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Cell Wall Metabolism in Developing Grape Berries

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A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

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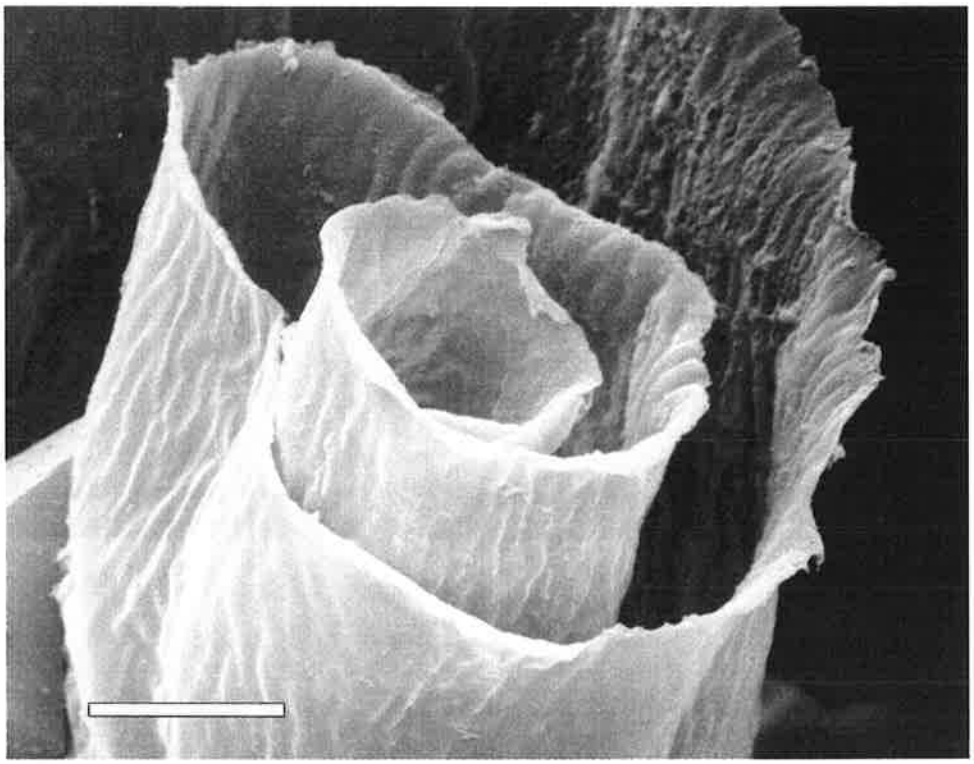

"Wallflower"

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Abstract

Softening is an important part of the ripening process in many fruit and changes in cell wall composition and associated enzyme activity are thought to play central roles in the softening process. In this study, research was focussed upon the phase of growth in which the grape berry (*Vitis vinifera* L.) softens.

A procedure was developed for the isolation of cell walls from mesocarp tissue of grape berries. This procedure included the use of filtration and phenol treatment to remove vascular tissue and adventitious proteins of cytoplasmic origin from the wall preparations. Compositional analysis of cell walls isolated from mature Gordo and Ohanez berries showed substantial differences in polygalacturonan and cellulose abundance. Subsequent analyses of the monosaccharide, polysaccharide and protein composition of walls isolated from Gordo berries at several stages of development revealed a number of changes that occurred during development. These included an increase in water-soluble pectins, a decrease in galactan/arabinogalactan-I and an increase in hydroxyproline-rich proteins.

The changes in cell wall polysaccharide composition during berry ripening suggested that a limited number of enzymes might be involved in the softening process. In an attempt to determine the importance of particular enzymes in berry softening, the activities of several enzymes were monitored throughout berry development. Overall, β-galactosidase and α-galactosidase activities were the most readily detectable and were found to increase markedly during berry softening. β-Galactosidase may be the enzyme responsible for the loss of galactan from cell walls as berries soften. Pectin methylesterase activity remained relatively low during berry development, consistent with the observation that the degree of esterification of pectins remained approximately the same during berry softening. Little or no activity was detected for polygalacturonase, galactanase, cellulase or xyloglucanase in ripening berries.

