, 1984). It is possible that *cel2* expression could account for these changes.

The expression of *cel2* is not fruit-specific and *Cel2* transcripts are also present in petiole, root and leaf tissue. This more widespread tissue-specificity of *cel2* compared to *cel1* indicates it participates in other processes in addition to fruit softening. The expression of *cel2* in young developing fruit and vegetative tissues suggests that Cel2 may also facilitate cell growth and expansion.

The situation in strawberry of multiple EGase genes differentially expressed and with different roles is paralleled in many other plants. Most work has been carried out on tomato where to date, seven different EGase genes have been identified, each with its own specific pattern of expression and hence involvement in different processes. The overlapping accumulation of different transcripts in the same tissue again suggests that multiple activities are required for the cooperative disassembly of the cell wall. Tomato *cel1* and *cel2* are comparable to strawberry *cel1* and *cel2* in that they are both expressed in ripening fruit (Lashbrook *et al.*, 1994). In fruit, tomato *Cel2* transcripts only accumulate in the latest stages of ripening in a manner similar to that of strawberry *Cel1* transcripts, whereas both tomato *cel1* and strawberry *cel2* are expressed in young developing fruit too. However, tomato *cel2* differs from strawberry *cel1* in that it is not truly fruit-specific and is also expressed in other tissues, including abscission zones, at lower levels. Strawberry *cel2* may be important in similar processes to tomato *cel4* (Milligan and Gasser, 1995; Brummell *et al.*, 1997b), *cel7* (Catala *et al.*, 1997)and *cel3* (Brummell *et al.*, 1997a) which are believed to be involved predominantly in cell expansion as they are expressed in tissues undergoing rapid expansion such as young

flower pistils, etiolated hypocotyls and young fruit (*cel4*) and elongating hypocotyls (*cel3* and *cel7*). Transcripts of tomato *cel5* and *cel6* were found to accumulate in abscission zones (del Campillo and Bennett, 1996) along with those of the tomato *cel1* gene (Lashbrook *et al.*, 1994) which therefore appears to have a role in both fruit ripening and abscission. In pepper, *cel1* is responsible for the EGase activity found in ripe fruit (Harpster *et al.*, 1997). However, it is also expressed in abscission zones although to a much lesser extent than either *cel2* or *cel3* (Ferrarese *et al.*, 1995). Interestingly, *Cel3* transcripts were also detected in developing fruit and may play a role in cell expansion similar to that proposed for strawberry *cel2* (Trainotti *et al.*, 1999b). The *ppEG1* gene of peach is predominantly expressed in abscission zones but is also present at a much reduced level in a very late stage of fruit ripening suggesting it may also be involved in fruit softening (Trainotti *et al.*, 1997). Similarly, avocado *cel1* is involved in both the ripening and abscission of avocado fruit (Tonutti *et al.*, 1995). The unique expression patterns of four EGase genes of sweet pea are observed to overlap in anthers throughout their development. Three of these genes have a second role as they are also expressed in stigma and style tissue, along with a further member that is not expressed in anthers (Neelam and Sexton, 1995). Of course, there are examples of plant EGase genes that, like strawberry *cel1*, so far appear to be involved in only one cell separation process in the plant. These include the bean abscission EGase gene (Tucker *et al.*, 1988), the pea *EGL1* gene involved in cell elongation (Wu *et al.*, 1996) and the *Arabidopsis cel1* gene which is also elongation-specific (Shani *et al.*, 1997).

Multigene families whose members are coordinately expressed to carry out a range of physiological processes are not limited to EGases. For example, six expansin genes have been characterized in tomato and each one has its own specific pattern of

expression during fruit growth and ripening. Expression of *Exp1* is fruit-specific and ripening-enhanced (Rose *et al.*, 1997) much like the strawberry *cel1* gene. *Exp3* is also fruit-specific but its transcripts accumulated to a higher level during earlier fruit growth and devopment than during ripening. Transcripts of *Exp4* and *Exp5* were present only during the earliest stages of fruit growth and development but were also detected in vegetative tissues. Finally, *Exp6* and *Exp7* mRNA was much less abundant than that of the other members and again was only found in young fruit. Hence more expansins are involved in green fruit development than in ripening (Brummell *et al.*, 1999c). Similarly, there are multiple expansin genes expressed in strawberry again each with their own expression profile (Harrison, McQueen-Mason and Manning, personal communication). Thus, many cell wall modifying proteins arise from multigene families and in this way a complex range of cell wall modifying proteins is produced such that specific requirements can be met for the various processes occuring in particular tissues. The complex nature of the cell wall and the subtle variations that may exist in its composition even within different tissues of the same plant (Carpita and Gibeaut, 1993; Brett and Waldron, 1996) necessitates the ability to fine-tune the mechanism for cell wall modification. This is provided for by the sets of genes, encoding different isoforms of different cell wall modifying proteins, that are expressed in both a temporally and spatially-specific manner.

CHAPTER 4.   PURIFICATION AND CHARACTERIZATION OF A

STRAWBERRY ENDO-β-1,4-GLUCANASE

4.1   INTRODUCTION

The changes in texture and firmness that accompany fruit ripening are considered to be the result of alterations in the composition and structure of the cell wall. The modification of cell wall polysaccharides is brought about by the coordinated activities of a range of cell wall hydrolases and wall-modifying proteins. The relative activities of cell wall hydrolytic enzymes in fruits and hence the changes that they effect in the cell wall differ with species or cultivar and may account for the differences in softening behaviour observed between different fruits.

In strawberry fruit, polyuronide solubility increases during ripening and occurs without enzymic depolymerization, consistent with the generally observed lack of endopolygalacturonase activity in the fruit (Neal, 1965; Barnes and Patchett, 1976; Huber, 1984; Abeles and Takeda, 1990). However, the hemicellulose component in strawberry is depolymerized during ripening and this is temporally correlated with softening (Huber, 1984). In contrast, the cellulose content of strawberry cell walls does not vary significantly during ripening although cellulase activity has been shown to increase throughout ripening and is temporally correlated with a loss in firmness of the fruit (Barnes and Patchett, 1976; Abeles and Takeda, 1990). The activity suggested by the term "cellulase" does not accord with the invariant cellulose content. The term cellulase more commonly refers to microbial enzymes that are able to hydrolyze cellulose. The β-1,4-glucan links found in cellulose also occur in other plant polymers

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including xyloglucans. The name endo-β-1,4-glucanase (EGase) is now often used in place of cellulase and refers to the type of bond cleaved by the enzyme rather than to the substrate. Indeed the EGase in crude extracts from strawberry fruit is unable to degrade insoluble cellulose and hence an involvement in the hemicellulose degradation has been suggested (Barnes and Patchett, 1976).

Taken together these findings indicate that EGase may play an important role in the softening of strawberry fruit. In order to determine more precisely what this role might be and to establish potential substrates within the cell wall, it was necessary to purify a ripening-related EGase. Endoglucanases have been purified from a range of plant species and tissues including the fruit of avocado (Awad and Lewis, 1980), pepper (Ferrarese *et al.*, 1995) and apple (Abeles and Biles, 1991), as well as bean leaf (Koehler *et al.*, 1981; Durbin and Lewis, 1988), sweet pea anthers (Sexton *et al.*, 1990), tobacco callus (Truelsen and Wyndaele, 1991), and elder leaf (Webb *et al.*, 1993). All these enzymes were isolated by affinity chromatography on cellulose columns and elution with a buffer containing cellobiose. The majority of these EGases have a basic isoelectric point and Durbin and Lewis (1988) noted that this method was more effective for purifying the basic rather than the acidic forms of bean EGase. The calculated pI of the polypeptide encoded by the *cel1* cDNA isolated here is also basic. Consequently, cellulose affinity chromatography was investigated for the purification of the corresponding ripening-related EGase, Cel1, from strawberry.

4.2   RESULTS

4.2.1   Optimization of EGase assay

4.2.1.1   Selection of optimum extraction method

Different methods for extracting protein from strawberry fruit were compared in terms of their ability to extract EGase activity. An standard buffer consisting of Buffer B (Appendix A2) with and without the addition of NaCl was compared with the Buffer A method described in section 3.2.3 and the acetone powder method described in section *3.2.9.1*. Powdered, frozen fruit tissue was extracted in 2 volumes of Buffer B with and without the addition of NaCl to 1 M at RT. The extract was centrifuged at 10 000xg for 10 min. The supernatant was then filtered through Miracloth and followed by a further centrifugation step as before. The extract was then assayed for EGase activity. Extractions were carried out in triplicate for each method. The EGase activities expressed per g FW are shown in Table. 4.1 as the mean ± standard error of the mean (SEM).

Table 4.1   Effect of extraction method on the release of EGase activity. EGase

activity is expressed as the mean of three replicates ± SEM

Extraction method	EGase activity $(\Delta\eta_{sp}^{-1}\,s^{-1}\;g\,FW^{-1})$
Buffer B (50 mM acetic acid, pH 5.0)	$9.7 \times 10^{-9} \pm 1.0 \times 10^{-9}$
Buffer B (50 mM acetic acid, pH 5.0) + 1 M NaCl	$8.7 \times 10^{-9} \pm 1.1 \times 10^{-9}$
Buffer A (CTAB extraction buffer)	$2.1 \times 10^{-7} \pm 2.1 \times 10^{-8}$
Acetone powder extraction	$1.3 \times 10^{-7} \pm 6.2 \times 10^{-9}$

EGase activity in extracts using Buffer B was only just detectable whereas the Buffer A and acetone powder methods produced activities that were an order of magnitude higher. The Buffer A method was more suitable for rapid, multiple extractions than the acetone powder method. The Buffer A method was therefore chosen to produce enzyme extracts for the reproducible assay of EGase.

4.2.1.2   Determination of optimum pH for assay

EGase activity has previously been measured in strawberry fruit using a viscometric assay (Barnes and Patchett,1976; Abeles and Takeda, 1990) in the range pH 5.0 to pH 7.0. Here, the optimum pH for the assay using a crude extract was determined by viscometric assay over the range pH 3.0 to 9.0. Activity was detected over a fairly broad range of pH with significant reduction only occurring at the extremes. The optimum was determined to be pH 5.0 in agreement with the previous reports (Figure 4.1) and this was used in all subsequent EGase assays.

4.2.1.3   Effect of enzyme amount and addition of cellobiose

Assays were carried out containing varying amounts of enzyme extract to determine if there was a linear relationship between the amount of EGase enzyme in the assay and the activity detected. The assays were carried out with and without the addition of 0.1 M cellobiose to determine if cellobiose carried over from the purification could affect the activity detected. A linear relationship validating the viscometric assay was demonstrated in both cases (Figure 4.2). The effect of cellobiose was considered negligible as the maximum concentration of cellobiose likely to be present is 10 mM.
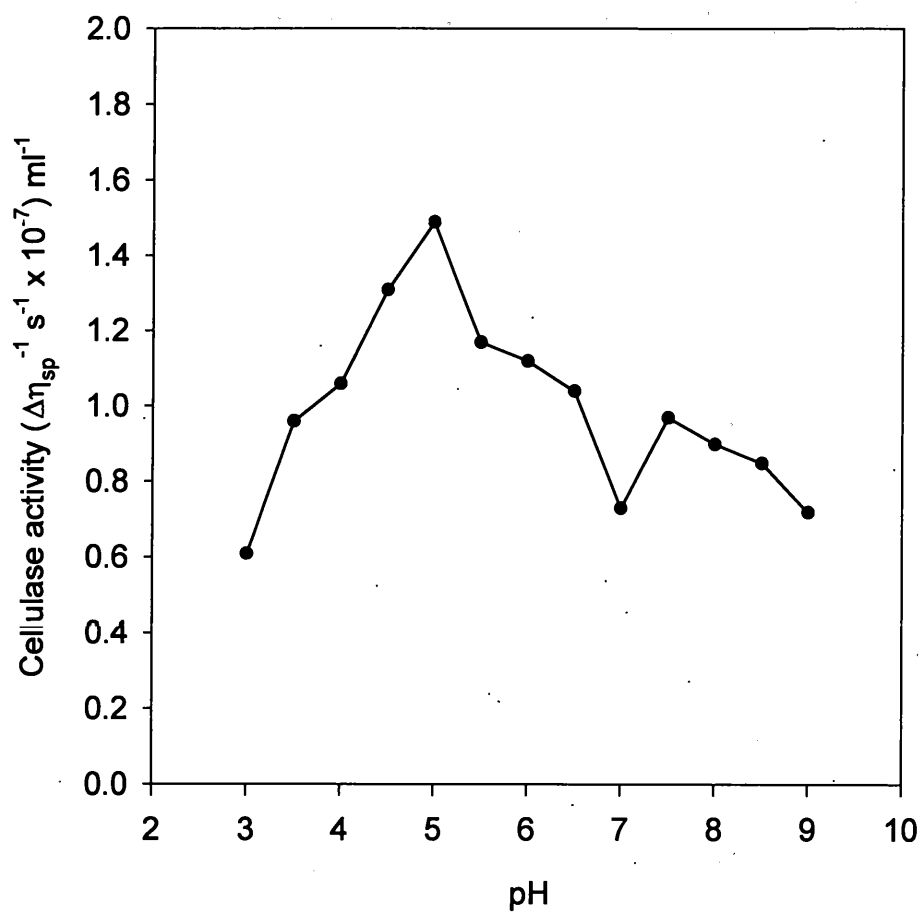
Figure 4.1   Determination of optimum pH for assay of EGase activity in crude
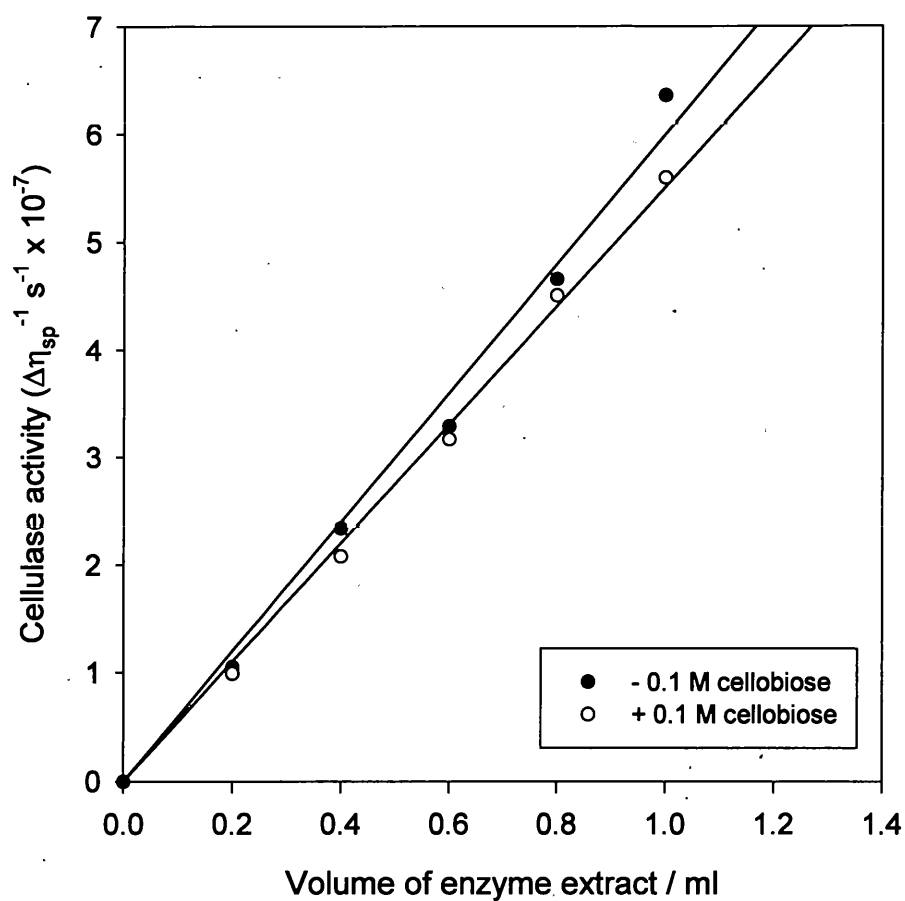
extracts

Figure 4.2   Effect of the amount of enzyme extract and cellobiose on the assay of

EGase

4.2.2 EGase activity in strawberry fruit throughout development

EGase activity was measured in strawberry fruit at various stages of development (Figure 4.3). EGase activity was low at the first three stages (small green to white with yellow achenes). Activity increased steadily from the turning stage reaching a maximum in over-ripe fruit. There was just over a 6-fold increase in EGase activity from the small green to the over-ripe stages. The largest increase was asociated with fruit as they became over-ripe.

4.2.3 Firmness of strawberry fruit throughout development

To study temporal changes in firmness strawberry fruit were sampled from the small green through to the over-ripe stage. Firmness was initially high in unripe fruit and then decreased by two orders of magnitude throughout fruit development (Figure 4.4). Two main phases of softening were observed. The first occurred in the early stages of development corresponding to the phase of growth and expansion of the receptacle. The second phase was associated with changes in the fruit between the ripe and over-ripe stages.
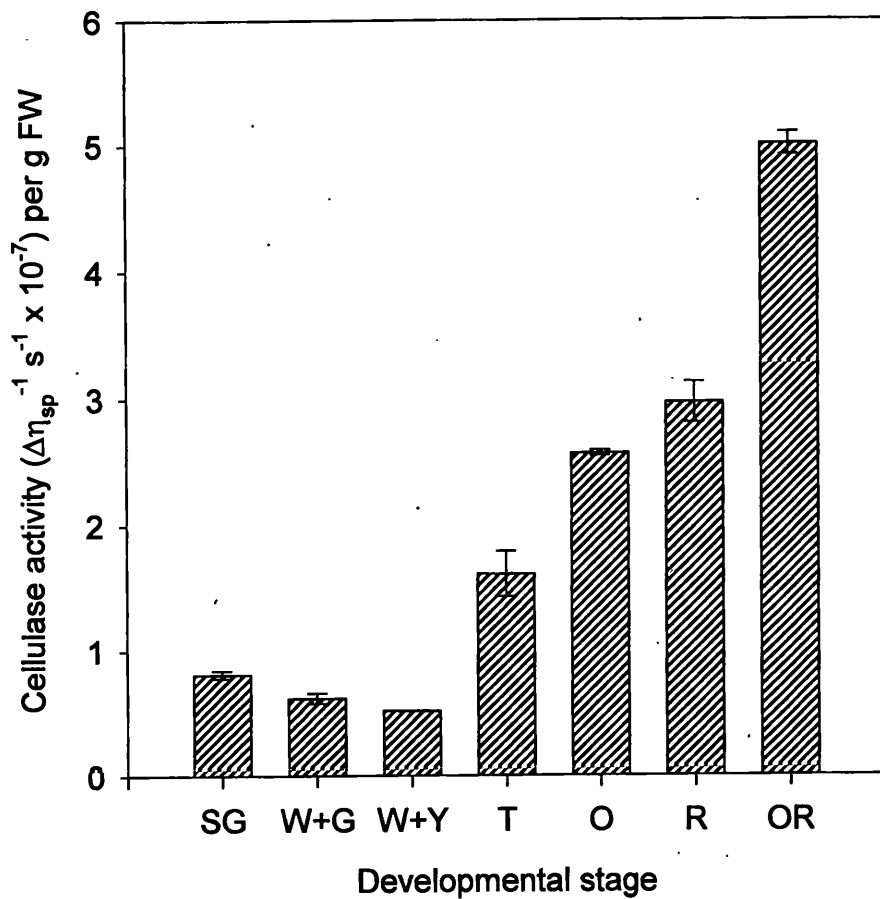
Figure 4.3   EGase activity in developing strawberry fruit

(SG, small green; W+G, white with green achenes; W+Y, white with

yellow achenes; T, turning; O, orange; R, red ripe; OR, over-ripe). EGase

activity is expressed as the mean of two replicate assays ± SEM

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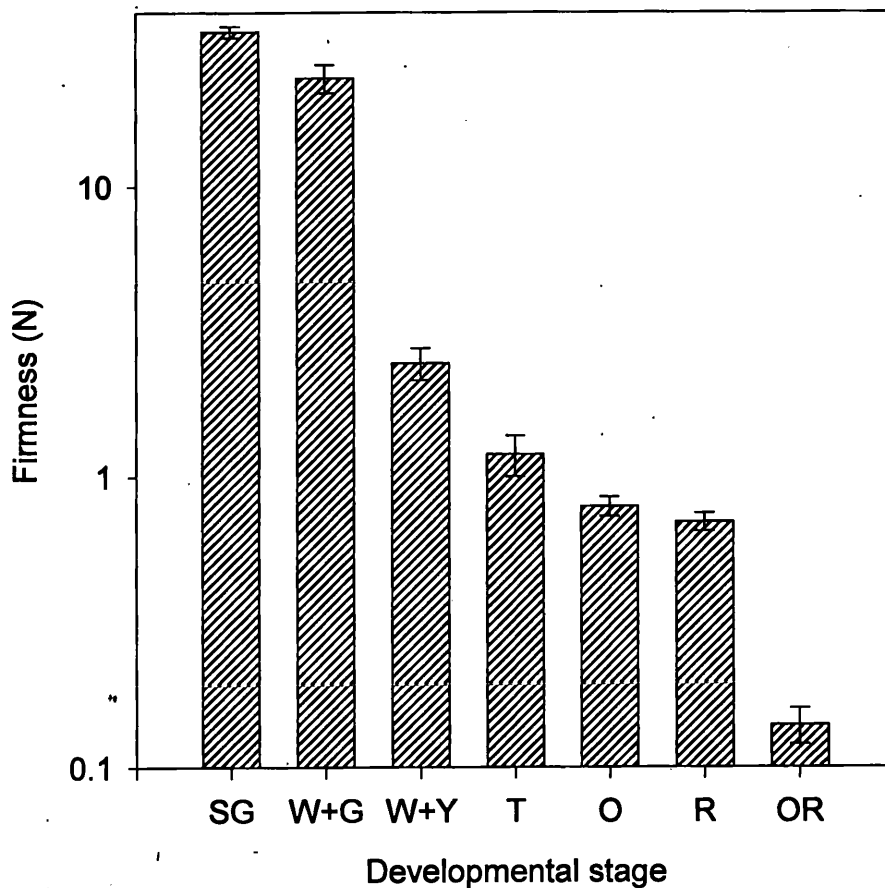
Figure 4.4   Firmness of developing strawberry fruit

(SG, small green; W+G, white with green achenes; W+Y, white with

yellow achenes; T, turning; O, orange; R, red ripe; OR, over-ripe).