To further investigate the possible roles of enzymes in grape berry development, cDNAs encoding a number of cell wall-modifying enzymes were isolated, either by screening a grape berry cDNA library or by the polymerase chain reaction. The cDNAs were used as probes to monitor steady-state mRNA levels of the respective genes in developing grape berries and other grapevine tissues. The detection of β-galactosidase mRNA was consistent with the presence of high levels of β-galactosidase activity and the decrease in galactan content in walls. Similarly, the presence of polygalacturonase mRNA was consistent with the increased solubility of pectic polysaccharides during ripening, although no polygalacturonase activity had been detected. The presence of xyloglucan endotransglycosylase mRNA also suggested that this enzyme plays some role in berry softening. Some pectin methylesterase mRNA was detected during berry development, consistent with the low levels of pectin methylesterase activity during berry ripening. Despite the pectin methylesterase activity, the degree of esterification of pectic polysaccharides remained constant during berry softening. In contrast, mRNA transcripts encoding cellulases were not detected in ripening berries, in agreement with the absence of detectable cellulase activity.

In summary, no gross changes in the cell wall composition occurred during berry development and no single enzyme could be assigned as the major cause for grape berry softening. This supports the general theory that fruit softening is a complex process involving relatively minor changes in different components of the cell wall that can subsequently have a large impact on the overall wall structure.

Statement of Authorship

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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May, 1999

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Publications

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Nunan KJ, Sims IM, Bacic A, Robinson SP, Fincher GB (1998) Changes in cell wall composition during ripening of grape berries. Plant Physiol 118: 783-792.

Abbreviations

ABA	Abscisic acid
AG-I	Arabinogalactan I
AG-II	Arabinogalactan II
AGP	Arabinogalactan protein
Ara*f*	L-Arabinofuranose
BME	β-Mercaptoethanol
CDTA	*Trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
dATP	2'-Deoxyadenosine triphosphate
DE	Degree of esterification
DIECA	Diethyldithiocarbamate
Dpa	Days post-anthesis
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
Fuc*p*	L-Fucopyranose
fw	Fresh weight
GalA*p*	D-Galacturonic acid
Gal*p*	D-Galactopyranose
GC-MS	Gas chromatography-mass spectrometry
GlcA*p*	D-Glucuronic acid
Glc*p*	D-Glucopyranose
GMA	Glycol methacrylate
GRP	Glycine-rich protein
HPGP	Hydroxyproline-rich glycoprotein
kDa	Kilodaltons
Man*p*	D-Mannopyranose
ORF	Open reading frame

PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque forming unit
PG	Polygalacturonan
PGA	Polygalacturonic acid
PGase	Polygalacturonase
PME	Pectin methylesterase
PMSF	Phenylmethylsulfonyl fluoride
PRP	Proline-rich protein
PVP	Polyvinylpyrrolidone
PVPP	Polyvinylpolypyrrolidone
RG-I	Rhamnogalacturonan I
RG-II	Rhamnogalacturonan II
Rha*p*	L-Rhamnopyranose
TBO	Toluidine Blue O
TCA	Trichloroacetic acid
T_m	Melting temperature
XET	Xyloglucan endotransglycosylase
XG	Xyloglucan
XGase	Xyloglucanase
Xyl*p*	D-Xylopyranose

Amino acids are denoted by standard 3-letter abbreviations.

Chapter 1

General Introduction

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1.1 Introduction

The increasing commercial importance of grape-based industries, including the wine, table fruit, dried fruits and grape juice sectors, has not been accompanied by an increased understanding of the basic composition and development of the grape berry (Dutruc-Rosset, 1998). The wine sector is experiencing a boom in Australia and, at an international level, wine production is again increasing after experiencing a 10 year decline from the mid 1980s to the mid 1990s (Dutruc-Rosset, 1998; Osmond and Anderson, 1998). Because cell walls are believed to affect grape texture, and cell wall-derived polysaccharides influence not only the final quality of the products, but also the ease of processing of fruit, the work described in this thesis was undertaken to define in detail the changes in cell walls that occur during the softening and ripening of the grape berry.

Polysaccharides of cell wall origin include pectic polysaccharides and other high molecular weight material that can have a major impact on the physicochemical properties of plant extracts. The polysaccharides are at least partly water-soluble, they are often highly asymmetrical in shape, and they therefore form solutions of high viscosity (Burchard, 1993; Jarvis, 1984). The presence of some polysaccharides can be detrimental in the process of winemaking because they can block filtration membranes (Brillouet *et al.*, 1989; Belleville *et al.*, 1991) or inhibit complete juice extraction (Robertson *et al.*, 1980). On the other hand, polysaccharides can be beneficial, such as in the prevention of wine haze formation (Waters *et al.*, 1994). As a result, wall polysaccharides, which may account for a relatively minor proportion of plant tissue, can exert a disproportionately large influence on clarification and filtration steps in winemaking processing and can affect quality characteristics such as clarity and storage life of the final product.

Earlier French studies on pectic polysaccharides from cell walls of grapes (Saulnier and Thibault, 1987a,b; Saulnier and Brillouet, 1989; Brillouet *et al.*, 1990) were

undertaken because of their importance in wine making and their influence on wine quality, but no comprehensive analyses of grape cell walls have been reported in the literature. Similarly, changes that occur to the cell wall and to the activity of cell wall-modifying enzymes as the grape softens during ripening, have not been described. In this project it was therefore proposed to develop a rapid procedure for the isolation of walls from grape berries and to use this procedure to isolate walls during ripening. Chemical analyses of the walls would subsequently be used to monitor changes that occur during berry development and enzymes responsible for these changes would be identified.

As background to this work, current information on grape berry morphology and physiology, general aspects of plant cell walls, and specific modifications that generally occur to cell walls of ripening fruit are now reviewed.

1.2 The Grape Berry

1.2.1 Morphology of the mature grape berry

The grape berry consists of the skin (or exocarp), the mesocarp and seeds (or traces of seeds)(Fig. 1.1). The skin is composed of the epidermis, which is covered by a waxy cuticle. Beneath the epidermis, the hypodermis, which is composed of 6-10 layers of small thick-walled cells (Hardie *et al.*, 1996), is located. The mesocarp tissue constitutes the major proportion of the berry and is composed of 25-30 layers of large thin-walled cells that are highly vacuolated (Kanellis and Roubelakis-Angelakis, 1993). These cells store most of the soluble compounds that are extracted as "juice" when the grapes are crushed. The number of seeds in a typical grape berry varies from zero to four due to the abortion of one or more of the four original ovules. Vascular tissue runs from the pedicel to the seeds, but also through the centre of the berry and under the skin around the periphery of the berry (Coombe, 1987).