Firmness is expressed as the mean of ten replicate measurements ± SEM

4.2.4 EGase activity in other fruit

EGase activity has been measured in fruit from many different species (Brummell et al., 1994) but it is difficult to compare relative activities. The methods used to extract and assay EGase activity vary and activities cannot be directly compared. In order to compare EGase activity in strawberry with that measured in other fruits, a common extraction and assay method was used. Fruits and tissues examined were avocado mesocarp, tomato (cv Ailsa Craig) pericarp, apple (cv Golden Delicious) cortex, red pepper fruit and raspberry fruit, all at the ripe stage of development. Assays were conducted on the extracts in duplicate (Table 4.2).

Table 4.2   EGase activity in strawberry compared to other fruit

Fruit	EGase activity $(\Delta\eta_{sp}^{-1}\,s^{-1}\ g\ FW^{-1})$	EGase specific activity $(\Delta\eta_{sp}^{-1}\,s^{-1}\ g^{-1}\ protein)$	Specific activity relative to strawberry
Strawberry	2.1×10^{-7}	2.0×10^{-3}	1
Avocado	7.0×10^{-5}	1.9×10^{-1}	95
Tomato	5.9×10^{-9}	4.8×10^{-5}	0.02
Apple	7.6×10^{-9}	1.7×10^{-4}	0.09
Red pepper	1.5×10^{-6}	8.4×10^{-3}	4
Raspberry	5.1×10^{-7}	5.8×10^{-3}	3

Avocado fruit contained very high levels of EGase activity, almost 100-fold more than that detected in strawberry. Pepper and raspberry fruit contained similar levels of EGase activity to strawberry whereas the activities detected in apple and tomato fruit were 10- and 50-fold lower than strawberry, respectively.

4.2.5   Isolation of a strawberry EGase

4.2.5.1   Preliminary experiments

Preliminary experiments were carried out to determine the ability of strawberry EGase to bind to a column of CF11 cellulose and be eluted by cellobiose. Proteins were extracted from 30 g FW receptacle tissue of ripe fruit as described in section 3.2.3. The protein pellet was dissolved in 10 ml Buffer B (Appendix A2), 1 ml was retained to assay initial activity in the extract and the rest was applied to a 1 ml (2.6 cm x 0.7 cm diameter) column of CF11 cellulose, equilibrated in Buffer B, in 1 ml aliquots. The column was washed with 5 column volumes of Buffer B in 1 ml aliquots. The column was eluted with 1 ml aliquots of Buffer B containing 0.1 M cellobiose. Fractions of 1 ml were collected individually from the column during the binding and elution steps and at the first and last washing steps and assayed for cellulase activity and total protein (Figure 4.5).

About 50% of the applied cellulase activity bound to the column from which about half was eluted with cellobiose. This equates to the elution of about 10% of the total activity applied. Different conditions were then tested in order to increase the proportion of EGase that bound to the CF11 column. Batch binding experiments were carried out in

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which aliquots of enzyme extract were incubated with excess CF11 cellulose for 0 to 150 min at RT or 4°C. After incubation, the mixtures were centrifuged and the supernatants were assayed for EGase activity. The amount of EGase activity bound to the CF11 as a percentage of the initial activity was plotted against incubation time (Figure 4.6). The results show that even after 2.5 h there is still only 50% of the initial EGase activity bound to the CF11. In fact most of the binding occurred within the first 20 min. Incubation at 4°C resulted in the same level of binding as that observed at RT. The extract prepared in the initial experiments was fairly crude. It is possible that insoluble contaminants were present which interfered with the binding process. To test the solubility of the protein in the extract, EGase activity was assayed in the extract before and after it was filtered through a glass fibre filter. Only 17% of the initial activity was present in the filtered extract confirming that the majority of the EGase protein was associated with insoluble contaminants that prevented it passing through the filter. Although this does not affect the ability to detect activity in the assay it may affect the ability of the enzyme to bind to the column. Consequently, a method for the extraction of soluble proteins from strawberry fruit as described by Given *et al*. (1988a) was used routinely to produce protein from which to purify EGase.
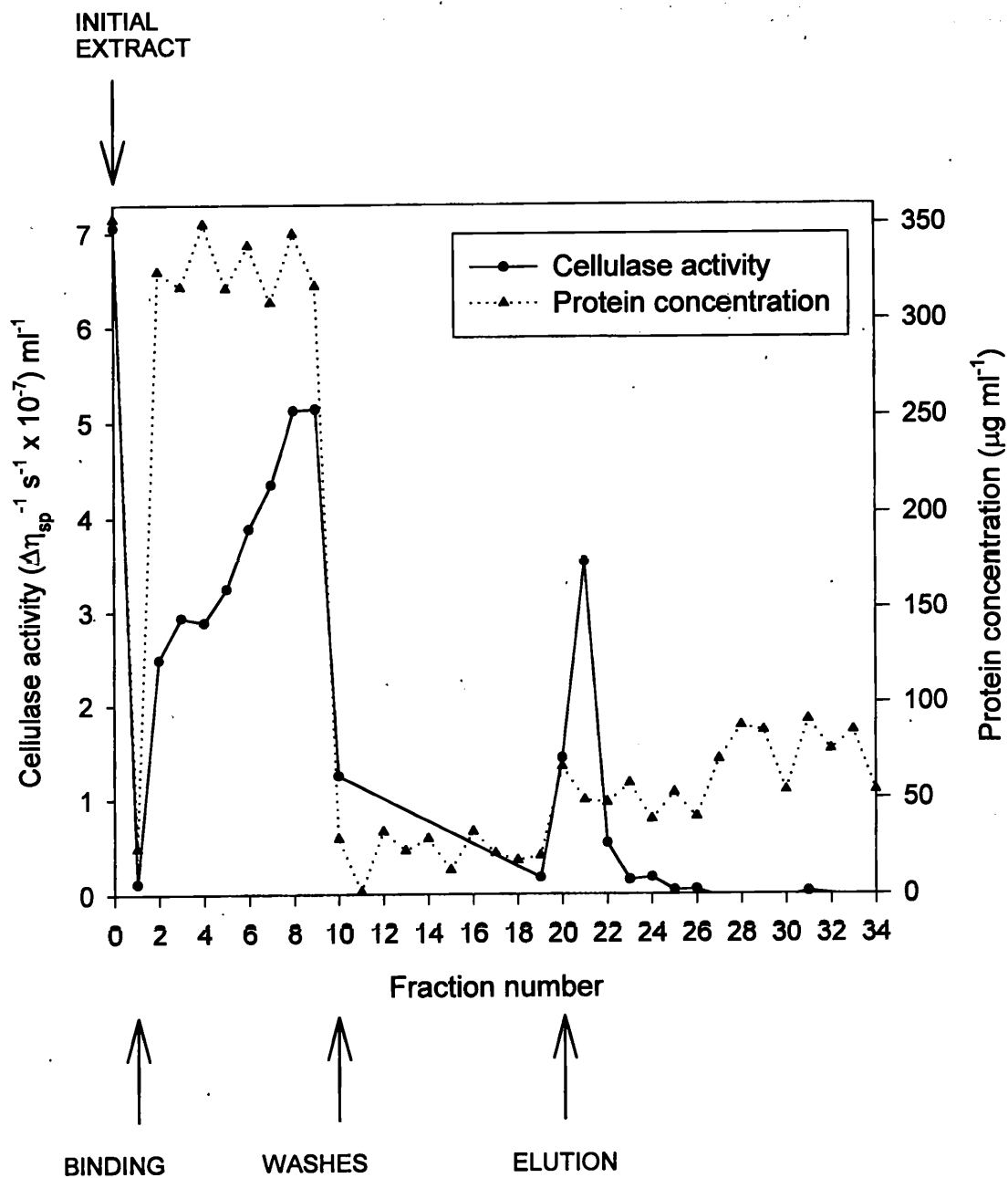
Figure 4.5   Binding and elution of EGase activity on a CF11 cellulose column under

conditions used initially. Arrows indicate the first fraction from each

stage

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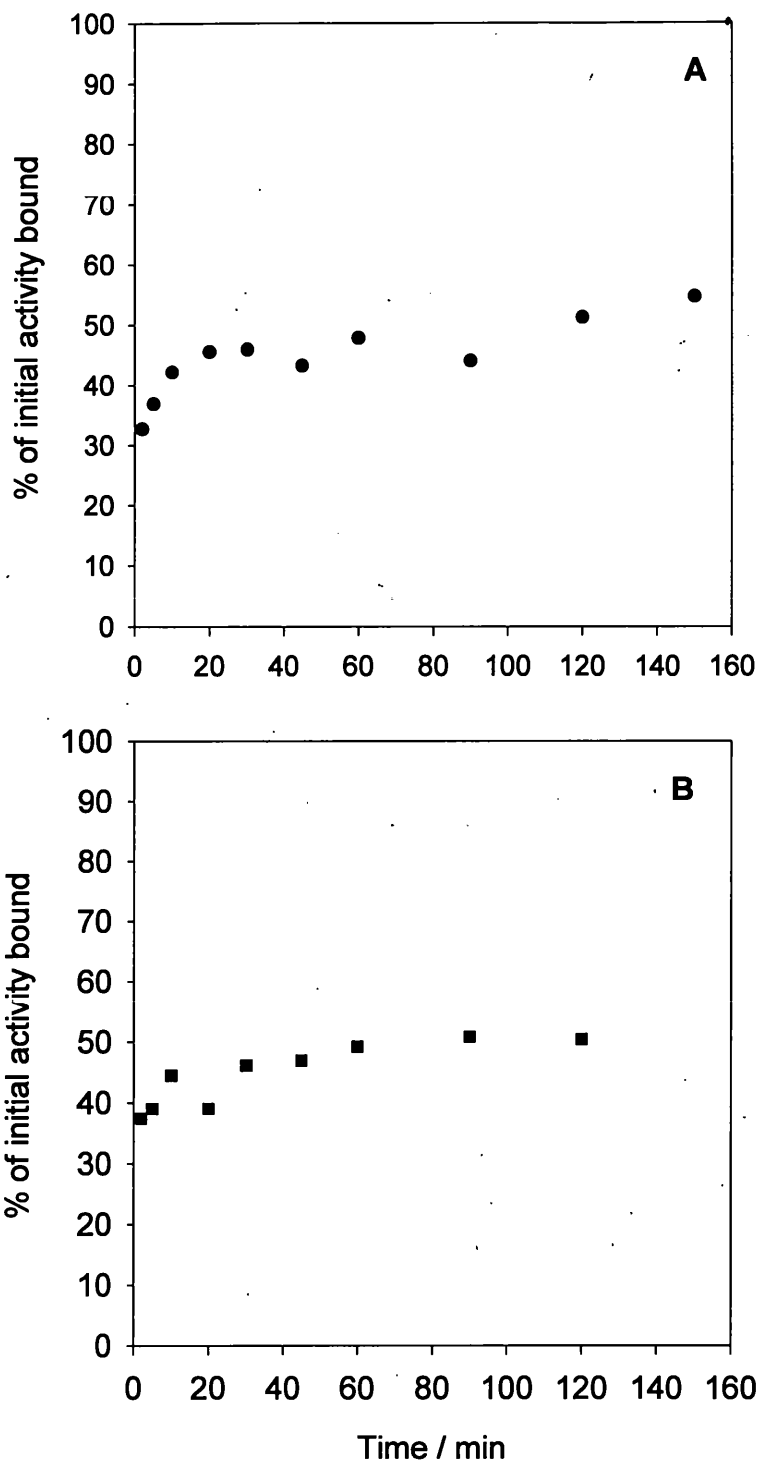
Figure 4.6   Binding of EGase activity to CF11 cellulose at RT (A) and 4°C (B)

Recovery of EGase activity bound to the column was low. The effect of pH and cellobiose, salt and detergent concentrations on the elution of EGase was examined. Experiments were carried out in which batches of CF11 cellulose to which EGase had been bound were incubated with various elution buffers at RT for 10 min. After centrifugation, the supernatant from each sample was assayed for the EGase activity eluted and compared with that eluted by the buffer used previously (Buffer B plus 0.1 M cellobiose). (Table 4.3).

Table 4.3   Effect of pH, salt and detergent on the elution of EGase from CF11 cellulose

Buffer	pH	Cellobiose	NaCl	Triton X-100	Total activity eluted ($\Delta\eta_{sp}^{-1} s^{-1}$)	Factor increase
Buffer B	5.0	0.1 M	0		2.7×10^{-8}	1
Buffer B	5.0	0.1 M	0.1 M		4.8×10^{-7}	18
Buffer B	5.0	0.1 M	1.0 M		5.9×10^{-7}	22
Buffer B	5.0	0.1 M	0	0.1 %	4.6×10^{-8}	1.7
50 mM CPT	7.0	0.1 M	0		7.7×10^{-7}	29
50 mM CPT	3.0	0.1 M	0		3.9×10^{-8}	1.5
50 mM CPT	9.0	0.1 M	0		9.1×10^{-7}	34
Buffer B	5.0	0.25 M	0		5.4×10^{-8}	2

The results show that adding the detergent Triton X-100 or increasing the cellobiose concentration above 0.1 M did not appreciably improve the elution of EGase. Addition of NaCl to 0.1 M gave an 18-fold improvement in yield. This increased further to 22-fold by using 1 M NaCl. Lowering the pH from pH 5.0 to 3.0 had little effect. However, increasing the pH from pH 5.0 to 7.0 resulted in a 29-fold improvement and pH 9.0 produced the greatest single effect on the elution with a 34-fold increase. The optimum conditions for eluting strawberry EGase were therefore 50 mM CPT, pH 9.0 containing 0.1 M cellobiose and 1 M NaCl.

4.2.5.3   *Purification of a strawberry EGase*

Soluble proteins were extracted from ripe strawberry fruit and applied to a column of CF11 cellulose in Buffer B. Elution with 50 mM CPT, pH 9.0 containing 0.1 M cellobiose and 1 M NaCl yielded a single peak of EGase activity almost immediately after application (Figure 4.7). The eluate obtained was then passed through two further columns of CF11 cellulose to remove as much contaminating protein as possible. The eluate from the third column contained a major protein species when run on SDS-PAGE and was designated the purified EGase protein (Figure 4.8). This purification is summarized in Table 4.4. The specific activity of the purified enzyme was 1.36×10^{-7} units μg^{-1} enzyme and it was purified 1030-fold relative to protein in the crude extract.

Table 4.4   Summary of the purification of strawberry EGase from ripe fruit

Purification step	Total EGase activity ($\Delta\eta_{sp}^{-1}\,s^{-1}$)	EGase activity ($\Delta\eta_{sp}^{-1}\,s^{-1}\,ml^{-1}$)	Protein ($\mu g\,ml^{-1}$)	Specific activity ($\Delta\eta_{sp}^{-1}\,s^{-1}\,\mu g^{-1}$ protein)	Purification factor
80% $(NH_4)_2SO_4$ precipitate	8.76×10^{-5}	1.99×10^{-6}	15100	1.32×10^{-10}	1
1st cellulose column + concentration	1.22×10^{-5}	6.96×10^{-6}	615	1.13×10^{-8}	86
2nd cellulose column + concentration	9.10×10^{-6}	1.30×10^{-5}	118	1.10×10^{-7}	833
3rd cellulose column + concentration	4.45×10^{-6}	6.64×10^{-6}	49	1.36×10^{-7}	1030
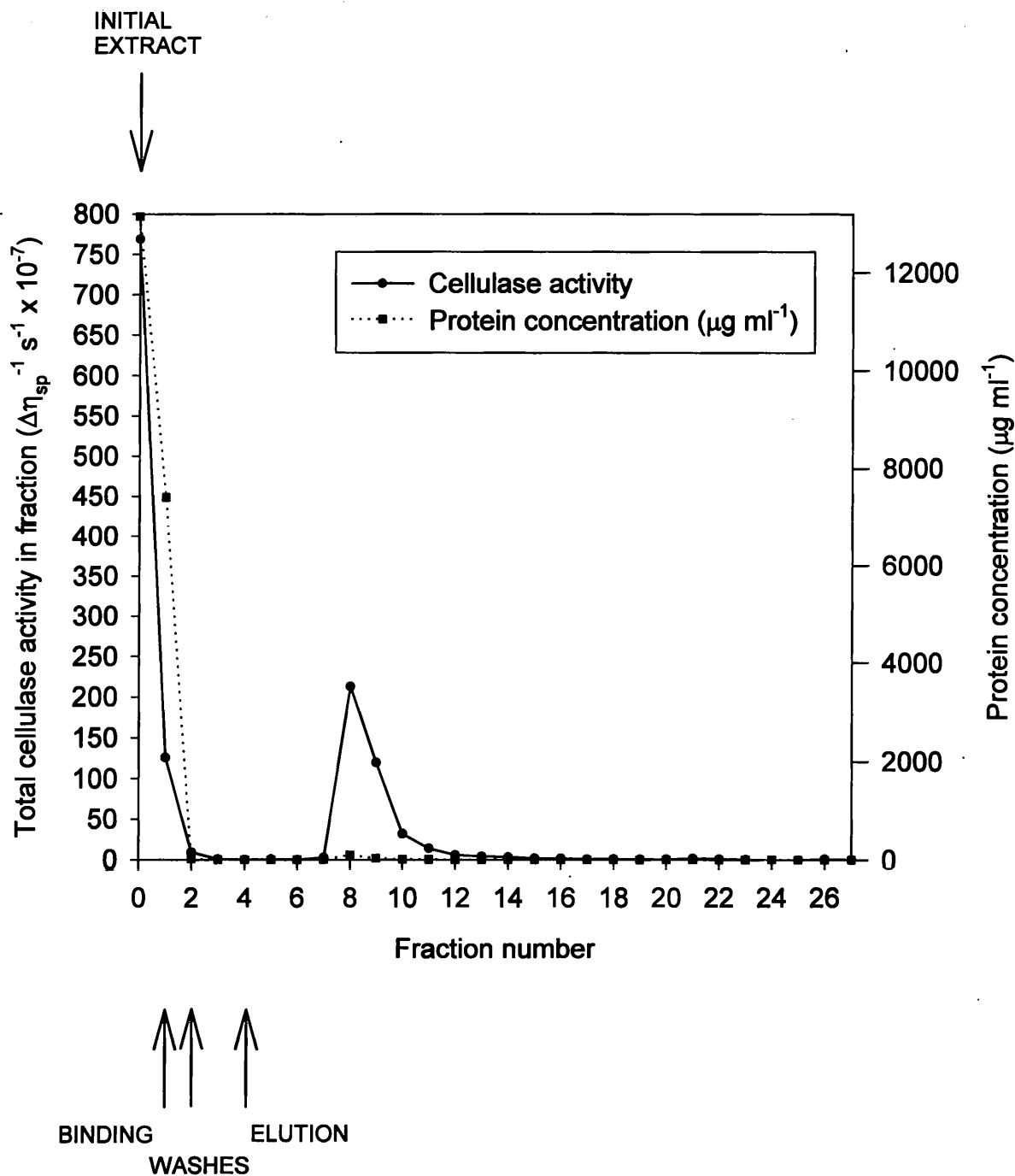
Figure 4.7   Binding and elution of EGase activity on the first CF11 cellulose column.

Arrows indicate each stage of the purification
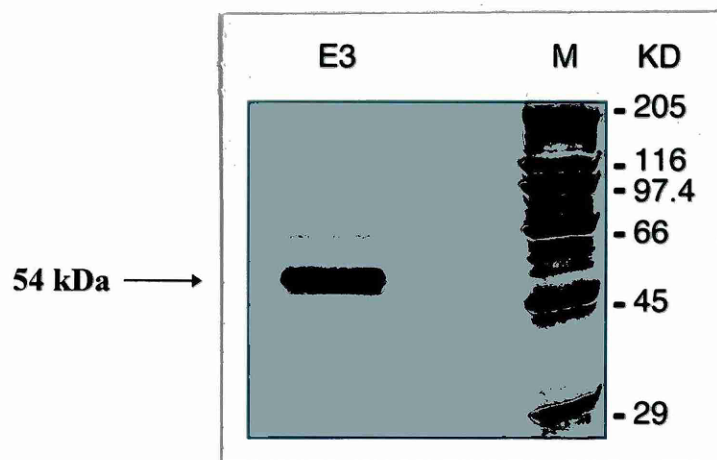
Figure 4.8   SDS-PAGE of strawberry EGase purified on CF11 cellulose. I = Initial

extract; E1, 2, 3 = Eluate from first, second, third columns respectively.

M = Molecular mass markers

4.2.6 Characterization of purified strawberry EGase

4.2.6.1 *Molecular mass*

The molecular mass of the EGase protein was estimated as 54 kDa from SDS-PAGE. This was consistent with the predicted molecular mass of 53 kDa deduced from the isolated *cell* cDNA. A minor protein band was present in some preparations of purified EGase with an estimated size of 66 kDa.

4.2.6.2 *Amino acid sequence*

The purified EGase protein was blotted onto PVDF membrane and 25 amino acids of N-terminal sequence were obtained (Table 4.5).