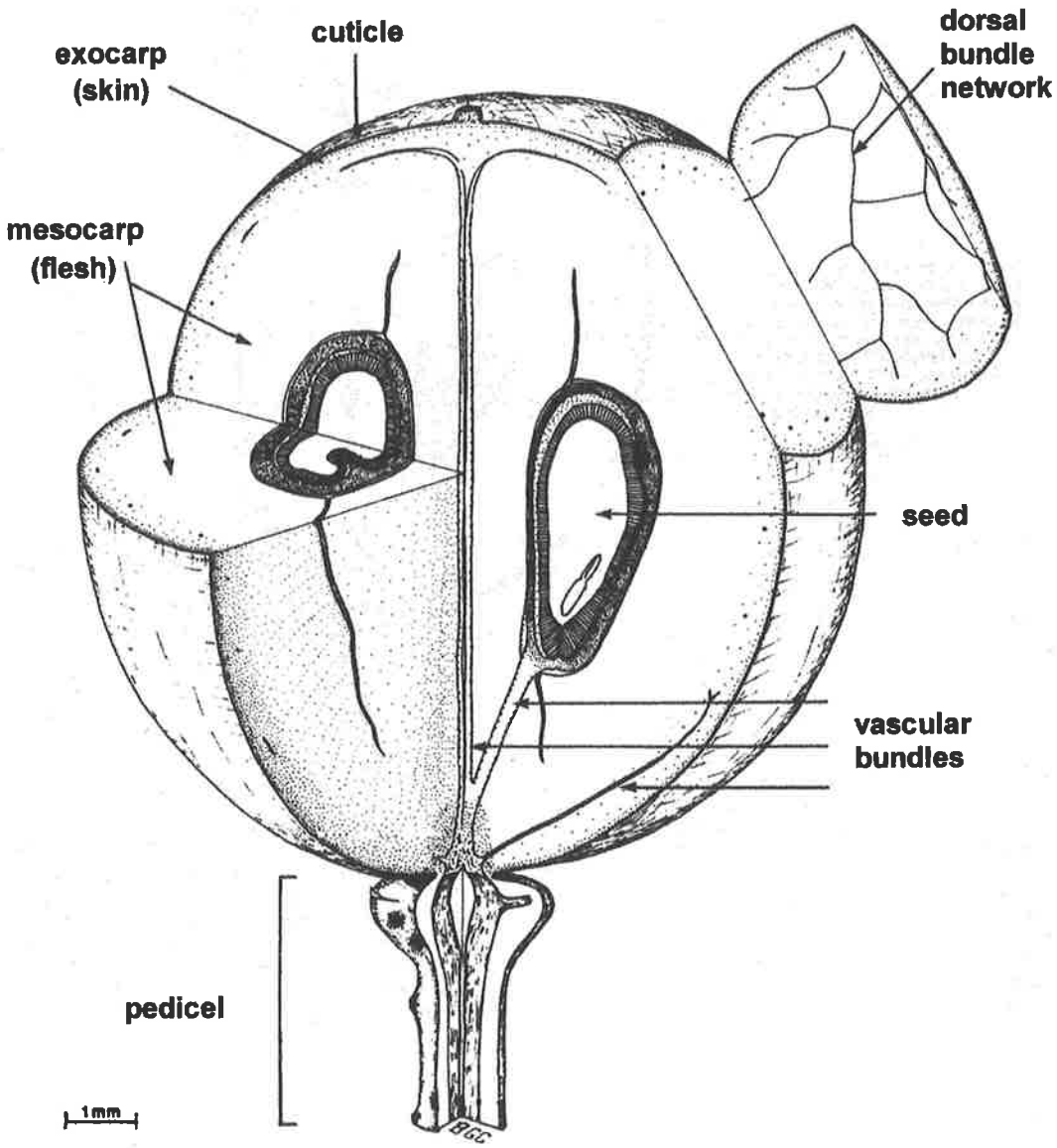

Figure 1.1 The anatomy of the grape berry. The exocarp consists of the epidermis and the hypodermis. (From Coombe, 1987)

1.2.2 Physiology of grape development

The grape berry is classified as a non-climacteric fruit. Non-climacteric fruit are defined by the absence of a large, transient increase in respiratory carbon dioxide evolution at the onset of ripening (Coombe, 1976) and do not usually show a distinct commencement of ripening (Mullins *et al.*, 1992). However, the grape berry is unusual, in that the transition into the ripening phase is quite distinct.

The grape berry shows the characteristic double-sigmoidal growth curve displayed by other berries and some fleshy fruits (Coombe, 1976). Growth can be divided into two phases, which are separated by a "non-growth" lag phase (Fig. 1.2). The first growth period varies from 45-60 days, depending on the grape cultivar and environmental factors (Kanellis and Roubelakis-Angelakis, 1993). After anthesis and fruit set, an increase in the size of the grape berry in the first 25 days is attributable to both cell division and cell expansion. The remainder of growth in the first phase is due solely to cell expansion (Harris *et al.*, 1968). Seeds attain their full size by the end of the first growth phase.

The grape berry subsequently goes through a lag phase of little or no growth. The length of this phase is variable between cultivars; it can be almost nonexistent or can extend for a number of weeks. Even within a cultivar, the length of the lag phase is variable. For example, the lag phase for the cultivar Muscat Gordo Blanco varies from 8 to 48 days (Coombe, 1973), depending on time of flowering, competition between clusters and the vine's environment.

The inception of the second growth phase is known as veraison, a French term originally used to describe the change in colour of the grape berry. However, veraison is now used by viticulturists to describe the onset of ripening. At veraison, a number of events are initiated, including colour development, softening, acceleration of growth (indicated by an increase in berry weight and volume), an increase in sugar content, a decrease in organic acids, a decrease in carbon dioxide production and an increase in activity of specific enzymes (Coombe, 1984). Normally, the start of grape berry

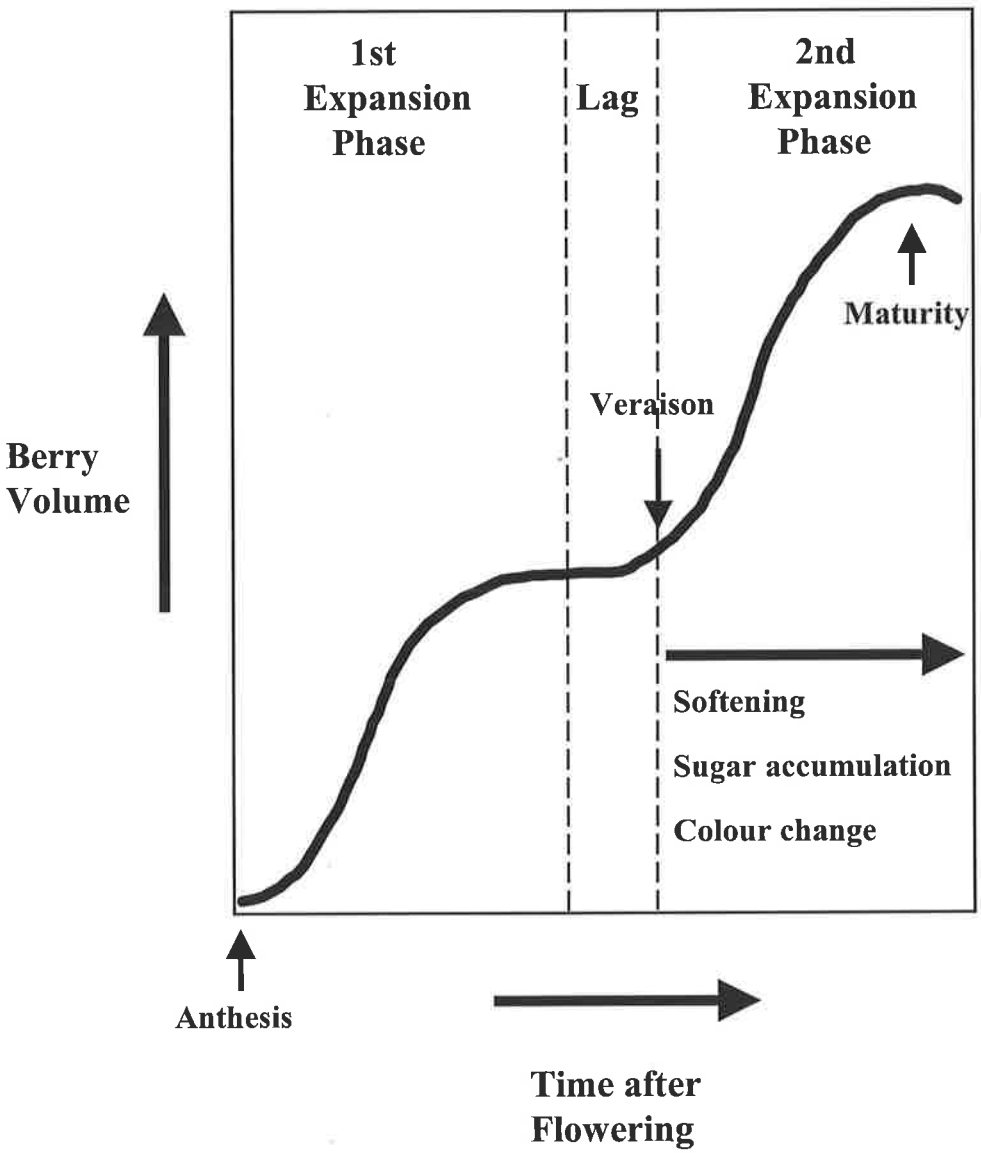

Figure 1.2 Change in grape berry size during development. The growth of the grape berry can be divided into three distinct stages once flowering has occurred – the first expansion phase, the lag phase and the second expansion phase. No scales are marked, because of large variations between cultivars.