Table 4.5   Amino acid N-terminus sequence of the purified strawberry EGase

1	2	3	4	5	6	7	8	9	10	11	12	13
I	D	Y	K	D	A	L	G	K	S	I	L	F

14	15	16	17	18	19	20	21	22	23	24	25
F	E	G	Q	R	S	G	K	L	P	N	N/S

The predicted N-terminus, after cleavage of the signal peptide, of the protein deduced from the *cell* cDNA was in close agreement with the N-terminus sequenced from the purified EGase. The N-terminal sequence showed strong similarity, but not identity, to the deduced amino acid sequence of strawberry *cell* cDNA and the proteins encoded by other recently identified EGase genes from different strawberry cultivars (Harpster *et al.*, 1998; Llop-Tous *et al.*, 1999) (Figure 4.9). Similarly, there was strong homology to the proteins encoded by EGase genes from *Arabidopsis* (Shani *et al.*, 1997), pepper (Trainotti *et al.*, 1998b) and tomato (Lashbrook *et al.*, 1994) (Figure 4.9).

4.2.6.3   *pH optimum*

The pH optimum of the purified EGase was determined over the range pH 3.0 to pH 9.0 in citrate-phosphate-Tris buffer. The enzyme exhibited at least 50% of its maximum activity over the whole pH range and over 90% in the range pH 5.0 to 7.0. The optimum pH was determined to be pH 7.0 (Figure 4.10). This is slightly shifted from the optimum pH of 5.0 that allowed maximum detection of EGase activity in a crude extract (Figure 4.1).

Sequence	I	D	Y	K	D	A	L	G	K	S	I	L	F	F	E	G	Q	R	S	G	K	L	P	N	N/S
Strawberry EGase protein	I	D	Y	K	D	A	L	G	K	S	I	L	F	F	E	G	Q	R	S	G	K	L	P	N	N/S
Translated *cell* cDNA (AF041405)	H	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	D
Strawberry Cel1 (AF074923)	H	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	D
Strawberry Cel1 (AF051346)	H	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	H	D
Strawberry Cel1 (AJ006348)	H	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	D
Strawberry Cel2 (AF054615)	H	.	.	G	A	.	.	S	.	.	.	.	.	.	.	.	A	.	.	.	.	V	.	H	N
Strawberry Cel2 (AJ006349)	H	.	.	G	A	.	.	S	.	.	.	.	.	.	.	.	A	.	.	.	.	V	.	H	N
Arabidopsis Cel1 (X98544)	H	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	P	D
Pepper Cel3 (X97189)	H	.	.	.	.	.	.	R	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	P	D
Tomato Cel2 (U13055)	H	.	.	.	.	.	.	R	.	.	.	.	Y	.	.	.	.	.	.	.	.	.	.	P	D

Figure 4.9   Comparison of the N-terminal amino acid sequence of the purified strawberry EGase with the deduced amino acid sequences of the isolated strawberry EGase cDNA, *cell*, other EGases from different strawberry cultivars and *Arabidopsis*, pepper and tomato. Dots (.) represent identical amino acids. (Database accession numbers are shown in brackets)
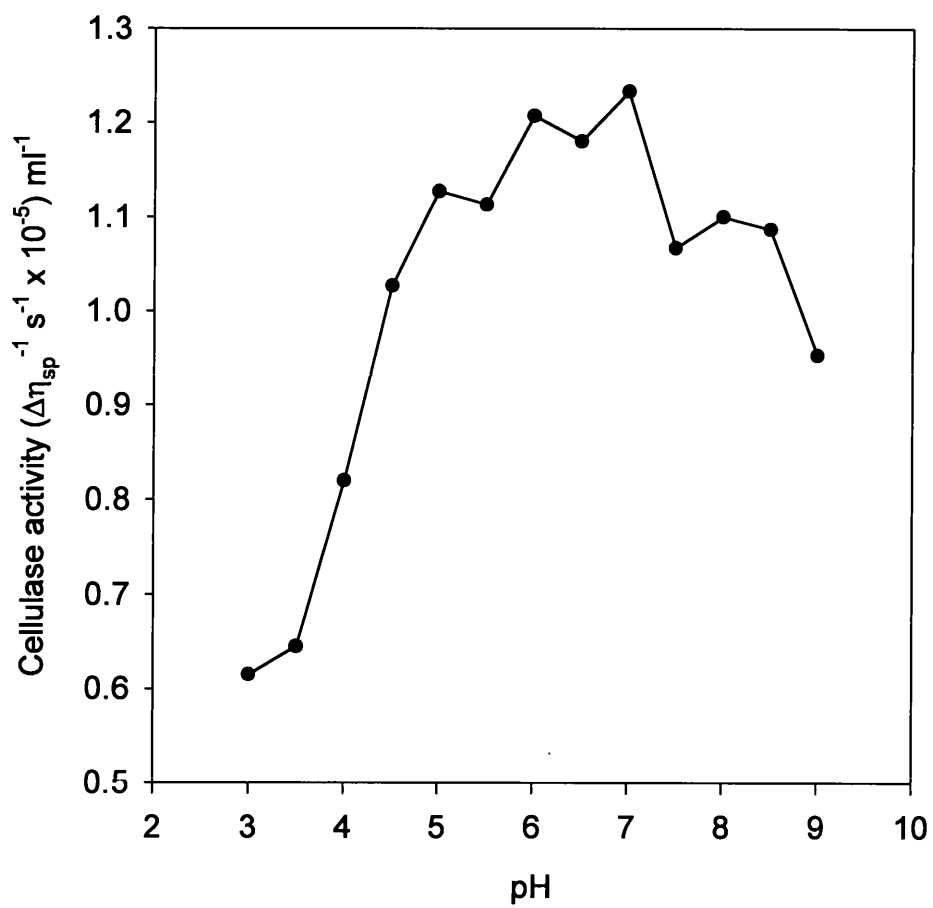
Figure 4.10   Determination of the optimum pH for EGase activity purified from

strawberry

4.2.6.4 K_m

The effect of substrate concentration on the activity of purified strawberry EGase was determined by reducing sugar assay using CMC as substrate. Endoglucanase activity is expressed as the amount of reducing sugars released from single reactions at each substrate concentration. The enzyme exhibited Michaelis-Menten kinetics with a K_m of 1.3 mg ml^{-1} and a V_{max} of 1 nmole min^{-1} μg^{-1} protein. At substrate concentrations above approximately 1.0% CMC a decrease in EGase activity was observed. This was manifested by an upward curvature at low values of 1 / [CMC] (Figures 4.11 and 4.12).

4.2.6.5   *Substrate specificity*

The substrate specificity of the purified EGase was examined using a range of polysaccharides. The reduction in viscosity of CMC suggests that EGase acts on internal β-1,4-glycosidic linkages present in a glucan. The action of the strawberry enzyme on a range of polysaccharides representing polymers found in plants with different sugar backbones and glycosidic linkages was investigated (Table 4.6).
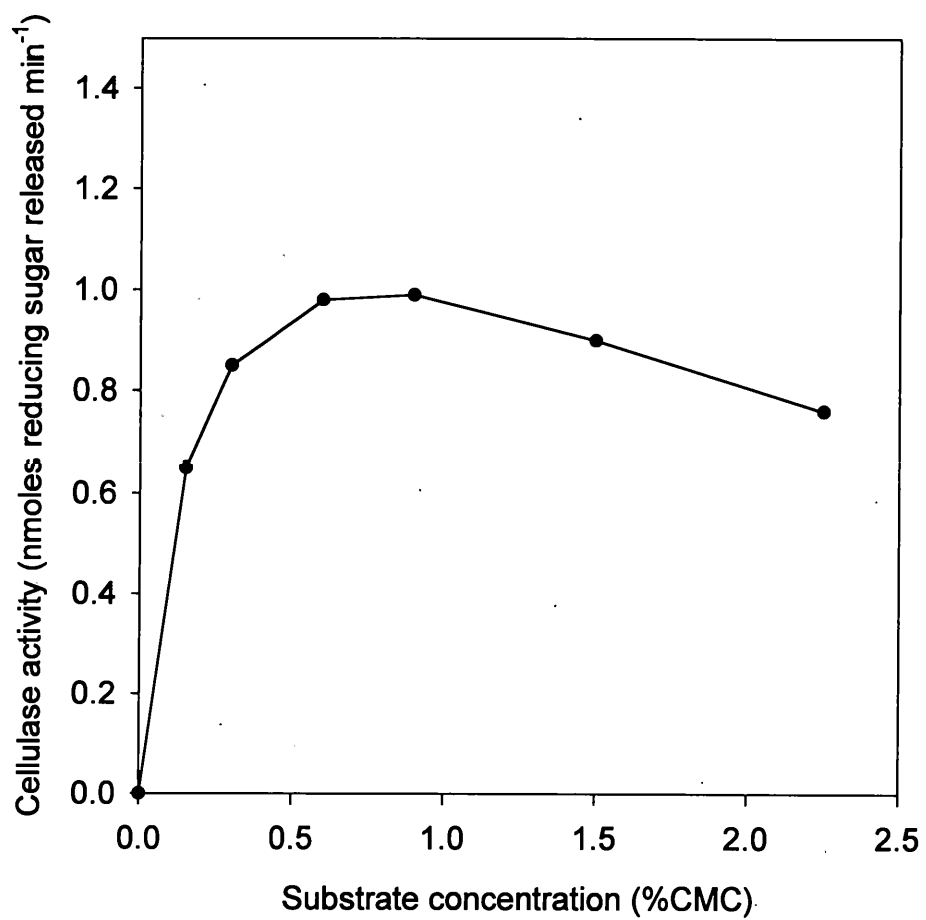
Figure 4.11   Effect of substrate (CMC) concentration on the activity of EGase purified

from strawberry
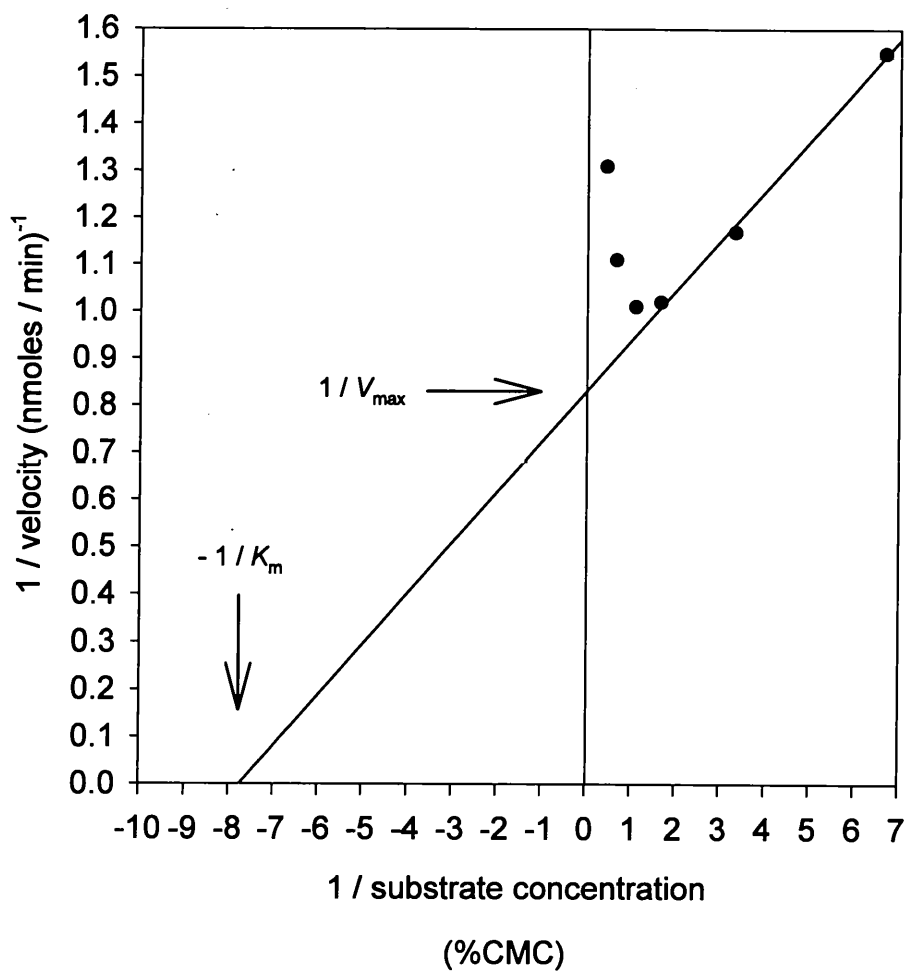
Figure 4.12   Lineweaver and Burk plot to determine K_m and V_{max} values for

purified EGase from strawberry

Table 4.6   Activity of purified strawberry EGase against a range of

polysaccharide substrates

Substrate	Sugar backbone	Linkage	EGase activity (nmoles reducing sugar released h^{-1})	Relative activity (%)
CMC	Glucose	β-1,4	25.7	100
CF11 cellulose (SN)	Glucose	β-1,4	0	0
CF11 cellulose (PPT)	Glucose	β-1,4	0	0
Xyloglucan	Glucose, xylose branches	β-1,4	11.2	44
Laminarin	Glucose	β-1,3	2.5	9
Pectin	Galacturonic acid	α-1,4	0.7	3
Galactan	Galactose	β-1,4	0	0
Xylan	Xylose	β-1,4	0	0
Starch	Glucose	α-1,4	0	0
Lichenan (SN)	Glucose	(β-1,3)(β-1,4)	2.2	8
Lichenan (PPT)	Glucose	(β-1,3)(β-1,4)	0	0

As expected, the enzyme showed greatest activity against CMC (the substrate used in the viscometric assay of EGase), a soluble cellulose derivative with carboxymethyl groups substituted on some of the β-1,4-linked glucose residues in the backbone. However, no activity was detected against insoluble CF11 cellulose which only comprises β-1,4-linked glucosyl residues. Activity was detected against xyloglucan (tamarind, amyloid, Ara:Gal:Xyl:Glc 3:16:36:45) containing a β-1,4-glucose backbone with β-1,6-xylose side chains. Very low activity was shown against lichenan (from *Cetraria islandica*) with a mixed β-1,3- and β-1,4-linked glucose backbone. No activity was detected against galactan (lupin, Gal:Ara:Rha:Xyl:GalUA 91:2:1.8:0.2:5), xylan (birchwood, >90% xylose) or starch (soluble potato), none of which contain β-1,4-linked glucosyl residues. Very low activities were detected against pectin (*Citrus*, partially methoxylated polygalacturonic acid) and laminarin (from *Laminaria digitata*) despite the absence of β-1,4-linked glucosyl residues. The activity against CMC was taken as 100% and the activities against the other substrates were calculated as a percentage of this maximum to give the relative activity (Table 4.6).

4.3   SUMMARY

EGase activity was measured in strawberry fruit using a viscometric assay with CMC as the substrate. Activity was initially very low in the early stages of fruit development. At the turning stage, when fruit begin to turn pink as anthocyanins accumulate, EGase activity started to increase and continued to do so up to the over-ripe stage. From the small green to over-ripe stages there was a 6-fold increase in EGase activity on a FW basis. The unripe fruit were very firm but as they developed there was a substantial

decline in firmness, in particular between the ripe and over-ripe stages, corresponding to the softening associated with ripening. In a comparison of ripe fruits, strawberries were found to contain similar levels of EGase activity to pepper and raspberry, 100-fold lower activity than avocado but 10- and 50-fold higher activity than apple and tomato fruit, respectively.

An EGase from strawberry was purified by affinity chromatography on successive cellulose columns. The bound EGase was eluted with a high pH buffer containing cellobiose and NaCl. When analyzed by SDS-PAGE the purified EGase protein ran as a major band estimated to have a molecular mass of 54 kDa. This was consistent with the predicted molecular mass of 53 kDa of the deduced polypeptide of the isolated *cell* cDNA. In some cases a minor band of about 66 kDa was also present.

The N-terminal amino acid sequence of the purified protein was determined. The predicted N-terminus of the deduced protein of the *cell* cDNA, after removal of the signal peptide, corresponded to the N-terminus of the purified protein. The N-terminal sequence showed a high degree of homology to the deduced amino acid sequence of the *cell* cDNA and exhibited strong homology to EGases from different strawberry cultivars and other plant EGases, confirming the identity of the purified protein. The enzyme retained 50% of its activity over the pH range 3.0 to 9.0 and has a pH optimum of 7.0. The K_m of the enzyme for CMC as substrate was 1.3 mg ml^{-1} and the V_{max} was 1 nmole min^{-1} µg^{-1} protein. Apart from CMC, xyloglucan was the only substrate against which the purified EGase had considerable activity.

4.4 DISCUSSION

ENDO-β-1,4-GLUCANASE ACTIVITY AND SOFTENING OF FRUIT

THROUGHOUT DEVELOPMENT IN STRAWBERRY

An efficient and rapid method for extracting EGase activity was required for the analysis of many samples of strawberry fruit. An extraction method using acetone powders (Given *et al.*, 1988a) overcomes many of the difficulties associated with the extraction of proteins from strawberry fruit tissue due to the high levels of phenols and pectin but the procedure is unsuitable for multiple extractions. A buffer consisting of 50 mM acetic acid/NaOH, pH 5.0 (Buffer B) with and without the addition of 1M NaCl, commonly used to release proteins associated with cell walls, released less than 5% of extractable EGase activity from ripe fruit. Proteins can be efficiently extracted by the anionic detergent SDS (Martinez-Garcia *et al.*, 1999) but activity is not usually preserved. However, the cationic detergent CTAB has been used for the electrophoretic analysis of protein molecular weight with retention of enzyme activity (Akins *et al.*, 1992) and is widely used to isolate nucleic acids free of contaminants from plant tissues. These properties of CTAB were utilized in a novel and highly effective method for extracting active EGase from strawberry fruit tissue for assay.

Fruit firmness is considered to have two components, skin strength and the firmness of the underlying flesh. Penetrometric methods have been used to assess the firmness of both raspberry (Sexton *et al.*, 1997) and strawberry (Hietaranta and Linna, 1999) fruit but in both cases the skin was left intact so that the measurement was not a true indication of flesh firmness. In this work, a motorized penetrometer was used but a

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layer of the fruit containing the skin and achenes was removed prior to measurement to provide a flat surface of receptacle tissue for the probe to penetrate. The measurements therefore represented the firmness of the receptacle tissue alone without any contribution from the skin or achenes. The maximum force recorded as the probe penetrated the tissue represents the yield point and was used as the measure of fruit firmness. This has been deemed the best parameter for the assessment of firmness by penetrometry (Hietaranta and Linna, 1999).

EGase activity was detected in strawberry fruit throughout development. Activity was low in unripe fruit and increased 6-fold to reach a maximum in over-ripe fruit on a fresh weight basis. The greatest increase was associated with fruit as they became over-ripe. This is in agreement with previous data (Barnes and Patchett, 1976; Abeles and Takeda, 1990) and parallels the pattern of *cell* expression in the ripening phase (section 3.2.4, Chapter 3). The softening of strawberry fruit occurred in two main phases. The first phase, early in development, coincides with the rapid growth and expansion of the receptacle whilst the second is associated with ripening of the fruit. There is a good correlation between the second phase of softening as the fruit turn from ripe to over-ripe and the observed increase in EGase activity.

The temporal correlation between EGase activity and softening reported here strongly suggests that EGase has a role in fruit softening in strawberry. When other cell wall changes that occur in ripening strawberry fruit are also considered, the case for a principal role for EGase in softening is strengthened. During ripening there is swelling and hydration of the cell wall and middle lamella and polyuronide solubility increases with loss of the neutral sugars arabinose, galactose and rhamnose from the wall (Woodward, 1972; Knee et al.,1977). However, there is no detectable depolymerization

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of polyuronide, as evidenced by a constant molecular weight profile throughout ripening, indicating that the observed solubilization is not the result of enzymic degradation of pectin polymers (Barnes and Patchett, 1976; Huber, 1984). This is in agreement with the generally observed lack of endopolygalacturonase (PG) activity in strawberry (Neal, 1965; Barnes and Patchett, 1976; Huber, 1984; Abeles and Takeda, 1990) and is also consistent with the findings that in tomato, PG is primarily responsible for pectin depolymerization but not solubilization (Hadfield and Bennett, 1998). It has been suggested that increased methylation of strawberry polyuronides during ripening is responsible for their solubilization by removing the sites available for Ca^{2+} cross-linking (Neal, 1965). This is consisent with the observed reduction in pectinmethylesterase (PME) activity in the later stages of ripening (Barnes and Patchett, 1976). Recently, the strawberry was the first fruit in which the ripening-enhanced expression of a pectate lyase gene was reported (Medina-Escobar *et al.*, 1997b). However, the presence of an enzyme that hydrolyses β-1,4-galacturonosyl bonds in pectin is difficult to reconcile with the lack of pectin depolymerization (Huber, 1984). In any case, work on tomato has indicated that PG-mediated pectin depolymerization is not necessary or sufficient for complete fruit softening (Hadfield and Bennett, 1998) implying that other cell wall modifying proteins are required for fruit softening.

The hemicellulosic polymers extracted from the cell walls of strawberry fruit undergo a marked shift from high molecular weight to low molecular weight as ripening progresses, a change which is temporally related to softening. This has been attributed to enzymic hydrolysis, although their neutral sugar content remains constant (Huber, 1984). The inability of the strawberry EGase purified here to degrade insoluble cellulose leads to the possibility that it is involved in hemicellulose degradation during fruit

ripening (Barnes and Patchett, 1976), a suggestion which has since been extended to embrace plant EGases as a whole (Hayashi, 1989). Taken together, these findings implicate EGase in the softening process. Subsequent to this work, other studies on strawberry fruit have reported a ripening-related increase in EGase activity, although a direct comparison with the data here is not possible as the fruits were not assayed at equivalent developmental stages (Harpster et al., 1998; Trainotti et al., 1999a). This increase also paralleled the accumulation of a cell transcript during ripening.

EGase is also proposed to play a role in the softening of many other fruits where its activity has been shown to increase during ripening. This is the case for blackberry which, like strawberry, does not contain any detectable PG activity (Abeles and Takeda, 1989). In common with strawberry, pepper fruit exhibit increased EGase activity (Ferrarese et al., 1995), an absence of PG activity and similar changes in cell wall polymers during ripening (Harpster et al., 1997; Gross et al., 1986). In some fruits, other cell wall hydrolase activities, such as PG, may act in conjunction with EGase to cause softening. EGase activity in avocado fruit was found to be directly correlated to a decrease in fruit firmness and the climacteric rise in respiration and ethylene production. Before the fruit became fully ripe, variations in EGase activity in different parts of the fruit were inversely related to the firmness of these parts (Pesis et al., 1978). The increase in EGase activity from the very beginning of the ripening phase in this fruit indicated that EGase was involved in the initial phase of softening while the subsequent increase in PG activity was related to the later softening events (Awad and Young, 1979). The depolymerization of xyloglucan and pectin (Sakurai and Nevins, 1997) and the ultrastructural changes observed in the walls of ripening avocado (Platt-Aloia et al., 1980) appear to involve both of these enzymes. The integrated action of EGase and PG

was also suggested for date (Hasegawa and Smolensky, 1971), mango (Roe and Bruemmer, 1981; Abu-Sarra and Abu-Goukh, 1992) and cherimoya (Sanchez *et al.*, 1998). In carambola fruit, increased EGase and PG activities in the later stages of ripening are coincident with the greatest decrease in firmness and marked depolymerization of hemicellulose and polyuronide (Chin *et al.*, 1999). Similarly, wall-bound EGase and PG activities increased when the decline in tissue firmness in ripening olive fruit was most rapid (Fernandez-Bolanos *et al.*, 1995). In sweet cherry, PME was also implicated in softening (Andrews and Li, 1995).

In some fruits, for example peach (Hinton and Pressey, 1974; Bonghi *et al.*, 1998), papaya (Paull and Chen, 1983) and guava (Mowlah and Itoo, 1983), an increase in EGase activity precedes a significant change in fruit firmness. The presence of high EGase activity early in the ripening process indicates that EGase is involved in the initial phase of tissue softening with pectin-degrading hydrolases becoming involved later on as fruit soften fully. A decrease in the molecular size of pectin and hemicellulose fractions from papaya cell walls occurred as the fruit softened. The changes in the pectin were not correlated with the early softening events (Paull *et al.*, 1999) supporting the involvement of EGase, rather than PG, in the initial softening phase. In peach there was a general decrease in molecular size of the more tightly bound xyloglucan fraction during the early stages of softening (Hegde and Maness, 1998) similar to that observed in strawberry. In kiwifruit, although there was no direct association with loss of firmness, EGase activity did increase in the later stages of softening, as did other cell wall degrading enzymes. Application of propylene to this climacteric fruit stimulated the fruit to soften and increased EGase activity (Bonghi *et al.*, 1996). Degradation of both hemicellulose and polyuronide polymers in kiwifruit

during ripening again indicates that a combination of enzymes are required in the softening process (Miceli *et al.*, 1995). Sexton *et al.* (1997) showed that there is a marked increase in EGase activity and extensive breakdown of mesocarp cell walls during softening of raspberry druplets. A substantial increase in three other hydrolases, PG, PME and β-galactosidase (β-gal) in addition to EGase during the ripening of raspberry has recently been reported, although only PG activity correlated with the rapid softening in red ripe fruit (Iannetta *et al.*, 1999). Studies on tomato have consistently shown that EGase activity is present in young fruit, declines during fruit swelling and then increases again during ripening. This has lead some workers to suggest that EGase is involved in the early stages of fruit development and that pectin-degrading enzymes whose activity increases in the later stages are responsible for softening (Babbitt *et al.*, 1973; Hobson, 1968). However, degradation of xyloglucan occurs in softening tomato fruit and this is associated with increased EGase activity when softening was most rapid (Sakurai and Nevins, 1993; Maclachlan and Brady, 1994). In transgenic tomato plants in which PG was down-regulated in wild-type fruit (Smith *et al.*, 1990a) and over-expressed in the non-softening *rin* mutant (Giovannoni *et al.*, 1989), polyuronide degradation by PG was not necessary or sufficient for fruit softening. This indicates that other cell wall degrading enzymes, possibly including EGase, are required for softening.

Finally, there are some fruit in which EGase activity does not increase during ripening or is not detected at all implying that in these cases other cell wall modifying proteins must be responsible for fruit softening. In apple fruit, EGase activity was detected in young expanding fruit and then decreased as fruit ripened. Endo-PG activity was not detected. This is perhaps not surprising considering that apple fruit maintain a steady, continuous loss of firmness. A role for EGase in growth rather than softening

was suggested although the possibility that the extraction technique was unable to release the enzyme from the cell wall was acknowledged (Abeles and Takeda, 1990; Abeles and Biles, 1991). However, there is no depolymerization of either polyuronide (Yoshioka *et al.*, 1992) or xyloglucan (Percy *et al.*, 1997) in apple cell walls during ripening suggesting that apple fruit are unique. Pear fruit soften more rapidly than apple and contain increasing PG activity which correlates with a decrease in soluble polyuronide molecular weight during softening (Yoshioka *et al.*, 1992). EGase activity has not been detected in pear (Knee *et al.*, 1991). In durian fruit EGase activity was unaltered during ripening suggesting that it may not be required for softening (Ketsa and Daengkanit, 1999).

The extent to which EGase is believed to play a role in fruit softening varies widely depending on species. In addition to EGase there is a range of cell wall hydrolases and modifying proteins active in ripening fruit and it is likely that a combination of these are required to bring about cell wall modification and hence fruit softening. Some or all of the possible enzymes and proteins are likely to participate to varying degrees in different species reflecting the underlying differences in the composition of the cell walls and the changes they undergo during ripening.

A variation in EGase activity detected in ripe fruit from different species is reported here. EGase activity has been assayed in fruit from many species (Brummell *et al.*, 1994) and very high levels have been reported in avocado fruit where the activity was more than 100 times higher than that in tomato and peach fruits (Awad and Lewis, 1980). These differences may reflect varying roles for EGase in the softening mechanism and may account for the differences in softening behaviour observed between the fruit of different species.

Correlations between EGase activity and the softening of strawberry fruit during ripening indicate that EGase may play a key role in the softening process. In order to identify a substrate for strawberry EGase and assess its role in fruit softening it was necessary to purify and characterize a ripening-related EGase from ripe fruit. Cellulose affinity chromatography has been used to purify EGases from a range of tissues. This approach was first used to purify the EGases from avocado fruit (Awad and Lewis, 1980) and bean leaf (Koehler *et al.*, 1981). In both cases the EGase was adsorbed to a column of CF11 cellulose and eluted with a buffer containing 0.1 M cellobiose. Passage of the enzyme through a second column resulted in a highly purified protein appearing as a single band after SDS-PAGE. The form of EGase that was selectively purified from bean leaf had a basic pI of 9.5 whereas the pI of the avocado enzyme was acidic. However, this method was not successful for purifying the acidic (pI 4.5) form from bean that could adsorb to the cellulose but not be adequately eluted. Likewise, the EGase from apple fruit (Abeles and Biles, 1991), also with an acidic pI, showed poor recovery. The other EGases that have been effectively purified by cellulose affinity chromatography, from pepper fruit (Ferrarese *et al.*, 1995), sweet pea anthers (Sexton *et al.*, 1990) and tobaco callus (Truelsen and Wyndaele, 1991), have basic pIs. The predicted polypeptide of the isolated *cell* cDNA from strawberry has a calculated basic pI of 9.18. From the available evidence, cellulose affinity chromatography appears to be most effective in purifying basic EGases and so this approach was investigated for the purification of the ripening-related EGase, Cel1, from strawberry.

Indeed, after optimization of the elution conditions, cellulose affinity chromatography alone resulted in a 1030-fold purification of EGase, relative to the crude enzyme extract. This was substantially higher than the 550- to 700-fold purification achieved for other affinity purified EGases. The resulting major protein band with a molecular mass of 54 kDa, as estimated by SDS-PAGE, was confirmed as a EGase by analysis of the N-terminal amino acid sequence. This indicated that the purified EGase was strawberry Cel1, the protein corresponding to the isolated *cell* cDNA. The molecular mass, N-terminus and N-terminal amino acid sequence of the purified protein were in close agreement with those of the deduced polypeptide of the *cell* cDNA. The minor differences between the predicted and analyzed N-terminal sequences may be due to the different cultivars used (Brighton and Elsanta, respectively). This variation was evident in the translated sequences of *cell* cDNAs isolated from the cvs Brighton, Chandler and Selva (section 3.2.2, Chapter 3). Variation in EGase sequences between cultivars has also been observed in avocado where the EGase present in ripe fruit possesses a slightly different pI and apparant molecular mass depending on the cultivar being studied (Brummell *et al.*, 1994). Alternatively, the differences may be due to the presence of multiple *cell* homoeologues in octoploid strawberry or the presence of another EGase gene. The molecular mass of the strawberry protein was similar to the value obtained from a western blot of total soluble proteins from strawberry using an antibody raised to a basic, 54 kDa, ripening-related EGase from peach (Trainotti *et al.*, 1999a). However, the value reported by Harpster *et al.* (1998), estimated from a western blot of total soluble proteins from strawberry using an antibody raised to a protein A/Cel1 fusion protein, was 62 kDa. This was significantly higher than the value for the mature Cel1 protein predicted from the cDNA but it was

suggested that aberrant migration on SDS gels or posttranslational modification could have accounted for these differences. The molecular mass of the purified protein here was similar to that reported for EGases from ripe fruit of pepper (Ferrarese *et al.*, 1995) and avocado (Kanellis and Kalaitzis, 1992) and elder leaf abscission zone (Webb *et al.*, 1993). It is also within the range of 46 to 70 kDa reported for most plant EGases that have been purified (Brummell *et al.*, 1994), the exceptions being EGases of 20 kDa from pea epicotyls (Byrne *et al.*, 1975), 29 kDa from nasturtium seeds (Edwards *et al.*, 1986) and 25 kDa from periwinkle (Smriti and Sanwal, 1999). The preferential purification of strawberry Cel1 over the second form, Cel2, by cellulose affinity chromatography could be due to the more basic pI of the Cel1 protein. Although the predicted pI of 9.9 for the Cel2 protein deduced from the *cel2* cDNA (Trainotti *et al*, 1999b) is more basic than that of Cel1, the pI of a second form of EGase in strawberry detected by activity on an isoelectric focusing gel of total proteins was 7.9 compared to a value of 9.0 for Cel1 (Trainotti *et al.*, 1999a). Values for pI deduced from primary sequence information do not take into account protein tertiary structure and hence may not correspond to the actual pI of the native protein. This is clearly demonstrated in the case of the bean abscission EGase which has an actual pI of 9.5 in contrast to the predicted value of 7.8 (Tucker and Milligan, 1991). Therefore, it is probable that the second strawberry EGase isoform detected with an actual pI of 7.9 was Cel2 suggesting that Cel2 may be more acidic than Cel1. It is interesting that in some preparations of Cel1 in this work a minor band of about 66 kDa was present on an SDS gel. This is consistent with the predicted size of the deduced polypeptide of the *cel2* cDNA isolated from strawberry (Trainotti *et al.*, 1999b) and it may be that on some occasions the purification yielded a small proportion of Cel2 protein in addition to the bulk of Cel1.

Unfortunately there was never enough of this minor band to allow it to be blotted and sequenced.

Characterization of the purified Cel1 revealed optimum activity at pH 7.0. This is in accordance with the pH optima described for most plant EGases and is likely to reflect the pH of the cell wall space where they act (Brummell *et al.*, 1994). The enzyme exhibited near maximum activity in a fairly broad pH range from 5.0 to 7.0, a feature that is often observed for plant EGases. A comparison of the pH profiles of the activity of purified Cel1 EGase and EGase present in a crude extract reveals a more acidic pH optimum for EGase activity in a crude extract. This suggests the presence of further form(s) of EGase in strawberry fruit, which are active in more acidic conditions, in addition to Cel1. Indeed, this was found to be the case with the discovery of Cel2.

The apparent K_m of the EGase was 1.3 mg ml^{-1} for CMC indicating that it had a similar affinity for CMC as did two EGases from poplar with K_ms of 1.0 mg ml^{-1} and 1.2 mg ml^{-1} (Nakamura and Hayashi, 1993; Ohmiya *et al.*, 1995). A comparison of the K_ms of two EGases from pea (3.5 and 3.6 mg ml^{-1}, Wong *et al.*, 1977; Hayashi *et al.*, 1984) and an EGase from periwinkle (0.44 mg ml^{-1}, Smriti and Sanwal, 1999) indicated that amongst the EGases the strawberry enzyme had an intermediate affinity for CMC. The affinities of the pea EGases for pea cell wall and amyloid seed xyloglucan were similar to those for CMC. However, another EGase from pea, specific for xyloglucan, had a much higher affinity for pea xyloglucan with a K_m of 0.64 mg ml^{-1} (Matsumoto *et al.*, 1997). The kinetic analysis of the EGase revealed that there was a decrease in activity at high substrate concentrations. This phenomenon is usually referred to as substrate inhibition where an excess of substrate available for binding somehow inhibits the catalytic activity of the enzyme. In this case, due to the nature of the substrate, it may be

that at high concentrations the increased viscosity of the CMC interferes with the

molecular interaction of the enzyme with the substrate and thus inhibits effective

catalytic activity. It is also possible that the accumulation of the reducing sugar end-

product of the reaction may have an inhibitory effect on the enzyme activity.

SUBSTRATE SPECIFICITY OF STRAWBERRY ENDO-β-1,4-GLUCANASE AND ITS POTENTIAL ROLE IN CELL WALL MODIFICATION DURING RIPENING

The purified strawberry Cel1 was most active against the soluble cellulose derivative

CMC, the substrate used in the viscometric assay of the enzyme. This substrate consists

of β-1,4-linked glucosyl residues that are the proposed site of action for EGases (Fry,

1995). In contrast, the enzyme was unable to degrade insoluble cellulose despite the

presence of the same sugar linkages. This absence of activity against insoluble cellulose

was previously reported for a crude enzyme extract from strawberry (Barnes and

Patchett, 1976) and is consistent with data that indicate that the cellulose content of

strawberry fruit cell walls does not vary significantly during ripening (Wade, 1964;

Neal, 1965). Plant EGases are generally believed to be unable to extensively hydrolyze

insoluble crystalline cellulose (Brummell et al., 1994). This has been reported for

several EGases including those isolated from bean (Durbin and Lewis, 1988) and coleus

(Wang et al., 1994b) leaf abscission zones and from avocado fruit (Hatfield and Nevins,

1986) where the cellulose content was also constant throughout ripening (Sakurai and

Nevins, 1997). However, ultrastructural analyses of avocado fruit cell walls have

revealed an apparent loss of cellulose fibrillar components and structural integrity of the

wall during ripening which was attributed to the action of EGase (Platt-Aloia et al.,

1980). Similar studies have noted that the generally observed lack of cellulose hydrolysis may in reality reflect degradation that does not generate detectable soluble products or that extensive hydrolysis is not required to effect the observed softening of the fruit. This work has lead to the suggestion that avocado fruit EGase can disrupt cellulose microfibril organization by limited hydrolysis at accessible sites in the noncrystalline regions of the fibrils. This would affect both microfibril structure and the interactions between cellulose and matrix polysaccharides thereby compromising the strength of the wall (O'Donoghue et al., 1994). This supports an earlier suggestion that avocado EGase, rather than acting to rapidly solubilize cell wall components, instead disrupts and loosens the cell wall matrix (Hatfield and Nevins, 1986). In contrast, it has been reported that EGases isolated from pea epicotyls (Wong et al., 1977) and periwinkle (Smriti and Sanwal, 1999) are able to hydrolyze both insoluble crystalline and swollen forms of cellulose, although at rates which are lower than those towards CMC. In this respect, they resemble microbial EGase systems which are able to effectively degrade native cellulose (Beguin, 1990).

Since most plant EGases are apparently inactive towards native cellulose, and given the correlation between EGase activity and xyloglucan degradation that is often observed, it has been suggested that the other cell wall component with β-1,4-glucosyl linkages, xyloglucan, is the true in vivo target of these enzymes (Hayashi et al., 1989). Indeed, activity against xyloglucan has been reported for EGases from tobacco callus (Truelsen and Wyndaele, 1991) and pea epicotyls (Hayashi et al., 1984). It is interesting to note that the tobacco EGase, whilst degrading native tobacco cell wall xyloglucan at a faster rate than CMC, was inactive against amyloid seed xyloglucan. The pea enzymes, however, were able to hydrolyze both native and amyloid forms equally effectively,

although the rate was slower than for CMC. Similarly, two EGases from poplar suspension culture cells hydrolyzed amyloid xyloglucan at a slower rate than CMC (Nakamura and Hayashi, 1993; Ohmiya *et al.*, 1995). In contrast, bean leaf abscission EGase was unable to degrade amyloid xyloglucan (Durbin and Lewis, 1988). The differences in relative activities have been attributed to the different degree of substitution of the β-1,4-glucosidic backbone of the substrates relative to the structural requirement of the enzymes for binding. The availability of the required sites to enable the enzyme access to the substrate obviously affects the rate at which it can be hydrolyzed by the enzyme. In the case of the pea EGases, they possess a binding site that recognizes at least six consecutive β-1,4-linked glucose units. The random limited substitution at the C_6 position in CMC does not interfere with the binding or hydrolysis of the substrate whereas the xyloglucan backbone is only hydrolyzable at every fourth glucose residue due to the substitution pattern. Such structural constraints are believed to account for the different relative activities of the enzymes towards these substrates (Hayashi *et al.*, 1984). Similarly, xyloglucans with different substitution patterns (Hayashi, 1989) may be differentially hydrolyzed by the same enzyme. Avocado EGase showed limited activity against soybean hypocotyl cell wall xyloglucan (Hatfield and Nevins, 1986) although no degradation of avocado fruit cell wall xyloglucan was detected *in vitro*, despite the observed depolymerization of xyloglucan during ripening. It was suggested that xyloglucan, when associated with cellulose *in vivo*, may satisfy the structural requirements for hydrolysis by EGase that are absent in the soluble xyloglucan *in vitro* (O'Donoghue and Huber, 1992). Activity of avocado EGase against xyloglucan can be reconciled with the ultrastructural studies suggesting it can modify cellulose (Platt-Aloia *et al.*, 1980; O'Donoghue *et al.*, 1994) in that degradation of hemicellulosic

components of the cell wall may result in the observed disorganization of fibrillar cellulose by disrupting the cellulose-xyloglucan network (Fischer and Bennett, 1991). Xyloglucan has previously been proposed as a substrate for strawberry EGase (Barnes and Patchett, 1976) and this is supported by the finding that, other than CMC, the only substrate the purified strawberry EGase showed appreciable activity towards was amyloid xyloglucan. For strawberry, the considerable activity of this EGase against xyloglucan and the decline in the average molecular weight of the hemicellulosic fraction of the cell wall during ripening which has been attributed to enzymic hydrolysis (Huber, 1984), together suggest that xyloglucan is the principal *in vivo* substrate for this enzyme. Xyloglucan, the predominant hemicellulose in dicotyledons (Brummell *et al.*, 1994), is believed to coat and form extensive cross-links with cellulose microfibrils. In doing so it produces the major load-bearing structure in the wall and hence plays an important role in maintaining the integrity of the cell wall (Rose and Bennett, 1999). The action of EGase on xyloglucan would disrupt the cellulose-xyloglucan network with the resultant loosening of the cell wall leading to fruit softening. Evidence of this disruption in strawberry has come from ultrastructural studies that show a progressive disorganization of the cellulose microfibrils, first apparent in white fruit (Trainotti *et al.*, 1999a). Some degradation of the hemicellulose fraction is evident in the fruit before the white stage (Huber, 1984). It may be that the second form of EGase in strawberry, Cel2, which is expressed in fruit from the earliest stages of development, acts on xyloglucan prior to the ripening-related induction of Cel1. These findings indicate that a synergistic interaction of both enzymes may be required for the subsequent softening of the fruit. It has been suggested that expansins, which are present in ripening strawberry fruit (Rose *et al.*, 1997), should also be included in the set of cell wall modifying proteins that act in

a coordinated manner to disassemble the cellulose-xyloglucan network and cause softening during fruit ripening (Rose and Bennett, 1999).

The presence of Cel2 in strawberry fruit early on in their development and the observed degradation of hemicellulose polymers at this time prior to the onset of fruit softening indicate that EGase is not solely involved in fruit softening. Young fruit undergo a period of cell growth and expansion and this may be facilitated by the action of Cel2. A role for EGases in cell growth and expansion in young rapidly-growing tissues has been proposed (Fry, 1989). The most studied of these EGases are the auxin-induced enzymes of pea (Hayashi *et al.*, 1984), but a correlation between increased EGase activity, xyloglucan degradation and cell growth has also been shown in soybean (Koyama *et al.*, 1981) and azuki bean (Hoson *et al.*, 1995).

The only other substrate tested that was degraded by strawberry Cel1 was lichenan which was also hydrolyzed by EGases from avocado fruit (Hatfield and Nevins, 1986), pea epicotyls (Wong *et al.*, 1977) and suspension-cultured poplar cells (Nakamura and Hayashi, 1993; Ohmiya *et al.*, 1995). Barley β-glucan with mixed 1,3;1,4-β-glucosidic linkages was hydrolyzed by tobacco callus EGase (Truelsen and Wyndaele, 1991). The weak activity of strawberry Cel1 against lichenan compared to the greater activities exhibited by the other EGases indicates that the β-1,3 linkages present in this polymer hinder access of the enzyme to the target β-1,4 linkages, again highlighting the differences in the specific requirements of individual EGases for binding. The specificity of the strawberry enzyme for a β-1,4-linked glucan polymer was confirmed by its lack of activity against substrates containing different glycosidic linkages and different sugar residues, as reported for the other plant EGases that have been characterized. The unexpected but low activities detected against pectin and

laminarin could be due to contamination by other polymers present in the commercial preparations of these substrates.