softening and accumulation of soluble sugars are detected at the same time, and decreases in the organic acid, malate, begin one day later (Coombe and Phillips, 1982). However, in some cases the onset of softening precedes the other ripening events by up to six days (Coombe and Bishop, 1980).

The second growth phase varies in length from five to eight weeks, and continues to the time of maturity (Winkler *et al.*, 1974). The increase in berry weight in the second growth phase has been attributed mainly to the accumulation of water, and the increase in berry size is due solely to cell expansion (Coombe and Bishop, 1980). Cell numbers are approximately the same for different-sized berries. The cell number of a berry apparently decreases slightly as the berry ripens, possibly because of cell fusion or cell rupture (Harris *et al.*, 1968; Jona *et al.*, 1983).

Despite these relatively distinct phases of berry development, there is considerable variation in berry growth and ripening, both between different berries in an individual bunch and between different bunches on an individual vine. Variations in flowering time can contribute to this asynchronous developmental pattern of berries (Coombe, 1980). The rate of increase in berry volume remains approximately constant for up to 25 days post anthesis, but berries that flower early in the season have slower and later periods of growth deceleration in the first phase, and shorter lag phases. This results in berries from early flowers being larger at the lag phase compared with berries from late flowers. Early flowering berries expand very slowly during the lag phase but later flowering berries show no expansion. The early berries also show a high growth rate during the second phase (Coombe, 1980). Asynchrony means that veraison can occur as much as 10 to 23 days apart for berries in the same bunch (Coombe and Bishop, 1980). Clearly, this variation in ripening results in significant variation in maturity of berries at harvest, which in turn affects the overall quality of the final grape product.

Growth and ripening of the grape berry appears to be mediated by phytohormones. Auxins, cytokinins, gibberellins, and abscisic acid (ABA) are believed to interact to effect development of the berry during the first growth phase (Coombe, 1973). ABA

concentration increases as the berry ripens, peaks and then declines by maturity. It is therefore thought that the accumulation of ABA may be a factor controlling the onset and rate of ripening (Coombe and Hale, 1973). Ethylene is the trigger for the ripening of climacteric fruit (Lelievre *et al.*, 1997), but does not appear to play a major role in ripening of the non-climacteric grape berry, in which ethylene levels actually decrease as the berry ripens (Coombe and Hale, 1973). However, treatment with ethylene can have a small effect on the ripening pattern of the grape berry, depending on the stage of berry development at which it is applied (Coombe and Hale, 1973). Treatment with the ethylene-generating chemical, 2-chloroethylphosphonic acid, early in development delays ripening, but when applied the week before veraison ripening is accelerated (Hale *et al.*, 1970). Despite these early studies on hormone levels, there is little real understanding of the physiological triggers for veraison and other events in the growth and ripening processes in grape berries.

1.3 Plant Cell Walls

Because the focus of the studies described in this thesis is on changes that occur in cell walls in grape berries during ripening, existing information on plant cell wall composition and structure is summarised in the sections below.

1.3.1 Function

The major function of the cell wall in plants is to provide strength and shape for individual cells; the cumulative strength of walls that surround individual cells provides the mechanical support that is essential to the plant overall (Brett and Waldron, 1996).

Plant cell walls have several additional functions and characteristics as summarised below.

- The wall is thought to control both the rate and extent of cell expansion (McNeil *et al.*, 1984; Brett and Waldron, 1996).