The differences observed in the substrate specificities of EGases isolated from different species suggest that plant EGases comprise a group of related enzymes that act on distinct polysaccharide substrates in different tissues. Thus individual EGases may have different roles in cell wall breakdown depending on the species and tissue that they exist in. It is also possible that an apparent lack of activity towards a particular substrate *in vitro* may be the result of a difference in the isolated substrate compared to its native state and may not reflect the true ability of the enzyme to degrade the substrate *in vivo*.

CHAPTER 5. GENERATION AND ANALYSIS OF ENDO-β-1,4-GLUCANASE TRANSGENIC STRAWBERRY PLANTS

5.1 INTRODUCTION

The modification of gene expression in plants by the introduction of a transgene has produced physiological and biochemical information on a range of metabolic pathways, aspects of development and the function of individual genes. It is also widely used to manipulate specific traits in plants with the aim of crop improvement. For example, the reduction of polygalacturonase (PG) activity in ripe tomato fruit (Smith *et al.*, 1988; Sheehy *et al.*, 1988) has resulted in enhanced resistance to mechanical damage, leading to improved shelf-life and flavour and improved processing properties (Gray *et al.*, 1994). The production of novel flower colours and pigmentation patterns has been achieved by the manipulation of levels of chalcone synthase (CHS), a key enzyme in the anthocyanin biosynthesis pathway, and the potential to alter the composition of fatty acids in seeds has been demonstrated (Bourque, 1995).

Suppression of endogenous gene expression can be achieved by the introduction of a homologous transgene in either the antisense or sense orientation and has been termed homology-dependent gene silencing. In some cases, the use of a transgene in the sense orientation causes both the endogenous gene and the transgene to be suppressed, a phenomenon referred to as cosuppression (Hamilton *et al.*, 1995). In this way there is the potential to determine the function of a particular gene by down-regulating its expression and examining the phenotypic effects on the transgenic plants produced. The ideal transformant for such studies would contain a single copy of the transgene that

would segregate as a stably inherited Mendelian trait and show uniform expression from one generation to the next. However, in reality, variability is generally observed from one transgenic plant to the next due to the inherent randomness of the integration of the transgene into the plant genome (Hansen and Wright, 1999). Thus the design and analysis of transgenic experiments must be carefully considered if any useful information is to be gained from them.

Antisense and sense suppression was used to specifically down-regulate *cell* expression in transgenic strawberry fruit. Fruit exhibiting reduced expression of *cell* were analyzed for changes in their ripening behaviour in an attempt to characterize the *in vivo* role of Cel1 in strawberry fruit ripening in relation to fruit texture.

5.2   RESULTS

5.2.1   Construction of transformation vectors

The isolated ripening-related endo-β-1,4-glucanase (EGase) full-length cDNA *cell* was used in the transformation vectors to produce both antisense and sense gene constructs. The vector pJR1Ri contains the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase (nos) 3' terminator and was used as an intermediate vector to provide the expression cassettes. The *cell* cDNA was cloned between the promoter and terminator in either orientation to generate the antisense and sense expression cassettes. The orientation of the cDNA in pJR1Ri was confirmed by DNA sequencing using a primer to the CaMV 35S promoter. In addition, a cassette consisting of only the promoter and terminator and no cDNA was used as a control. The expression cassettes

were then excised from pJR1Ri using *Eco*R I and *Hin*d III and directionally cloned into the corresponding sites of the multiple cloning site of the binary transformation vector pBINPLUS (van Engelen *et al*, 1995). This produced three transformation vectors with the 35S promoter of the expression cassette (antisense, sense or control) adjacent to the right border of the T-DNA and the plant kanamycin resistance gene (non-mutant *nptII*) adjacent to the left border as shown in Figure 5.1.
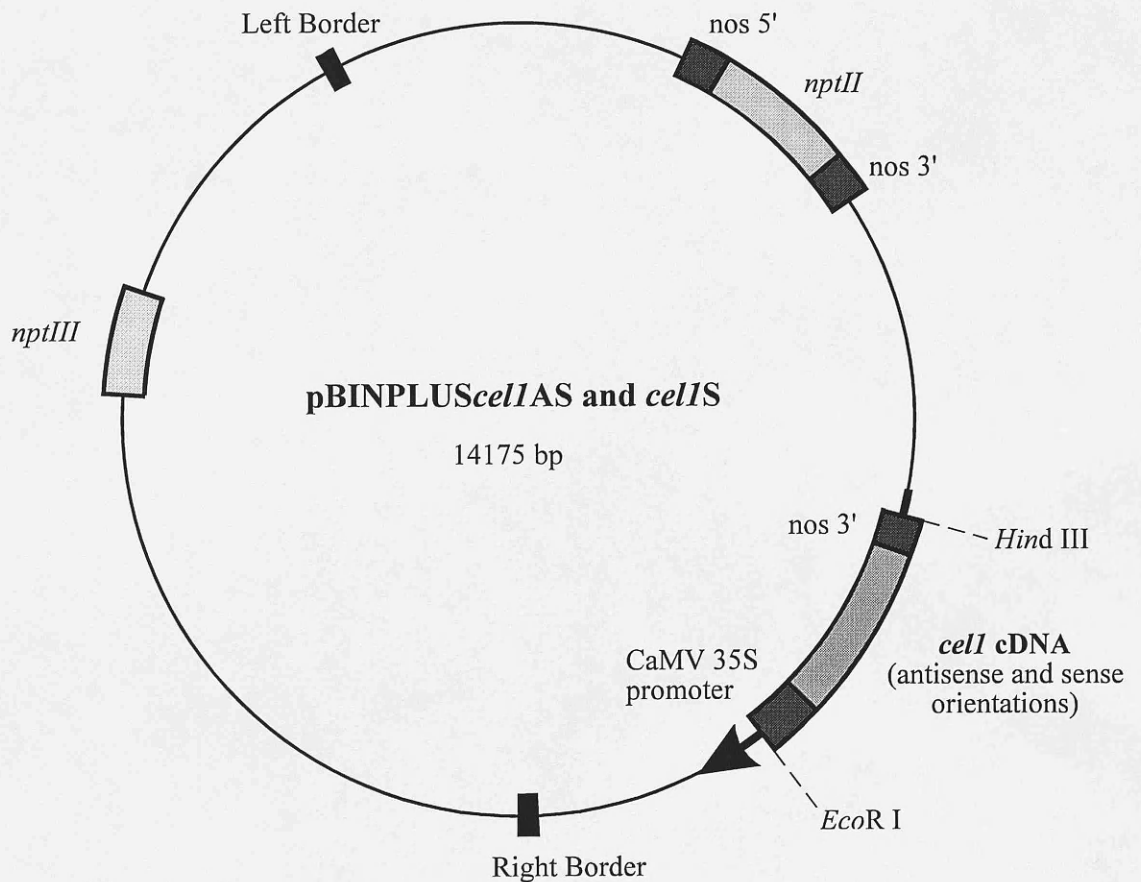

Figure 5.1   Map of the transformation vector used to transform strawberry cv Calypso plants. The CaMV 35S/nos expression cassette alone (control) or containing the *cell* cDNA (antisense and sense) was inserted into the binary transformation vector pBINPLUS

5.2.2   PCR analysis of putative transformants

PCR analysis was used for the preliminary confirmation of the transformation status of the 90 plants that had survived the kanamycin selection. Only six of these plants did not produce the 475 bp PCR product. These six negative plants were maintained as non-transformed controls.

5.2.3   Presence of the transgene in EGase transgenic strawberry plants

A total of 71 out of the 90 plants that resulted from the transformations grew to maturity and produced fruit for analysis. These comprised 21 control-transformed lines and 25 each of antisense and sense *cel1*-transformed lines. The remaining 19 plants showed a variety of abnormal phenotypes, including stunted growth without flowers and proliferation of small flowers that failed to pollinate, and this was likely to be the result of events occurring during the regeneration of the plants in tissue culture. The antisense and sense lines were re-tested by Southern analysis to confirm the presence and nature of the transgene in their genomes. Genomic DNA from each of these 50 independent lines was digested with *Hinc* II which only cuts at one site in the CaMV 35S promoter (at position 117) and at one site in the *cel1* transgene (at position 744) (Figure 5.2). Only 5 lines, plant numbers W1 29, 106, 133, 139 and 161, were found not to contain the vector (non-transformed) as indicated by the absence of a hybridizing fragment of 1466 bp or 1169 bp. Three of these had tested negative in the PCR analysis, but the remaining two had given a PCR product of the correct size. Unexpectedly, many of the positive transformed lines thought to be sense transformants did not contain the expected 1169

bp hybridizing fragment. A total of 37 plants were confirmed as antisense lines by the presence of a 1466 bp band but only 8 were confirmed as sense lines by the presence of a 1169 bp band. It appears that a labelling error occurred during the process of shoot regeneration, resulting in mature plants with incorrect identities. As expected, no hybridizing fragments were observed in the wild-type strawberry cv Calypso plants. Detection of the transgene by Southern analysis of single digests of genomic DNA extracted from each *cell*-transformed line is shown in Figure 5.3.

425 bp   744 bp   1169 bp   sense

425 bp   1041 bp   1466 bp   antisense
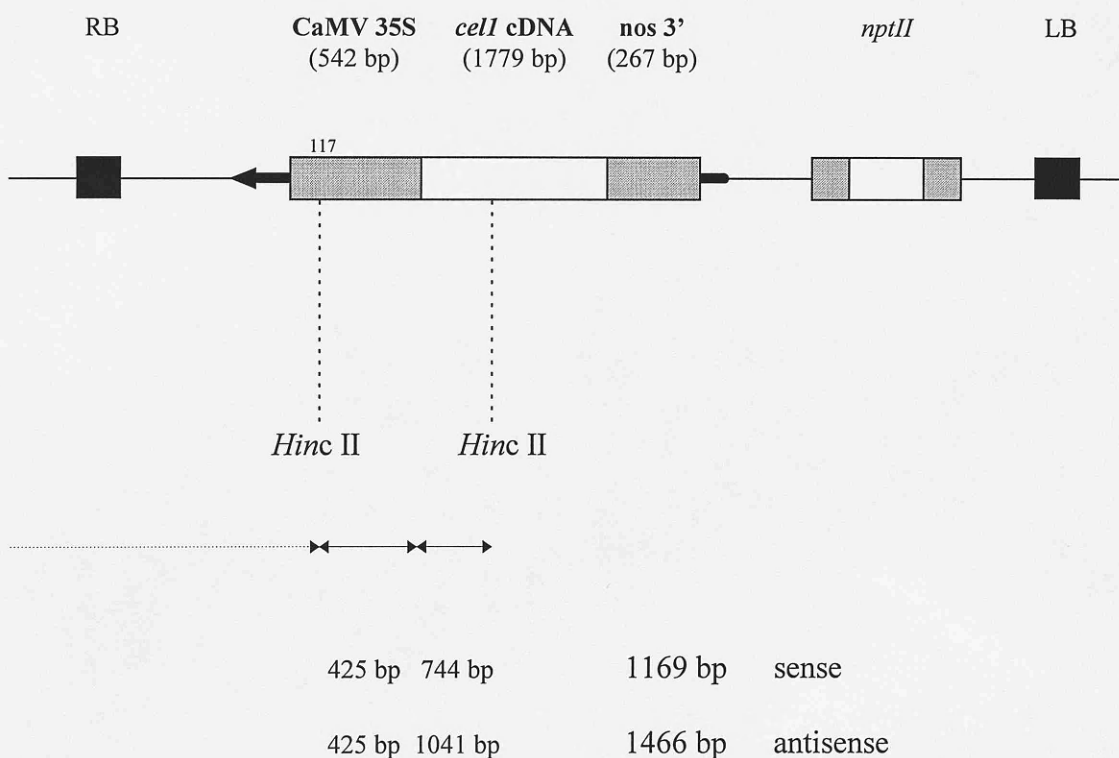
Figure 5.2   Representation of the *cell* transgene in the binary vector pBINPLUS.

Restriction with *Hin*c II produces the fragments shown which hybridize

to the CaMV 35S probe in Southern analysis

215
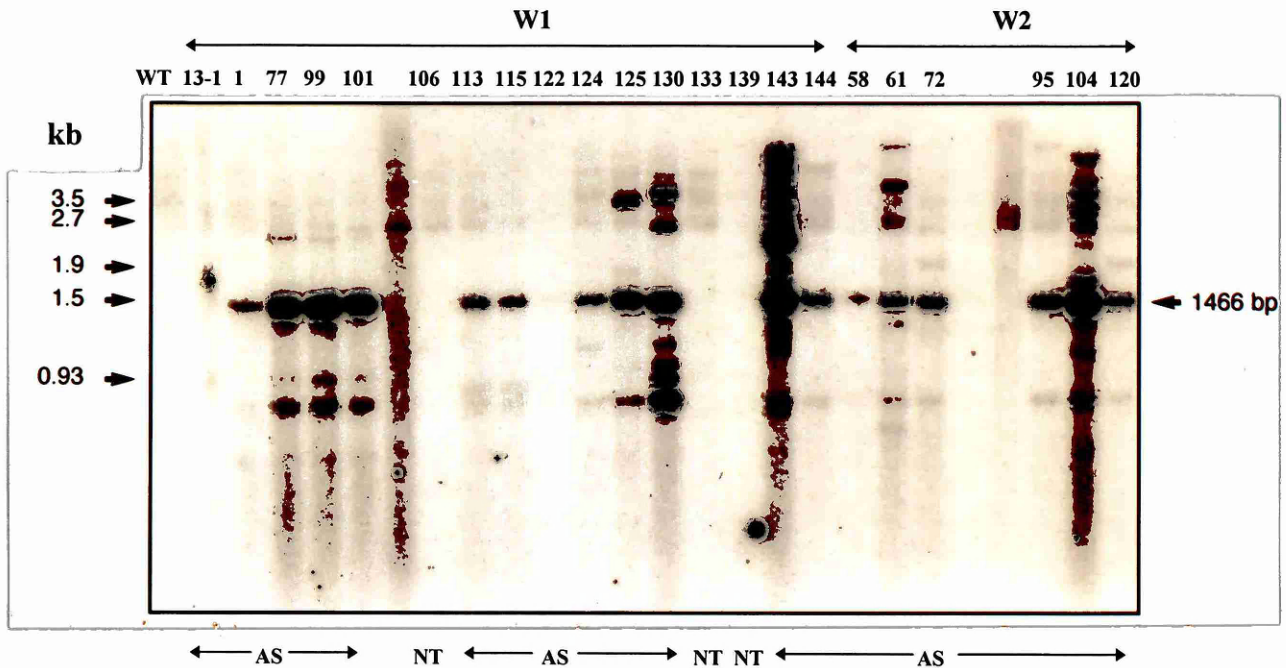
Figure 5.3   Detection of the transgene by Southern analysis. Each plant number (eg. W1 101, W2 58) is an independent transformation line. WT indicates an untransformed wild-type strawberry cv Calypso control plant. AS and S represent antisense and sense *cel1*-transformed lines respectively. NT (non-transformed) represents lines that were not successfully transformed. The positions of the hybridizing fragments (bp) are shown on the right. Sizes of DNA molecular mass markers (kb) are shown on the left (continued overleaf)
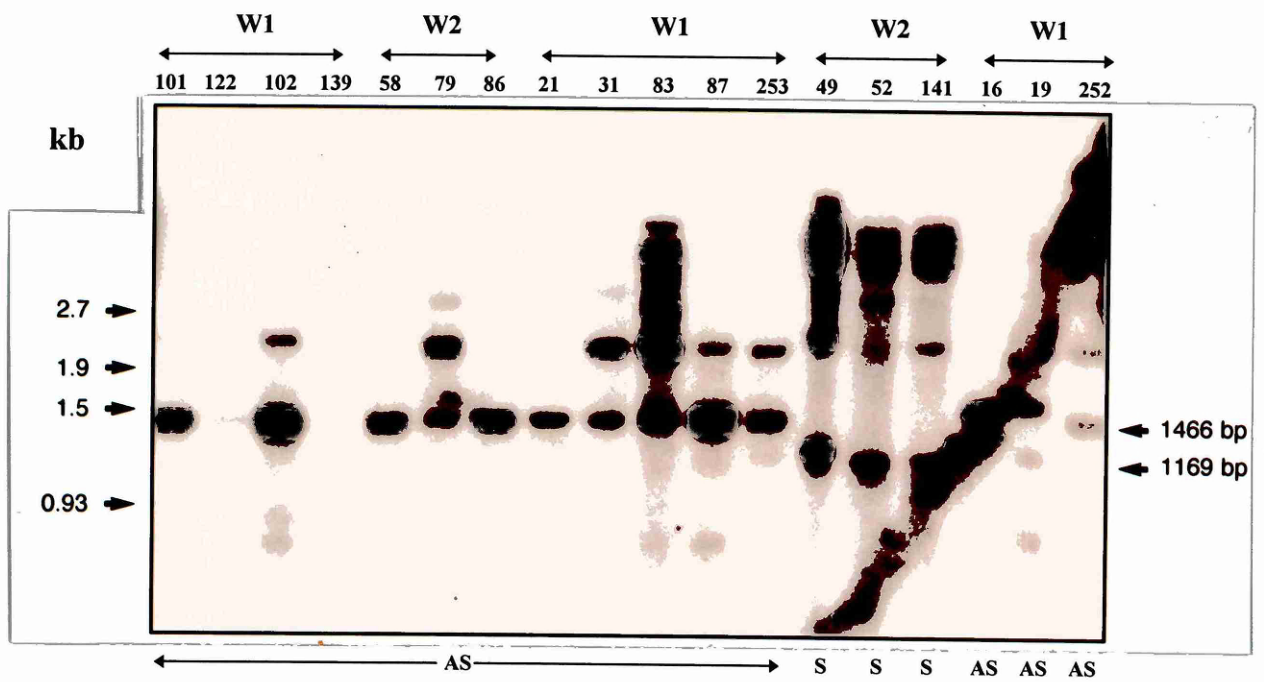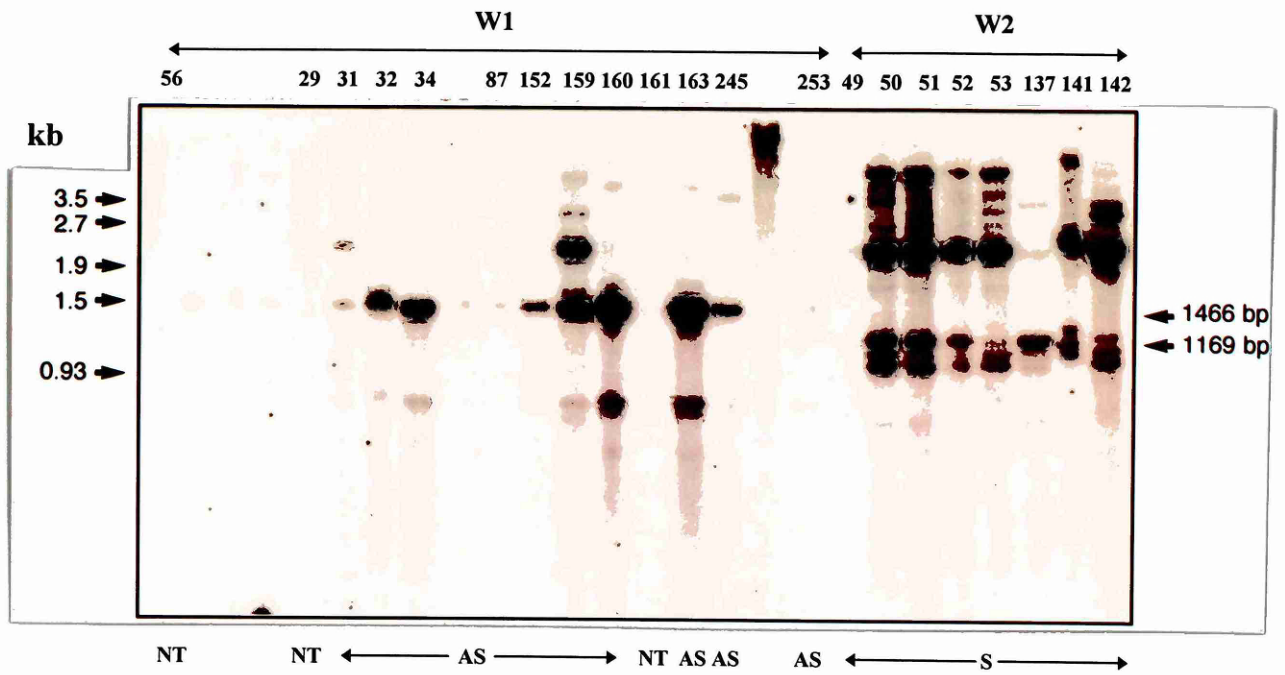
Figure 5.3   Detection of the transgene by Southern analysis, continued

5.2.4   Analysis of *cel1* and *cel2* expression in EGase transgenic strawberry plants

Duplicate northern blots of total RNA extracted from each primary transformant were hybridized with *cel1* and *cel2* cDNA probes and exposed to X-ray film for the same length of time. The levels of expression of *cel1* varied over a wide range between the different *cel1*-transformed lines and the control plants (Figure 5.4). In ripe fruit from several independent antisense and sense *cel1*-transformed lines (plant numbers W1 31, 159, 160, 163, W2 86 and W2 49, 50, 51, 52, 53, 137 respectively) there were no detectable *Cel1* transcripts. These plants represented approximately 25% of the confirmed *cel1*-transformed lines. In further *cel1*-transformed lines (antisense W1 77, 83, 143, W2 72, 79, 95, 104 and sense W2 141) the accumulation of *Cel1* mRNA in ripe fruit was strongly suppressed compared to that in the control-transformed lines, non-transformed and untransformed wild-type control plants. In fruit from one sense line (W2 142), *cel1* expression was higher than in the control plants. It should be noted that the *cel1* cDNA probe used should hybridize to both the endogenous *Cel1* transcripts and the transcripts derived from the *cel1* transgene. The endogenous *cel1* gene produces a transcript of size 1.9 kb whereas the *cel1* transgene should produce a transcript of size 1779 bp, corresponding to the size of the *cel1* cDNA used to construct the transgene. The sizes of these transcripts are too similar to allow their corresponding hybridizing bands to be distinguished from each other on the northern blot. However, the absence of any detectable transcripts in the lines described indicates that the expression of both the endogenous *cel1* gene and the transgene were concomitantly down-regulated. In ripe fruit the down-regulation of *cel1* expression did not affect the expression of *cel2*, which remained relatively constant in all plants (Figure 5.5).
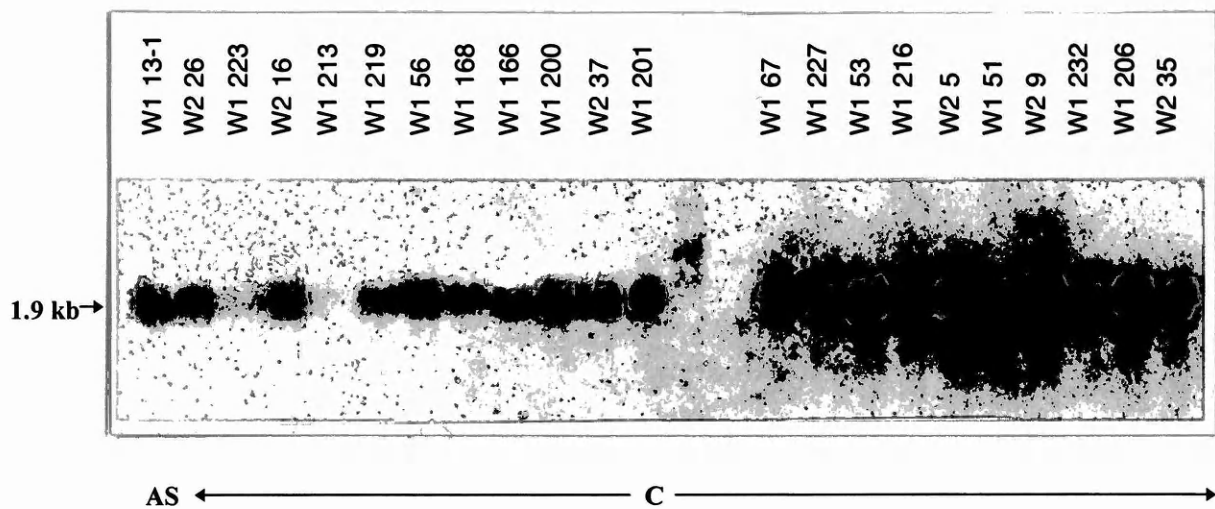
218

Figure 5.4   Northern analysis of *cell* expression in transgenic strawberry plants.

Each plant number is an independent transformation line. WT indicates

an untransformed wild-type strawberry cv Calypso control plant. AS and

S represent antisense and sense *cell*-transformed lines respectively. NT

(non-transformed) represents lines that were not successfully

transformed. The size of the transcript is shown on the left (kb)

Figure 5.4   Northern analysis of *cell* expression in transgenic strawberry plants,

continued

Figure 5.5   Northern analysis of *cel2* expression in transgenic strawberry plants.

Each plant number is an independent transformation line. WT indicates

an untransformed wild-type strawberry cv Calypso control plant. AS and

S represent antisense and sense *cel1*-transformed lines respectively. NT

(non-transformed) represents lines that were not successfully

transformed. The size of the transcript is shown on the left (kb)

2.6 kb →

All AS except for * which are NT

2.6 kb →

S NT AS NT AS AS AS AS AS S S AS AS   AS AS S AS S AS AS S AS AS S AS S

Figure 5.5   Northern analysis of *cel2* expression in transgenic strawberry plants,

continued

5.2.5   Phenotype of EGase transgenic strawberry plants

There were no observed transgene-related phenotypic differences between the *cel1*-transformed strawberry plants and the control-transformed, the non-transformed and the untransformed wild-type control plants (Figure 5.6).

Figure 5.6   Phenotypes of control-transformed and *cel1*-transformed strawberry cv Calypso plants

5.2.6 Effect of down-regulation of *cel1* on EGase activity in EGase transgenic strawberry plants

Endoglucanase activity was measured viscometrically in ripe fruit from each antisense, sense and control-transformed line along with non-transformed and untransformed wild-type control plants. Substantial differences were observed in the specific enzyme activities between the plants. Three *cel1*-transformed lines (plant numbers W1 19, 122 and W2 79) showed markedly lower EGase specific activities than any of the other lines and two showed higher levels (plant numbers W2 95 and 142). However, there was no statistically significant difference in the activity of any of the *cel1*-transgenic lines when compared to the control transgenic lines and the non-transformed and untransformed wild-type control plants (Figure 5.7).

5.2.7 Effect of down-regulation of *cel1* on fruit firmness of EGase transgenic strawberry plants

The firmness of ripe fruit from each antisense, sense and control-transformed line along with non-transformed and untransformed wild-type control plants showed lower variation between plant lines than did the EGase specific activities. None of the lines showed markedly lower or higher fruit firmness values than any of the other lines. No statistically significant differences in texture, as measured with a penetrometer, were found between *cel1*-transformed lines and control-transformed lines and non-transformed and untransformed wild-type control plants (Figure 5.8).
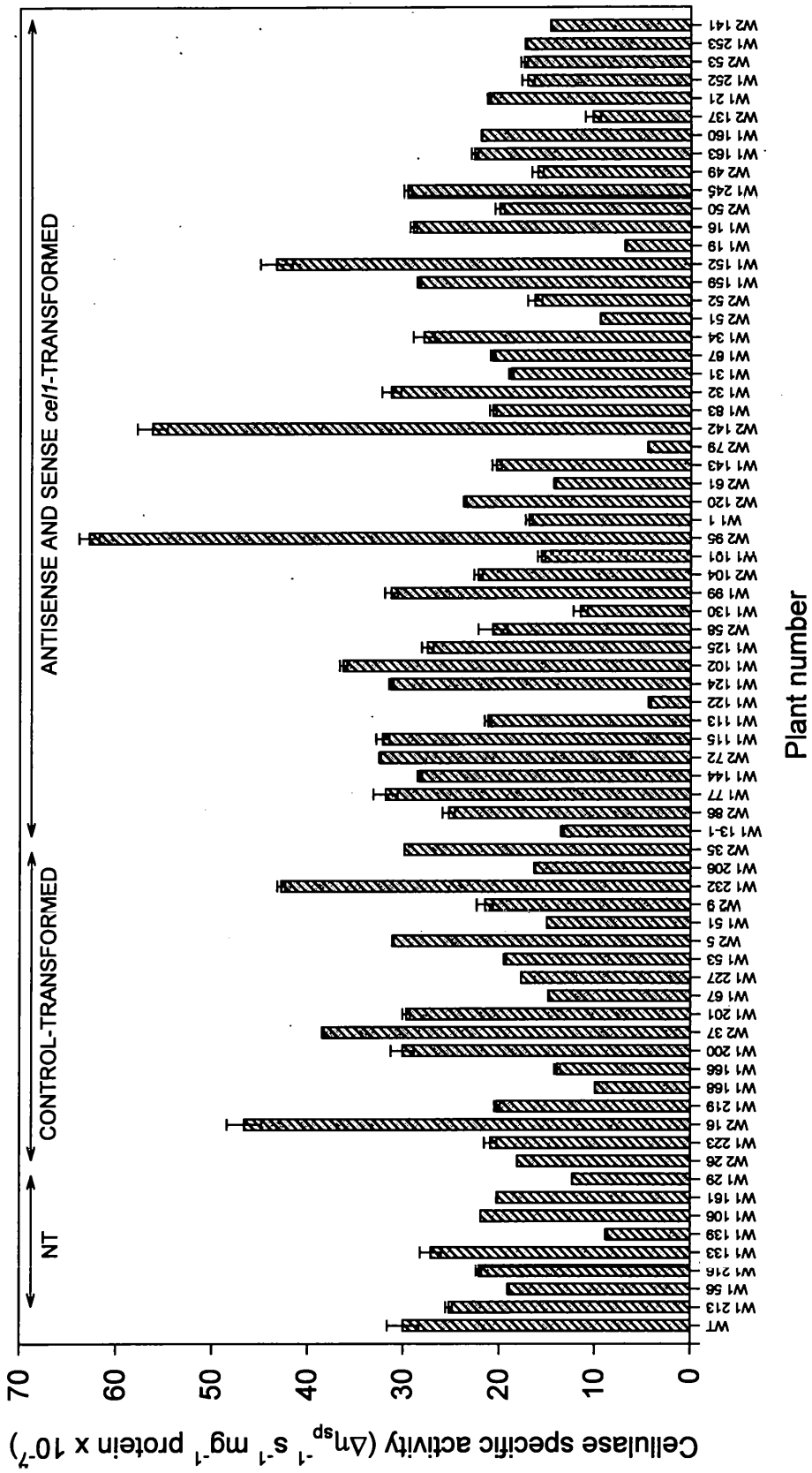
Figure 5.7   EGase specific activity in ripe fruit of transgenic strawberry plants. Each plant is an independent line. WT indicates an untransformed wild type strawberry cv Calypso plant. NT (non-transformed) represents lines that were not successfully transformed. Specific activity is expressed as the mean of two replicate assays ± SEM
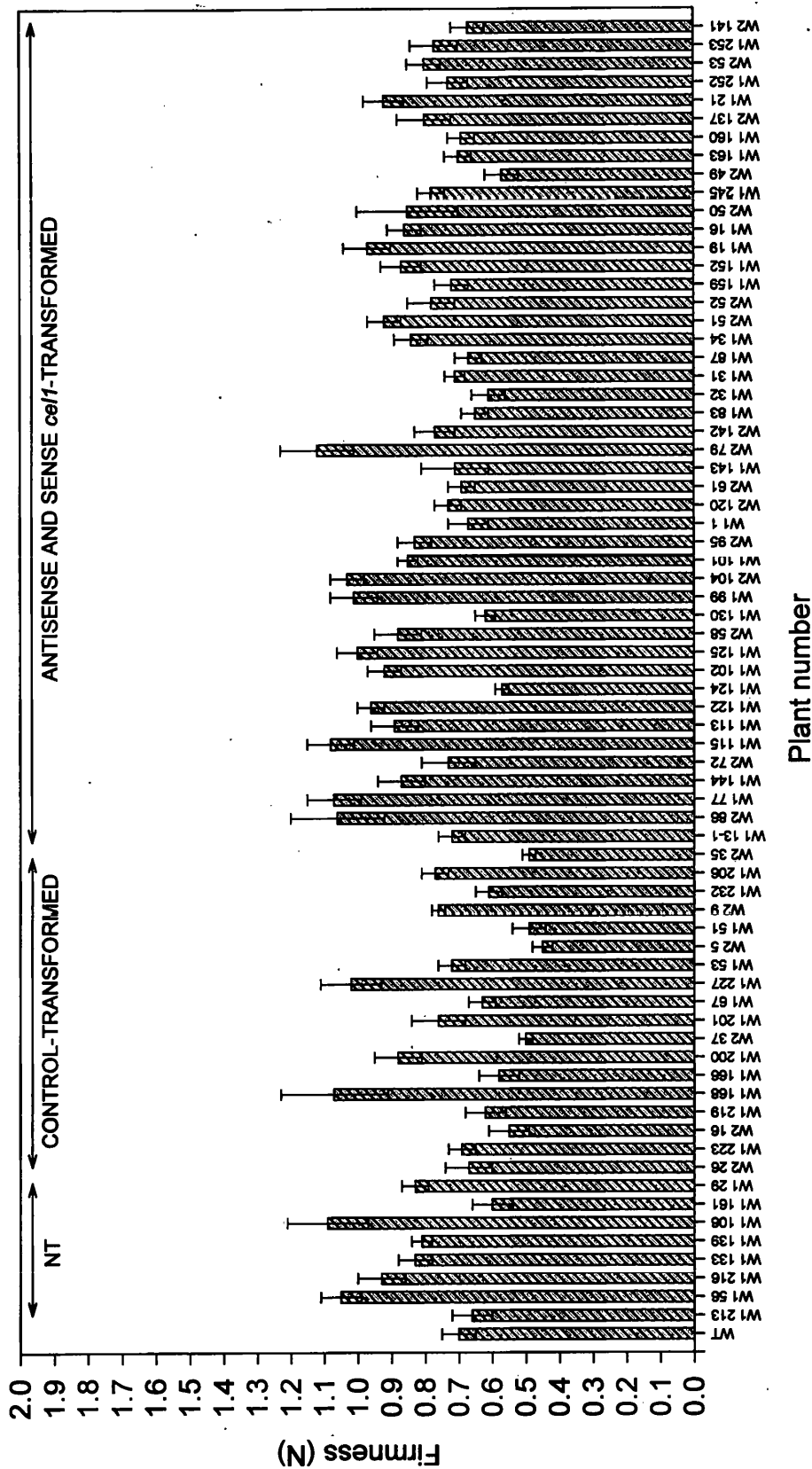
Figure 5.8   Firmness of ripe fruit of transgenic strawberry plants. Each plant number is an independent line. WT indicates an untransformed wild type strawberry cv Calypso plant. NT (non-transformed) represents lines that were not successfully transformed. Firmness is expressed as the mean of ten replicate measurements ± SEM

5.3   SUMMARY

The isolated ripening-related EGase cDNA *cel1* was used in the binary transformation vector pBINPLUS to produce both antisense and sense transgene constructs to modify the expression of strawberry *cel1*. Strawberry cv Calypso plants were successfully transformed with the constructs and also with a control construct lacking the *cel1* transgene. Southern analysis was used to confirm the presence of the transgene in 45 independent *cel1*-transformed lines. Of these, 37 lines contained the antisense transgene and the remainder contained the sense transgene.

Northern analysis showed no detectable *Cel1* transcripts were present in ripe fruit of 25% of the *cel1*-transformed lines. In further lines the accumulation of *Cel1* mRNA in ripe fruit was strongly suppressed compared to that in the control plants. The down-regulation of *cel1* did not affect the expression of *cel2*, which remained relatively constant in all plants.

There were no apparent phenotypic differences in the *cel1*-transformed plants or their fruit compared to any of the controls. Similarly, no statistically significant differences in the EGase activity or firmness of ripe fruit were found between *cel1*-transformed lines and the control plants.

5.4   DISCUSSION

GENERATION OF ENDO-β-1,4-GLUCANASE TRANSGENIC STRAWBERRY PLANTS

Strawberry plants were successfully transformed with both an antisense and sense *cell* transgene under the control of the constitutive CaMV 35S promoter in the binary vector pBINPLUS. This vector has the plant selectable marker gene *nptII* for kanamycin resistance adjacent to the left T-DNA border and the transgene expression cassette adjacent to the right T-DNA border. Since transfer of the T-DNA from *Agrobacterium* to plant cell occurs from right to left (Sheng and Citovsky, 1996) kanamycin-resistant transformed plants should contain the transgene.

Agrobacterium-mediated transformation procedures have been reported for different cultivars of octoploid strawberry, *Fragaria x ananassa* Duch. (James *et al.*, 1990; Nehra *et al.*, 1990a,b; Mathews *et al.*, 1995; Barcelo *et al.*, 1998) and also for the diploid *Fragaria vesca* (El Mansouri *et al.*, 1996; Haymes and Davis, 1998). The transformation frequencies reported vary depending on the cultivar and transformation procedure used. The cultivar used in this work, *Fragaria x ananassa* Duch. cv Calypso, was chosen for its high transformation efficiency compared to the other cultivars studied (D. J. James, personal communication). A limited selection strategy was used for the selection of transformed tissue. A period of 3 weeks selection on kanamycin was followed by 6-8 weeks without kanamycin. This allows a compromise between minimizing the number of escapes and reducing the regeneration time of transformed tissue (James *et al.*, 1990; Barcelo *et al;.*, 1998). The initial antibiotic selection reduces

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the proliferation of non-transformed cells which can lead to the regeneration of non-transformed shoots or chimaeras containing both transformed and non-transformed cells, known as escapes. The removal of selection then allows the regeneration of shoots, the majority of which should be transformed, without antibiotics present to interfere with their development. A minimal regeneration time is preferable to reduce the possibility of somaclonal variation in the plants. A final period of regeneration on kanamycin provides a further round of selection.

Southern analysis confirmed that the transgene was present in 45 of the 50 putative independent antisense and sense *cell*-transformed lines. The 5 lines that did not contain the transgene were considered escapes. The differences in relative intensities of the predicted hybridizing bands from these lines suggests that some transformants contain a low number of transgene insertions (eg. W1 152 and 115) whilst others may contain several insertions (eg. W1 87 and 163). The presence of multiple hybridizing bands in some lines (eg. W1 130 and 143) indicates that rearrangement of the inserted transgene may have occurred (Register, 1997).

DOWN-REGULATION OF *CEL1* EXPRESSION IN TRANSGENIC STRAWBERRY PLANTS

To address the role of Cel1 in fruit softening, strawberry plants were transformed to down-regulate *cell* expression. Northern analysis revealed considerable differences in *Cell* transcript levels between independently transformed lines. This observed variation is characteristic of primary transformants and is believed to be a result of the random nature of the integration of the transgene into the plant genome (Hansen and Wright,

1999). Both the chromosomal location of the insertion and the number and arrangement of the copies will vary between independent primary transformants such that one or more intact or rearranged transgene copies are integrated at one or more unlinked loci (Vaucheret *et al.*, 1998). These factors may affect the degree of expression of the transgene and consequently the expression of the target endogenous gene, resulting in primary transformants with different levels of down-regulation. Indeed the Southern analysis suggested the presence of different copy numbers of the transgene in the independent *cel1* primary transformants, although the sites of insertion could not be determined from this particular analysis.

Position effects have been demonstrated in experiments in which polygalacturonase (PG) was down-regulated by an antisense transgene in tomato (Smith *et al.*, 1990a). Transformed plants that contained a single copy of the transgene had 50-95% lower PG activity, indicating that the integration site of the transgene must have influenced the degree of PG suppression. However, selfed progeny of these plants that were homozygous for the transgene exhibited a further reduction in PG enzyme expression, down to 1% of wild-type activity, clearly demonstrating a gene dosage effect (Smith *et al.*, 1990a). Transgenic petunia plants exhibited no correlation between the number of antisense chalcone synthase (CHS) transgene copies inserted and the level of antisense mRNA, indicating the influence of the transgene insertion site on the level of gene expression. In turn, the level of antisense mRNA did not correlate with the flower phenotype achieved (van der Krol *et al.*, 1988). In transgenic petunia in which a sense dihydroflavonol-4-reductase (DFR) gene was introduced, again no correlation was observed between transgene copy number or transcript levels and the degree of endogenous gene suppression or the resulting flower phenotype (van der Krol *et al.*,

1990). Many transgenic experiments have demonstrated that there is little or no correlation between the level of down-regulation and the transgene copy number or expression level (Bourque, 1995).

The *cell* primary transformants exhibited expression of *cell* that ranged from wild-type levels to complete suppression in 25% of the lines. In lines in which *cell* expression was strongly down-regulated but not totally suppressed the residual transcripts could have resulted from expression of either the endogenous gene or the transgene, or both. The sizes of the two transcripts are too similar to allow a reliable distinction between them from the northern analysis. In the antisense lines the use of single-stranded antisense or sense probes would have enabled the specific detection of the endogenous transcript or transgene transcript, respectively. However in the sense lines, only a probe designed to the 5' sequence of the *cell* gene, which is absent in the transgene, could have distinguished both transcripts.

In every *cell*-transformed line which showed total down-regulation of *cell* expression there was concomitant suppression of the expression of both the endogenous *cell* gene and the *cell* transgene. Such coordinate down-regulation was first observed when homologous CHS genes were introduced into petunia. Transcripts from both the endogenous CHS gene and the introduced CHS gene were suppressed demonstrating the phenomenon of cosuppression of homologous genes in trans (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). This effect has also been observed in transgenic tomatoes where a chimaeric sense transgene, homologous to both PG and pectinesterase (PE), caused simultaneous down-regulation of both endogenous PG and PE genes and the transgene itself (Seymour *et al.*, 1993). Down-regulation of PG alone has also been achieved in tomato by cosuppression (Smith *et al.*, 1990b).

Thus cosuppression is generally used to describe the suppression of endogenous gene expression by the introduction of a homologous (sense) transgene which itself is concomitantly silenced (Stam *et al.*, 1997; Depicker and Van Montague, 1997). However, of the 25% of *cell*-transformed lines that exhibited down-regulation of both the endogenous gene and transgene, only about half of them were transformed with the sense transgene. The other half contained the antisense transgene indicating that cosuppression can occur regardless of the orientation of the introduced transgene. There are a few instances in which limited cosuppression has been observed in antisense plants (Hamilton *et al.*, 1995). In transgenic tomatoes containing an antisense PG transgene, reduction in endogenous PG expression correlated with a reduction in the expression of the antisense transgene (Smith *et al.*, 1988). In a similar experiment, Sheehy *et al.* (1988) also reported that levels of an antisense PG transcript may have been decreased in tomato plants in which endogenous PG was down-regulated. However there are numerous reports of antisense suppression of endogenous genes where the antisense transcript is clearly still present. It may be that the phenomenon of cosuppression occurs less frequently and with greater variation in antisense plants than in sense plants (Hamilton *et al.*, 1995). The observation that cosuppression can occur in antisense plants and sense plants has lead to the suggestion that similar mechanisms of suppression may operate in both situations (Hamilton *et al.*, 1995; Bruening 1998).