- The wall provides a level of protection for the cell against microbial attack, both as a passive physical barrier and by active responses such as the elicitation of phytoalexin production by cell wall fragments (McNeil *et al.*, 1984; Brett and Waldron, 1996), and the deposition of lignin (Brett and Waldron, 1996).
- The cell wall is selectively porous, and is therefore important for the control of intercellular transport (Brett and Waldron, 1996).
- The walls can act as carbohydrate reserves in cotyledons and endosperms (Fry, 1989; Brett and Waldron, 1996).

From these properties and functions, it can be concluded that the plant cell wall is not an inert structure, but is in fact a dynamic component of the plant. Many modifications to cell walls occur during cell growth, development, and differentiation, and these result in changes in their composition, in cell shape and in physical properties of the walls.

1.3.2 Components of the cell wall

Following nuclear division, the middle lamella is deposited in the cell plate region and persists as a layer between walls of adjacent cells. The middle lamella consists mainly of pectic polysaccharides and possibly callose (Selvendran, 1985). The primary cell wall is deposited as the cells expand. In some cases, the primary wall is the only additional layer but in other cases a secondary cell wall may be deposited (Brett and Waldron, 1996). The classifications of primary and secondary cell walls are not well defined. The primary cell wall is generally associated with actively growing tissue (Albersheim *et al.*, 1994), whilst the secondary cell wall is normally defined as wall which is deposited when expansion has ceased. However, this is not always the case. Schubert *et al.* (1973) showed that cotton lint fibers contained substantial secondary wall thickening while still elongating. Lignification is not necessarily an indicator of secondary cell walls, because there are many tissues where secondary cell walls are not lignified (Gane *et al.*, 1994).

The two major components of plant cell walls are polysaccharides and proteins (Varner and Lin, 1989); the latter include glycoproteins. Phenolics are an important subsidiary component in both primary and secondary cell walls (Bacic *et al.*, 1988). Specialised cells contain other components such as lignins, cutin, suberin, waxes, sporopollenins, inorganic compounds and additional wall-associated molecules such as gibberellins and amines (Bacic *et al.*, 1988). Primary cell walls are generally composed of about 90% polysaccharide and 10% protein (McNeil *et al.*, 1984) but this varies greatly depending on the plant and tissue type. Because parenchymatous tissue of the fruit mesocarp is composed mainly of primary cell walls (Selvendran and O'Neill, 1987), the following discussion will focus on the major constituents of primary cell walls in dicotyledonous plants in general.

1.3.3 Cell wall polysaccharides

Primary cell walls of higher plants are composed predominantly of a heterogeneous mixture of polysaccharides, consisting of cellulosic microfibrils embedded in an essentially amorphous matrix phase. Secondary networks of protein may also be present (Carpita and Gibeaut, 1993) There are variations between dicotyledons, monocotyledons and gymnosperms (Bacic *et al.*, 1988). The types of polysaccharides found in plant cell walls have been thoroughly reviewed over many years and the material presented below has been obtained from reviews by Darvill *et al.* (1980), McNeil *et al.* (1984), Selvendran (1985), John and Dey (1986), Bacic *et al.* (1988), Carpita and Gibeaut (1993) and Brett and Waldron (1996).

1.3.3.1 Non-cellulosic polysaccharides

The major non-cellulosic polysaccharides in dicotyledonous primary cell walls are pectic polysaccharides, heteroxylans, xyloglucans and glucomannans (Selvendran, 1985). Certain non-cellulosic polysaccharides may be closely associated with cellulose (see Section 1.3.5).

The primary walls of growing cells and parenchymatous tissue may consist of up to 50% pectic polysaccharides (Selvendran, 1985). Pectic polysaccharides are composed mainly of *polygalacturonan* (PG), which includes homogalacturonan and rhamnogalacturonan I. *Homogalacturonan* is comprised mainly of unbranched chains of (1→4)-linked α-D-galacturonosyl (GalA*p*) residues that may be methyl esterified. In carrot roots, specific antibodies were used to show that methyl-esterified GalA*p* residues are spread evenly through the walls, whereas unesterified GalA*p* residues tend to be located in the middle lamella, the inner surface of the wall and the outer surface of the walls at the intercellular spaces (Knox *et al.*, 1990). To extract homogalacturonan from walls, treatment with chemicals that may break covalent bonds is required, but it is not known whether homogalacturonan exists as a separate entity, or whether it is covalently attached to other polysaccharides such as rhamnogalacturonan I (McNeil *et al.*, 1984).