RELATIONSHIP BETWEEN *CEL1* EXPRESSION, ENDO-β-1,4-GLUCANASE
ACTIVITY AND FIRMNESS IN FRUIT OF TRANSGENIC STRAWBERRY
PLANTS

Ripe fruit from each of the transformed lines were analyzed for EGase activity and fruit firmness. Although variation was observed in both parameters, down-regulation of *cel1* expression, even to undetectable levels, had no statistically significant effect on the EGase activity or firmness of ripe fruit from any of the *cel1*-transgenic lines when compared to the control plants.

In fruit where *cel1* expression was not completely suppressed the reduced transcript level may still allow normal levels of Cel1 enzyme to accumulate such that levels of EGase activity remain unchanged. This was observed when antisense transgenes to thylakoid membrane proteins in tobacco successfully modified the transcript levels, in some cases down to 10% of untransformed levels, but not levels of the corresponding proteins (Palomares *et al.*, 1993). However, a close correlation is usually observed between the reduction in transcript levels and the reduction in enzyme activity. In transformed tomato, the expression of PG mRNA was reduced to 15-20% of wild-type levels and PG activity was also reduced to 20% (Smith *et al.*, 1990a). In transformed petunia, down-regulation of CHS resulted in an almost complete loss of both endogenous CHS mRNA and protein (van der Krol *et al.*, 1990).

The unchanged level of EGase activity in fruit with reduced or undetectable *cel1* expression may be explained by the presence of a second EGase gene (*cel2*) in strawberry, which was identified after this work was initiated. The *cel1* transgene used should specifically down-regulate *cel1* as *cel1* and *cel2* are only 59% homologous at the

nucleotide level. In this case, the activity of Cel2 must increase to compensate for the expected loss of Cel1 activity. The extent of the expected increase in Cel2 cannot be predicted as the relative contributions of Cel1 and Cel2 to the total activity are unknown. The assay for EGase detects total activity and does not differentiate between the different isoforms. However, northern analysis showed that the expression of *cel2* was essentially unaffected in the *cel1*-suppressed plants indicating there was little compensation by the *cel2* gene. In addition, contribution of, and possible compensation by, any as yet undetected EGase isoforms must also be considered. Physiological compensation for a deficient enzyme by the production of a functionally equivalent protein has been reported in tobacco plants. Class I β-1,3-glucanase activity, which is believed to be involved in plant defense responses to pathogen attack, was down-regulated by the introduction of an antisense transgene. Induction of the enzyme after pathogen infection was inhibited but was compensated for by the production of a distinct β-1,3-glucanase (Beffa *et al.*, 1993).

Given the unchanged EGase activity as a result of *cel1* suppression it is not surprising that there was no apparent effect on fruit firmness. Although the role of Cel1 in fruit softening has not been revealed, the absence of any effect of *cel1* suppression on fruit texture implicates the involvement of other cell wall proteins, including Cel2 in the softening process. In cases where a cell wall modifying enzyme has been successfully down-regulated these do not always provide information about its role in fruit softening. When a substantial reduction in the corresponding enzyme activity is achieved there may be several possible reasons why an effect on cell wall metabolism and fruit softening is not observed: a) the residual enzyme activity may be sufficient to maintain normal function. b) the method chosen for analysis of fruit texture may not be able to

detect any underlying changes that have occurred in cell wall metabolism. c) other isoforms of the enzyme may be dominant in the softening process. d) the coordinate action of several cell wall modifying enzymes may be required for softening, none of which by themselves are fully responsible for changes in fruit texture. e) the enzyme is not involved in softening and may have an alternative role in the cell.

Extensive transgenic studies on the cell wall metabolism of ripening tomato fruit have clearly demonstrated the potential difficulties that can be encountered. Down-regulation of PG enzyme activity in transgenic tomato plants containing an antisense PG transgene had no effect on fruit softening as measured by fruit compressibility (Smith *et al.*, 1988). However, ripe fruit from the progeny of these plants did show reduced depolymerization of the soluble pectin, although pectin solubilization was unaffected and the compressibility of these fruit was also unchanged. It was suggested that, in the case of tomato fruits, compressibility is only one aspect of softening and that other physical parameters that were not measured may have changed (Smith *et al.*, 1990a). In fact, although softening of these fruits appeared indistinguishable from that of wild-type fruit, their pectin metabolism was affected and this resulted in altered characteristics that were only discovered after further study. The fruit were found to be more resistant to mechanical damage and produced pastes with higher viscosity and increased soluble solids, characteristics which are commercially valuable (Gray *et al.*, 1994). The residual low PG activity in the transgenic plants may have been sufficient to maintain normal levels of pectin solubilization (Smith *et al.*, 1990a). This demonstrates the importance of the type of analysis performed on fruit with altered levels of cell wall enzymes and highlights the difficulties with interpretation that can occur when enzyme activity has not been completely suppressed.

The expression of a fruit-specific pectinmethylesterase (PME) antisense transgene in tomato greatly reduced the activity of the group I isoforms of PME present in fruit. It had no effect on the activity of the group II isoforms present in either fruit or vegetative tissues. The absence of any noticeable effects on growth and development led to the suggestion that the group I PME isoforms were not involved in fruit development and ripening. However, the possibility that the different isoforms have different modes of action on pectins or that the action of group II isoforms may substitute for group I isoforms in their absence was also considered. The conclusion was that the role of PME could not be determined by studying a specific isoform alone and that a study of the effect of manipulating the other isoforms in transgenic plants was also necessary (Gaffe *et al.*, 1994). As in the case of the PG antisense tomatoes, further work was able to reveal an effect of the suppressed enzyme. The fruit-specific PME enzyme did not affect fruit firmness during ripening, but was shown to cause an almost complete loss of tissue integrity during fruit senescence (Tieman and Handa, 1994).

The roles of specific EGases in tomato fruit ripening have been investigated in a similar way as the role of Cel1 has been studied in strawberry, by the down-regulation of a single EGase gene in transgenic plants. In tomato there are at least seven different isoforms of EGase resulting from a divergent gene family. The expression of two members of this gene family, *cel1* and *cel2*, increases during fruit ripening (Lashbrook *et al.*, 1994). The pattern of tomato *Cel2* mRNA accumulation in fruit parallels that of strawberry *Cel1* transcripts, whilst the expression of tomato *cel1* in fruit is more similar to that of strawberry *cel2*. However, the expression of both tomato *cel1* and *cel2* is not restricted to fruit and so in this respect they are more similar to strawberry *cel2* than to strawberry *cel1*, which is fruit-specific. In separate studies the expression of tomato *cel1*

and *cel2* genes was independently down-regulated by antisense suppression. The constitutive expression of an antisense *cel1* transgene resulted in the reduction of *Cel1* transcripts to trace levels in ripening fruit of transgenic tomato plants. Transgenic fruits in which *cel1* expression was suppressed to less than 0.1% of wild-type levels exhibited normal growth and softening behaviour, indicating that Cel1 is not a primary determinant of cell expansion in early fruit development or softening of tomato fruit. This may be expected as *cel1* is only transiently expressed in immature green fruit and reaches a maximum in pink fruit before declining in over-ripe fruit (Lashbrook *et al.*, 1994). However, as already discussed, the authors noted that the absence of an effect of Cel1 on softening as measured by fruit compressibility may not reflect a lack of any effect on cell wall structure (Lashbrook *et al.*, 1998).

In similar experiments, *Cel2* mRNA abundance was reduced by >95% in ripe fruit of transgenic tomato plants. The softening of fruit in which *cel2* was suppressed was indistinguishable from that of control fruit as determined by measurement of fruit firmness. Hence the role of Cel2 in softening or textural changes in fruit during ripening suggested by its considerable accumulation in the later stages of ripening, was not revealed by the suppression of *cel2* expression (Brummell *et al.*, 1999a).

However, the level of the corresponding EGase enzyme activity in the transgenic fruit was not determined in either case. It may be that although both *Cel1* and *Cel2* transcript levels were reduced, the level of EGase activity remained constant as one isoform compensated for the lack of the other, as discussed for the strawberry *cel1*-suppressed plants. It may be, therefore, that the suppression of both isoforms together is required to observe any effect on softening or fruit quality characteristics.

Parallels can be drawn from the results from the suppression of specific EGase genes in tomato and those obtained here from the down-regulation of *cel1* in strawberry. It is clear that suppressing an individual EGase gene in fruit where other members of the EGase gene family are expressed does not neccessarily reveal its role in fruit softening. At the very least, it is likely that transgenic strawberry plants reduced in the ability to express all fruit-specific EGase genes will be required to determine the role of EGase in softening.

However, it is possible that even this approach may not be sufficient. Although fruit of the tomato non-softening *rin* mutant lack both *Cel1* and *Cel2* mRNA they are also deficient in mRNAs encoding a whole range of cell wall modifying enymes including PG, expansins and β-galactosidase. They also have reduced levels of activity of xyloglucan endotransglycosylase (XET), β-galactanase and probably other enzymes (Brummell *et al.*, 1999a). Thus softening is likely to be the result of the synergistic activity of many cell wall modifying enzymes all of which may play a role to varying extents in the cell wall disassembly that leads to fruit softening. It is therefore possible that even the suppression of all isoforms of one particular enzyme may not have a distinct, measurable effect on fruit softening due to the presence of other enzyme activities that contribute to the overall softening process. Any observed effect on softening may depend on whether a particular enzyme affects the cell wall either directly or influences the action of other cell wall modifying proteins. In one recent report it has been demonstrated that modification of expansin activity in transgenic tomato plants had a direct effect on fruit softening. Fruits in which Exp1 protein accumulation was suppressed to 3% of wild-type levels resulted in firmer fruit whilst overexpression of *exp1* enhanced fruit softening. The results indicate a direct role for expansin in cell wall

disruption but also that expansin action may indirectly affect changes in the cell wall brought about by cell wall hydrolases (Brummell *et al.*, 1999b).

It can be seen therefore that elucidation of the roles of cell wall modifying proteins in fruit softening may require the coordinate suppression of multiple isoforms of one or more different proteins. This reflects the complex nature of the action of the enymes and proteins that effect the cell wall disruption leading to fruit softening. This approach appears to be feasible as the down-regulation of two (or more) non-homologous genes has already been demonstrated in transgenic tomato plants by the use of single chimaeric transgenes (Seymour *et al.*, 1993; Jones *et al.*, 1998b; Simons and Tucker, 1999).

GENERAL DISCUSSION AND FURTHER WORK

ROLE OF ENDO-β-1,4-GLUCANASES IN PLANT DEVELOPMENT

The results of this study indicate a role for EGases in the softening associated with strawberry fruit ripening. The increase in EGase activity in strawberry fruit throughout development parallels increases in the expression of the two strawberry EGase genes identified, *cel1* and *cel2*. The coincidence of maximum expression of *cel1* and *cel2* just prior to the time of greatest EGase activity and ripening-associated loss of fruit firmness strongly suggests that the products of these genes, Cel1 and Cel2, have roles in fruit softening. Purified strawberry Cel1 was found to be capable of hydrolyzing xyloglucan and thus the role of strawberry Cel1, at least, in fruit softening may be to disrupt the cellulose-xyloglucan network and cause loosening of the cell wall by its action on xyloglucan. Down-regulation of *cel1* expression in transgenic strawberry plants implicated the involvement of other cell wall modifying proteins, including Cel2, in the softening process. A role for specific EGases in fruit softening has been reported for many other plants, the most well-studied being tomato Cel1 and Cel2 (Lashbrook *et al.*, 1994), avocado Cel1 (Cass *et al.*, 1990) and pepper Cel1 (Harpster *et al.*, 1997).

In addition to their role in fruit softening, plant EGases are involved in a range of physiological processes that require cell wall modification including cell expansion and abscission, events in which cell wall loosening or cell separation is necessitated. The expression of strawberry *cel2* in young developing fruit and vegetative tissues, in addition to ripening fruit, indicates that Cel2 may also facilitate cell growth and

expansion by loosening the cell wall. The *Arabidopsis cel1* gene has been reported to show strong expression in the elongating zone of flowering stems but transcripts were undetectable in fully expanded leaves. Transgenic tobacco plants containing the *Arabidopsis cel1* promoter driving expression of the *gus* reporter gene showed the promoter was active in shoot and root elongating zones suggesting an involvement of Cel1 in cell expansion in *Arabidopsis* (Shani *et al.*, 1997). Similarly, the pea *EGL1* gene is thought to have a role in cell elongation. The transcript was found to accumulate predominantly in flowers and young pods undergoing rapid growth but levels declined rapidly in pods when they were no longer elongating. A high level of expression of this gene was also detected in elongating epicotyls (Wu *et al.*, 1996).

Abscission-related EGases have also been well characterized in many plants and are implicated in developmental processes involving the abscission of a variety of different organs (Brummell *et al.*, 1994). Expression of the avocado EGase Cel1 was associated with the abscission of mature fruits as well as their softening. Transcripts of this gene accumulated in activated fruit abscission zones, as well as ripe mesocarp, and an antibody raised against the EGase protein isolated from ripe fruit cross-reacted with a protein in extracts from fruit abscission zones (Tonutti *et al.*, 1995). Two isoforms of EGase were detected in fruit abscission zones of orange by isoelectric focusing. The corresponding genes were expressed in activated fruit abscission zones and in activated leaf and floral abscission zones (Burns *et al.*, 1998). Similarly, fruit abscission in peach is associated with two EGase isoforms and these are also present in leaf abscission zones, although at considerably higher levels (Bonghi *et al.*, 1992). In contrast, although bean leaf abscission zones contain two isoforms of EGase, only the basic form is involved in abscission (Tucker *et al.*, 1988). Three of the multiple tomato EGase genes,

cel1, *cel5* and *cel6*, were expressed predominantly in floral abscission zones and the accumulation of their transcripts was correlated with flower shedding (del Campillo and Bennett, 1996). *Cel5* mRNA has also been detected in leaf abscission zones (Kalaitzis *et al.*, 1999).

Thus, plant EGases from many different species have been linked to a variety of physiological processes either by the identification of EGase genes and their temporal and spatial expression patterns or by the purification of EGase isoforms and characterization of their activities. All of these processes in which EGases are reported to have a role involve cell wall modifications, either leading to loosening of the wall or to complete degradation. However, further work is required to fully understand the mechanism of action and the extent to which EGases are involved in these processes.

FURTHER WORK

This study has started to explore the role of EGases in strawberry fruit development by examining the ripening-related EGase Cel1. The confirmation that there is a multigene family for EGase in strawberry necessitates investigation into the role of each isoform in fruit development and how the different isoforms may interact to modify strawberry fruit cell walls and cause softening. The following are suggestions for further work which should provide a fuller understanding of the role of EGases in strawberry fruit development.

a) Strawberry endo-β-1,4-glucanase genes

So far two distinct EGase genes have been identified in strawberry. The multiple gene families for EGase in other plants and the results of the Southern analysis here indicating the presence of a small gene family in strawberry, suggest that further EGase genes may be present in strawberry. This possibility could explain the results from the *cell*-transformed plants and any future experiments to down-regulate EGase activity in strawberry fruit would have to take this into account. A lower stringency screening of the ripe fruit cDNA library may identify additional EGases that are present in ripe fruit. However, a more comprehensive approach to identify all EGases in strawberry would be to use degenerate primers designed to the conserved regions of EGase genes to perform PCR with genomic DNA as template. Alternatively, degenerate primers could be used in RT-PCR with RNA isolated from a range of strawberry tissues to examine EGases specifically expressed in these tissues. This would allow the identification of genes that may be involved in aspects of strawberry development other than fruit softening, the expression patterns of which could then be characterized. The octoploid nature of strawberry must be taken into account during the identification of further genes. The multiple copies, or homeologues, of a particular gene in a polyploid species are not always identical. Thus, any further EGase genes identified in octoploid strawberry that share just less than 100% sequence identity with others most likely represent different homeologues of the same locus and not divergent members of the gene family. Individual homeologues may not share the same expression patterns and there may be considerable difficulties in distinguishing the expression of genes with highly similar sequences. Indeed a *cell* genomic clone has been isolated from cv Brighton whose

sequence in the coding region differs by 3 nucleotides from the *cell* cDNA isolated from cv Brighton here (Manning, personal communication) and it is possible that the genomic clone represents a different homeologue to the cDNA clone.

b) Antibodies to strawberry Cel1

The generation of antibodies raised to Cel1 would allow the level of Cel1 protein throughout fruit development to be determined and correlated with the increase in *cell* expression and the total EGase activity in the fruit. More importantly it would allow levels of Cel1 protein to be determined in fruit from the *cell*-transformed plants. If, as may be expected, in transformed lines in which *cell* expression was completely suppressed in the fruit there was no Cel1 protein, then the unchanged EGase activity in these fruit must be the result of compensation by other EGase isoforms.

c) Activity of strawberry Cel1 against strawberry cell walls

The substrate specificity of strawberry Cel1 was tested against a range of cell wall polymers found in plants. However, it would be relevant to determine the activity of Cel1 against the cell walls of strawberry fruit at different stages of development and also specific fractions of the cell wall. This would provide further information about the true *in vivo* substrate of the enzyme and at which developmental stage the substrate is susceptible to degradation by the enzyme. This may reveal whether the substrate has been modified in any way before Cel1 is able to act on it and hence give a better

understanding of the coordinated activities of multiple cell wall modifying proteins that may be required to cause cell wall degradation and fruit softening.

d) Strawberry Cel2

Purification of the second strawberry EGase isoform, Cel2, would allow its substrate specificity to be determined as for Cel1. This may reveal differences in the preferred substrates of the two enzymes which could help in understanding the different roles of the two isoforms in fruit development and how they may cooperate to modify the cell wall. A different strategy to purify Cel2 would be required from the one successfully used for Cel1. An alternative approach would be to examine the properties of recombinant Cel2 protein. However, as the native protein is likely to be glycosylated the recombinant protein would need to be processed correctly.

e) Further studies in transgenic plants

The aim of down-regulating *cel1* expression in strawberry plants was to produce fruit which contained no EGase activity. However, this approach was unsuccessful due to the presence of a further EGase gene, *cel2*, in strawberry which has since been identified and possibly the existence of further EGase genes in strawberry, as yet unidentified. It would be of interest to purify the EGase from transgenic plants in which Cel1 was completely suppressed. In order to determine the role of EGases in strawberry fruit softening, it is likely that plants in which the expression of all EGase genes is

suppressed will be required. Only then could plants be obtained in which no detectable EGase actvity was present and the result of this on fruit softening could be assessed.

A marked inhibition of fruit softening may only be possible if several cell wall associated proteins are down-regulated together. These might include pectinmethylesterase(s), expansin(s), xyloglucan endotransglycosylase(s) and β-galactosidase(s). The role of EGases in the softening of strawberry fruit may be revealed more clearly if the influence of other synergistically acting proteins is elucidated.

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APPENDIX A. COMPOSITION OF MEDIA, BUFFERS AND SOLUTIONS

A1. MOLECULAR BIOLOGICAL REAGENTS

BACTERIAL MEDIA

Terrific Broth (TB) medium

12 g bactotryptone, 24 g yeast extract, 0.4 ml glycerol, 23.14 g KH_2PO_4 and 16.43 g $K_2HPO_4.3H_2O$ were dissolved in distilled water and made up to 1 l. The medium was sterilized by autoclaving and stored at $4^{\circ}C$.

TYN medium

10 g bactotryptone, 5 g yeast extract and 5 g NaCl were dissolved in distilled water and made up to 1 l. The medium was sterilized by autoclaving and stored at $4^{\circ}C$.

TYN plates

15 g agar was added to 1 l TYN medium before autoclaving.

TYN top agarose

0.7 g agarose was added to 100 ml TYN medium before autoclaving. The medium was cooled to $50^{\circ}C$ and sterile 1 M $MgCl_2$ was added to a final concentration of 10mM.

SOB medium

20 g bactotryptone, 5 g yeast extract and 0.5 g NaCl were dissolved in distilled water and made up to 1 l. The medium was sterilized by autoclaving. Before use, 10 ml each of sterile 1 M $MgCl_2$ and 1 M $MgSO_4$ were added.

SOC medium

2 ml sterile 20% (w/v) glucose were added to 100 ml SOB medium prior to use.

LB (Luria-Bertani) medium

10 g bactotryptone, 5 g yeast extract and 10 g NaCl were dissolved in distilled water and made up to 1 l. The medium was sterilized by autoclaving and stored at 4°C.

LB plates

20 g agar was added to 1 l LB medium before autoclaving. After the medium had cooled to 50°C, antibiotics were added where required at the appropriate concentration.

YEP medium

10 g yeast extract, 10 g bactopeptone and 5 g NaCl were dissolved in distilled water and made up to 1 l. The medium was sterilized by autoclaving and stored at 4°C.

ANTIBIOTIC STOCK SOLUTIONS

Ampicillin

The sodium salt of ampicillin was dissolved in sterile distilled water at a concentration of 25 mg ml^{-1}. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

Tetracycline

Tetracycline hydrochloride was dissolved in ethanol/sterile distilled water (50% v/v) at a concentration of 12.5 mg ml^{-1}. The solution was sterilized by filtering through a 0.2 μm filter and stored in the dark at -20°C.

Kanamycin

Kanamycin sulphate was dissolved in sterile distilled water at a concentration of 50 mg ml^{-1}. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

Streptomycin

Streptomycin sulphate was dissolved in sterile distilled water at a concentration of 50 mg ml^{-1}. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

Cefotaxime

The sodium salt of cefotaxime was dissolved in sterile distilled water at a concentration of 100 mg ml^{-1}. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

PLANT TISSUE CULTURE MEDIA

S5 Proliferation medium

4.4 g Murashige & Skoog (MS) basal salt mixture (Sigma) and 30 g sucrose were dissolved in 900 ml sterile distilled water, adjusted to pH 5.7 and made up to 1 l. 7.5 g agar powder (Merck) was added and the medium was autoclaved and allowed to cool to 35-45°C. The following sterile solutions were added :

1.0 ml 1 mg ml^{-1} 6-benzyladenine purine (BAP)
0.1 ml 1 mg ml^{-1} 3-indolebutyric acid (IBA)
0.2 ml 1 mg ml^{-1} giberellic acid 3 (GA$_3$)
1.0 ml 1000 x strawberry vitamins

and the medium poured into sterile Coulter pots (10 ml per pot).

R13 Rooting medium

4.4 g MS basal salt mixture and 30 g sucrose were dissolved in 900 ml sterile distilled water, adjusted to pH 5.7 and made up to 1 l. 7.5 g agar powder was added and the medium was autoclaved. After cooling, 3 ml 1 mg ml^{-1} IBA was added and the medium poured into sterile Coulter pots.

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<u>R37 Rooting medium</u>

2.2 g MS basal salt mixture and 30 g sucrose were dissolved in 900 ml sterile distilled water, adjusted to pH 5.7 and made up to 1 l. 7.5 g agar powder was added and the medium autoclaved and poured into sterile honey jars (100 ml per jar).

<u>1 mg ml^{-1} 6-Benzyladenine purine (BAP)</u>

0.1 g BAP was dissolved in 1 ml 1 M NaOH and made up to 100 ml in sterile distilled water. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

<u>1 mg ml^{-1} 3-Indolebutyric acid (IBA)</u>

0.1 g IBA was dissolved in 1 ml 1 M NaOH and made up to 100 ml in sterile distilled water. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

<u>1 mg ml^{-1} Gibberellic acid 3 (GA$_3$)</u>

0.1 g GA$_3$ was dissolved in 1 ml 1 M NaOH and made up to 100 ml in sterile distilled water. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

<u>1000 x Strawberry vitamins</u>

0.05 g nicotinic acid, 0.05 g pyridoxine-HCl and 0.01 g thiamine-HCl were dissolved in 100 ml sterile distilled water. The solution was sterilized by filtering through a 0.2 μm filter and stored at -20°C.

<u>MS20 solution</u>

4.4 g MS basal salt mixture and 20 g sucrose were dissolved in distilled water and made up to 1l. To each 1 ml of MS20 solution, 1 μl each of acetosyringone (AS) and betaine

phosphate (BP) solutions were added just before use. The pH was adjusted to 5.2 and the solution was sterilized by filtering through a 0.2 μm filter.

Acetosyringone (AS)

0.098 g AS was dissolved in 5 ml 100% ethanol. The solution was sterilized by filtering through a 0.2 μm filter and stored at 4°C.

Betaine phosphate (BP)

1.076 g BP was dissolved in 5 ml sterile distilled water. The solution was sterilized by filtering through a 0.2 μm filter and stored at 4°C.

Wash solution

0.05 g augmentin and 0.02 g cefotaxime were dissolved in 100 ml sterile distilled water. The pH of the solution was ajusted to 5.2 and sterilized by filtering through a 0.2 μm filter and stored at 4°C.

ZN102 medium

4.4 g MS basal salt mixture and 10 g sucrose were dissolved in 900 ml sterile distilled water, adjusted to pH 5.7 and made up to 1 l. 2.5 g gelrite (Sigma) was added and the medium was autoclaved. After cooling, 1 ml 1 mg ml^{-1} thidiazuron (TDZ) and 200 μl 1 mg ml^{-1} α-naphthaleneacetic acid (NAA) were added.

BUFFERS AND SOLUTIONS

TE Buffer, pH 7.5/8.0 (10 mM Tris, 1 mM EDTA)

1.211 g Tris and 0.372 g disodium ethylene diamine tetraacetate.2H$_2$O (Na$_2$EDTA.2H$_2$O) were dissolved in 800 ml distilled water, adjusted to pH 7.5/8.0 and made up to 1 l. The solution was sterilized by autoclaving.

10 mM Tris, pH 8.0

1.211 g Tris base was dissolved in 800 ml distilled water, adjusted to pH 8.0 with HCl and made up to 1 l. The solution was sterilized by autoclaving.

0.5 M EDTA, pH 8.0

186.1 g $Na_2EDTA.2H_2O$ was dissolved in 800 ml distilled water, adjusted to pH 8.0 with NaOH and made up to 1 l. The solution was sterilized by autoclaving.

Resuspension buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0)

0.45 g glucose, 0.186 g $Na_2EDTA.2H_2O$ and 0.151 g Tris were dissolved in 40 ml sterile distilled water, adjusted to pH 8.0 with HCl and made up to a final volume of 50 ml. The solution was sterilized by filtering through a 0.2 μm filter and stored at 4°C. Before use, lysozyme was added to a concentration of 2 mg ml^{-1}.

Lysis Buffer (0.2 M NaOH, 1% (w/v) SDS)

2 ml 5 M NaOH and 5 ml 10% (w/v) sodium dodecyl sulphate (SDS) were made up to 50 ml in sterile distilled water just before use.

50 x Tris-Acetate (TAE) buffer (2 M Tris, 0.05 M EDTA, pH 8.0)

242.3 g Tris and 18.6 g $Na_2EDTA.2H_2O$ were dissolved in 800 ml distilled water, adjusted to pH 8.0 with glacial acetic acid and made up to 1 l. The solution was sterilized by autoclaving. The stock solution was diluted 50 times to give a 1 x working solution.

TAE-agarose gel

1 g agarose was melted by heating in 2 ml 50 x TAE buffer and 98 ml sterile distilled water.

10 x DNA Sample Loading Buffer (50% (w/v) glycerol, 1 mM EDTA, 0.4% (w/v) bromophenol blue (BPB), pH 8.0)

25 ml glycerol, 0.1 ml 0.5 M EDTA, pH 8.0 and 0.2 g BPB were dissolved in distilled water to a final volume of 50 ml.

CTAB DNA Extraction Buffer (2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, pH 8.0, 2% (w/v) PVP-40, 1% (v/v) 2-mercaptoethanol)

100 ml 10% (w/v) hexadecyltrimethylammonium bromide (CTAB), 40.91 g NaCl, 3.7 g $Na_2EDTA.2H_2O$, 50 ml 1M Tris, pH 8.0 and 10 g polyvinylpyrrolidone-40 (PVP) were dissolved in distilled water to a final volume of 500 ml. Just before use 5 ml 2-mercaptoethanol was added.

CTAB Wash Buffer (76% (v/v) ethanol, 10 mM ammonium acetate)

380 ml 100% ethanol and 0.83 ml 6 M ammonium acetate were made up to a final volume of 500 ml in distilled water.

Phage Dilution Buffer (PDB) (50 mM Tris, 100 mM NaCl, 0.01% (w/v) gelatin, pH 7.9)

5.844 g NaCl, 6.057 g Tris base and 0.1 g gelatin were dissolved in 800 ml distilled water, adjusted to pH 7.9 with HCl and made up to 1 l. The solution was sterilized by autoclaving, cooled to RT and sterile 1 M $MgCl_2$ added to a final concentration of 10mM.

Buffer 1 (0.1 M maleic acid, 0.15 M NaCl, pH 7.5)

11.608 g maleic acid and 8.768 g NaCl were dissolved in 800 ml distilled water, adjusted to pH 7.5 and made up to 1 l. The solution was sterilized by autoclaving.

10% (w/v) Blocking Reagent

10 g Blocking reagent (Boehringer Mannheim UK) were dissolved in 100 ml Buffer 1 and autoclaved.

Blocking Buffer 2

10% Blocking reagent was diluted 10-fold in Buffer 1 to give a final concentration of 1%.

Buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5)

12.11 g Tris, 0.844 g NaCl were dissolved in 800 ml distilled water and adjusted to approximately pH 9.5. 10.165 g $MgCl_2$ were dissolved in water first then added to the solution. The pH was adjusted to pH 9.5.

RNA extraction buffer (0.2 M boric acid/Tris, 10 mM EDTA, pH 7.6)

12.366 g boric acid and 3.722 g $Na_2EDTA.2H_2O$ were dissolved in distilled water, adjusted to pH 7.6 with Tris and made up to 1 l. The solution was sterilized by autoclaving. Before use, 20 ml 25% (w/v) SDS and 20 ml 2-mercaptoethanol were added.

10 x MOPS buffer (0.2 M MOPS, 50mM sodium acetate, 10 mM EDTA, pH 7.0)

41.86 g MOPS free acid, 6.8 g sodium acetate and 3.72 g $Na_2EDTA.2H_2O$ were dissolved in 800 ml distilled water, adjusted to pH 7.0 with NaOH and made up to 1 l. The solution was sterilized by autoclaving.

Denaturing RNA gel

1 g agarose was melted by heating in 88.2 ml sterile distileed water and allowed to cool to approximately 50°C. 10 ml 10 x MOPS buffer and 1.85 ml 11.9 M formaldehyde solution were added.

RNA loading buffer

RNA samples were dissolved in buffer containing final concentrations of 1 x MOPS buffer, 2.2 M formaldehyde, 50% formamide, 10 μg ml^{-1} ethidium bromide, 5% glycerol, 0.1 mM EDTA, 0.04% BPB.

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<u>20 x SSC</u> (3 M NaCl, 0.3 M trisodium citrate, pH 7.0)

175.3 g NaCl and 88.2 g trisodium citrate were dissolved in 800 ml distilled water, adjusted to pH 7.0 with NaOH and made up to 1 l. The solution was sterilized by autoclaving.

<u>10 x SSPE</u> (3 M NaCl, 0.2 M sodium phosphate, 20 mM EDTA, pH 7.4)

87.65 g NaCl, 13.8 g $NaH_2PO_4.H_2O$ and 3.7 g $Na_2EDTA.2H_2O$ were dissolved in 800 ml distilled water, adjusted to pH 7.4 with NaOH and made up to 1 l. The solution was sterilized by autoclaving and filtered through a 0.2 μm filter.

<u>5 M Potassium Acetate, pH 4.8</u>

24.535 g potassium acetate were dissolved in 50 ml distilled water. Glacial acetic acid was added until the solution was pH 4.8. The solution was sterilized by filtering through a 0.2 μm filter and stored at RT.

<u>1 M Magnesium Chloride</u>

20.33 g $MgCl_2.6H_2O$ was dissolved in sterile distilled water and made up to 100 ml. The solution was sterilized by filtering through a 0.2 μm filter and stored at 4°C.

<u>1 M Magnesium Sulphate</u>

24.65 g $MgSO_4.7H_2O$ was dissolved in sterile distilled water and made up to 100 ml. The solution was sterilized by filtering through a 0.2 μm filter and stored at 4°C.

<u>20% (w/v) Maltose or Glucose</u>

20 g maltose or glucose was dissolved in sterile distilled water and made up to 100 ml. The solution was sterilized by filtering through a 0.2 μm filter and stored at 4°C.

<u>13% (w/v) PEG in 1.6 M NaCl</u>

13 g polyethylene glycol (PEG) 8000 and 9.352 g NaCl were dissolved in sterile distilled water, made up to 100 ml and sterilized by filtering through a 0.2 μm filter.

<u>20% (w/v) PEG in 2 M NaCl</u>

200 g PEG 8000 and 116.9 g NaCl were dissolved in distilled water and made up to 1 l.

<u>Denaturing Solution</u> (0.5 M NaOH, 1.5 M NaCl)

20 g NaOH and 87.66 g NaCl were dissolved in distilled water and made up to 1 l.

<u>Neutralizing Solution</u> (0.5 M Tris, 1.5 M NaCl, pH 7.4)

87.66 g NaCl and 60.57 g Tris were dissolved in 800 ml distilled water, adjusted to pH 7.4 with HCl and made up to 1 l.

<u>HYBSOL</u> (0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, 7% (w/v) SDS, 10% (w/v) PEG, 100 μg ml^{-1} herring sperm DNA, 250 μg ml^{-1} heparin, pH 7.2)

8.75 g NaCl, 1.56 g $NaH_2PO_4.2H_2O$, 0.37 g $Na_2EDTA.2H_2O$, 70 g SDS and 100 g PEG 8000 were dissolved in 800 ml sterile distilled water and adjusted to pH 7.2 with NaOH. 10 ml 10 mg ml^{-1} herring sperm DNA and 0.25 g heparin were added and the volume made up to 1 l. Before use, the solution was sterilized by filtering through a 0.2 μm filter.

<u>DNase I</u>

DNase I was dissolved in sterile distilled water to a final concentration of 10 mg ml^{-1}, dispensed into aliquots and stored at -20°C.

<u>RNase A</u>

RNase A was dissolved in sterile distilled water to a final concentration of 10 mg ml^{-1}. The solution was heated to 100°C for 15 min, dispensed into aliquots and stored at -20°C.

Phenol:Chloroform:Isoamyl alcohol (IAA) (25:24:1)

500 µl phenol, 480 µl chloroform and 20 µl IAA were mixed by vortexing in a microfuge tube. The mixture was centrifuged at low speed in a microcentrifuge for 1 min, the upper phase discarded and the lower phase was used for extractions.

Phenol:Chloroform (1:1)

Equal volumes of phenol and chloroform were mixed by vortexing and centrifuged at 2300 g for 5 min or low speed for 1 min in a microcentrifuge. The lower phase was used for extractions.

2% (w/v) Isopropyl-1-thio-β-D-galactopyranoside (IPTG)

0.2 g IPTG was dissolved in 10 ml sterile distilled water, sterilized by filtering through a 0.2 µm filter and stored at -20°C.

2% (w/v) 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-Gal)

0.2 g X-gal was dissolved in 10 ml dimethylformamide (DMF) and stored at -20°C.

50 x Denhardt's Solution (1% (w/v) each of Ficoll, PVP, BSA)

5 g Ficoll (Type 400, Sigma), 5 g polyvinylpyrrolidone (PVP) and 5 g bovine serum albumen (BSA Fraction V, Boehringer Mannheim UK) were dissolved in sterile distilled water and made up to 500 ml. The solution was sterilized by filtering through a 0.2 µm filter and stored at -20°C.

Salmon Sperm DNA

Salmon sperm DNA (Type III sodium salt, Sigma) was dissolved overnight in sterile distilled water at a concentration of 10 mg ml^{-1}. The NaCl concentration was adjusted to 0.1 M and the DNA extracted with an equal volume of phenol, followed by an equal volume of phenol:chloroform (1:1). Each time the solutions were mixed by vortexing, centrifuged at 20 800 g for 10 min and the upper aqueous phase was collected. The

DNA was sheared by passing it 12 times rapidly through a 17-gauge needle. The DNA was precipitated by adding 2 volumes ice-cold 100% ethanol and collected by centrifugation at 10 000 g for 10 min. The pellet was dried and redissolved in sterile distilled water to a concentration of approximately 10 mg ml^{-1}. The OD$_{260}$ was determined and the exact concentration was calculated before storade in aliquots at -20°C. Before use, the DNA was denatured by heating at 100°C for 10 min then cooling rapidly on ice/ethanol.

A2.   BIOCHEMICAL REAGENTS

Buffer A (CTAB extraction buffer: 10 mM MOPS, 0.5% (w/v) CTAB, 30% (w/v) glycerol, pH 7.0)

2.093 g MOPS free acid, 5 g CTAB and 300 g glycerol were dissolved in 800 ml distilled water, adjusted to pH 7.0 with NaOH and made up to 1 l.

Buffer B (50 mM acetic acid/NaOH, pH 5.0)

3 g acetic acid was made up to 800 ml with distilled water, adjusted to pH 5.0 with NaOH and made up to 1 l.

Enzyme Extraction Buffer (76 mM Na$_2$HPO$_4$, 27 mM NaH$_2$PO$_4$, 9.2 mM Na$_2$B$_4$O$_7$, 13 mM H$_3$BO$_3$, 5 mM EDTA, 5 mM DTT, 0.25 mM PMSF, 5% (w/v) PVPP, pH 7.6)

A 2 x concentrate was prepared by dissolving 27.054 g Na$_2$HPO$_4$.2H$_2$O, 7.45 g NaH$_2$PO$_4$.H$_2$O, 7.016 g Na$_2$B$_4$O$_7$.10H$_2$O, 1.606 g H$_3$BO$_3$ and 3.724 g Na$_2$EDTA.2H$_2$O in 800 ml distilled water. The pH was adjusted to pH 7.6 and the volume made up to 1 l. Before use the final buffer was prepared with 150 ml 2 x buffer, 1.5 ml 1 M

dithiothreitol (DTT), 750 μl 100 mM phenylmethanesulphonyl fluoride (PMSF) and 15 g polyvinylpolypyrrolidone (PVPP) made up to 300 ml, per 100 g FW of original tissue.

0.4 M Citrate-Phosphate-Tris (CPT) buffer

58.8 g trisodium citrate monohydrate, 27.6 g $NaH_2PO_4.H_2O$ and 24.2 g Tris base were dissolved in 400 ml distilled water, adjusted to pH 9.0 with HCl and made up to 500 ml. The solution was diluted to 50 mM in working solutions.

Resolving Gel Buffer (1.5 M Tris, pH 8.8)

181.71 g Tris base was dissolved in 800 ml distilled water, adjusted to pH 8.8 with HCl and made up to 1 l.

Stacking Gel Buffer (0.5 M Tris, pH 6.8)

30.29 g Tris base was dissolved in 400 ml distilled water, adjusted to pH 6.8 with HCl and made up to 500 ml.

20% (w/v) Sodium Dodecyl Sulphate (SDS)

20 g SDS was dissolved in distilled water, made up to 100 ml and heated until dissolved.

10% (w/v) Ammonium Persulphate

0.1 g ammonium persulphate was dissolved in 1 ml distilled water just before use and stored at 4°C for a maximum of 1 day.

SDS-PAGE Resolving and Stacking Gels

Stock Solution	10% Resolving Gel	3.75% Stacking Gel
Resolving gel buffer	3.75 ml	-
Stacking gel buffer	-	3.75 ml
30 % Acrylamide (Bio-Rad)	5.0 ml	1.875 ml
20% (w/v) SDS	75 µl	75 µl
10% (w/v) Ammonium persulphate	75 µl	75 µl
TEMED	7.5 µl	15 µl
H$_2$O	6.1 ml	9.21 ml
Total volume (for 2 gels)	15 ml	15 ml

2 x Laemmli Sample Buffer (125 mM Tris, 20% (v/v) glycerol, 4% (w/v) SDS, 0.01% (w/v) bromophenol blue, pH 6.8)

6.25 ml 1 M Tris, pH 6.8, 10 ml glycerol, 20 ml 10% (w/v) SDS and 0.5 ml 1% (w/v) BPB in 50 mM NaOH were made up to 50 ml with distilled water.

10 x SDS-PAGE Running Buffer (0.25 M Tris, 1.92 M glycine, 1% (w/v) SDS)

30.3 g Tris base, 144.2 g glycine and 10 g SDS were dissolved in 1 l distilled water. The pH was checked and should be approximately pH 8.3 without adjustment.

20 mM CAPS Buffer

4.426 g CAPS was dissolved in 800 ml distilled water, adjusted to pH 11.0 with NaOH and made up to 1 l.

Transfer Buffer (10 mM CAPS, 10% (v/v) methanol)

500 ml 20 mM CAPS buffer was combined with 100 ml methanol and 400 ml water.

Carboxymethylcellulose (CMC) Substrate (1.875% (w/v) CMC)

500 ml sterile distilled water was mixed on slow speed in a sterile Waring blender with 18.75 g CMC. Further water was added until the blender was nearly full and the solution was mixed for approximately 2 min. The solution was poured into a 1 l volumetric flask and stored at 4°C overnight to allow the bubbles to settle out. The solution was allowed to reach RT, made up to 1 l and centrifuged in sterile Oakridge tubes at 36 900 g for 20 min at RT. The solution was poured off into a sterile bottle and stored at 4°C for up to 6 months.

Ferricyanide Reagent

0.25 g $K_3Fe(CN)_6$, 14 g K_2HPO_4 and 4.2 g K_3PO_4 were dissolved in 100 ml distilled water.

100 mM PMSF

0.871 g PMSF was dissolved in 50 ml methanol and stored at 4°C.

1 M DTT

3.855 g DTT was dissolved in 25 ml distilled water and stored at -20°C in aliquots.

Coomassie Protein Reagent (0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid)

0.05 g Coomassie Brilliant Blue G-250 was dissolved in 25 ml ethanol, 50 ml 85% (w/v) phosphoric acid and made up to 500 ml with distilled water. The solution was filtered through a 0.45 μm filter before use.

Coomassie Blue Stain (0.05% (w/v) Coomassie Brilliant Blue R-250, 25% (v/v) methanol, 8% (v/v) acetic acid)

0.25 g Coomassie Brilliant Blue R-250 was dissolved in 125 ml methanol. 40 ml acetic acid was added and the volume made up to 500 ml with distilled water.

Destain (25% (v/v) methanol, 8% (v/v) acetic acid)

250 ml methanol and 80 ml acetic acid were made up to 1 l with distilled water.

Fix/Stop Solution (10% (v/v) acetic acid)

50 ml acetic acid was made up to 500 ml with distilled water.

Stain Solution (0.1% (w/v) silver nitrate, 0.05% (v/v) formaldehyde)

0.025 g $AgNO_3$ was dissolved in distilled water and made up to a volume of 25 ml. 37.5 μl 37% formaldehyde was added and the solution was stored in the dark.

Developer (3% (w/v) sodium carbonate, 2 μg ml^{-1} sodium thiosulphate, 0.05% (v/v) formaldehyde)

3 g Na_2CO_3 was dissolved in distilled water, made up to 100 ml and stored at 4°C. Just before use 4 μl 10 mg ml^{-1} sodium thiosulphate and 30 μl 37% formaldehyde were added to 20 ml developer solution.

10 mg ml^{-1} Sodium Thiosulphate

0.1 g Na thiosulphate was dissolved in 10 ml distilled water and stored at 4°C.