Rhamnogalacturonan I (RG-I) is a heteropolymer with a backbone of alternate (1→4)-linked α-D-galacturonosyl and (1→2)-linked α-L-rhamnosyl residues (Lau *et al.*, 1985). Numerous sidechains of neutral sugar residues can be attached through the rhamnosyl residues of RG-I, mainly through the C(O)4 position. The most common sidechains consist of D-galactosyl, L-arabinosyl and L-fucosyl residues. These residues can be attached as single sugars or as neutral polysaccharides such as galactans, arabinans and arabinogalactans (Bacic *et al.*, 1988). *Galactan* consists of a (1→4)-linked β-D-galactopyranose backbone, and is thought to be commonly linked to RG-I. When this galactan backbone is substituted with α-arabinofuranosyl sidechains, the galactan is referred to as *arabinogalactan I* (AG-I). *Arabinogalactan II* (AG-II) is a structurally distinct polysaccharide characterized by (1→3)- and (1→3,1→6)-linked β-D-galactopyranosyl residues with α-L-arabinosyl substituents. There is some debate as to whether AG-II is a true component of the primary cell wall or if it is associated with the extracellular space and plasma membrane (Bacic *et al.*, 1988). *Arabinan* is made up of (1→5)-linked α-L-arabinofuranosyl residues with branching of α-L-arabinofuranosyl residues through the C(O)2 or C(O)3 position, or both.

Rhamnogalacturonan II (RG-II) is another type of pectic polysaccharide found in primary cell walls. RG-II has a rhamnogalacturonan backbone but contains a complex array of monosaccharides branching from the backbone (Stevenson *et al.*, 1988). Many of the monosaccharides present in RG-II, such as 2-O-methyl-fucosyl, 2-O-methyl-xylosyl, apiosyl, 3-C-carboxy-5-deoxy-L-xylosyl and 3-deoxy-manno-octulosonyl residues, are rarely found in other polysaccharides. The proportion of RG-II in plant cell walls is relatively low, usually making up 4% or less by weight in walls of dicotyledons (O'Neill *et al.*, 1996).

Xyloglucans (XG) generally account for about 20% of the non-cellulosic polysaccharides of primary cell walls (Dey and Brinson, 1984), although this value can be highly variable. XG contains a backbone of (1→4)-linked β-D-glucosyl residues with (1→6)-linked α-D-xylosyl residue substituents, to which other neutral sugars such as galactose, arabinose and fucose may be attached.

Heteroxylans constitute a small portion of dicotyledonous walls and are composed of a backbone of (1→4)-linked β-D-xylosyl residues, with highly variable substituents or branches originating from the C(O)2 or C(O)3 position of the xylosyl residues. These substituents and sidechains consist of combinations of arabinosyl, glucuronosyl (or its 4-O-methyl derivative), xylosyl and galactosyl residues (Bacic *et al.*, 1988).

Mannan is generally associated with thick, unlignified, secondary cell walls and is the major component of endosperm cell walls of some seeds, but is present in very small amounts in other tissues. It has a linear chain of (1→4)-linked β-D-mannopyranosyl residues of varying degrees of polymerisation (Bacic *et al.*, 1988). *Galactomannan* has a (1→4)-β-mannan backbone that is generally substituted at C(O)6 positions with single α-galactopyranosyl residues and is mainly found in the endosperm cell walls of legumes. *Glucomannans* and *galactoglucomannans* are polysaccharides that consist of (1→4)-linked β-D-mannopyranosyl and β-D-glucopyranosyl residues arranged randomly in a single chain. In galactoglucomannans, main chain residues are

substituted with single α-D-galactopyranosyl residues at the C(O)6 position. These polysaccharides are only minor cell wall components of dicotyledons (Bacic *et al.*, 1988).

β-Glucans include the (1→3)- and (1→3,1→4)-linked β-D-glucans. The (1→3)-β-glucans are known as callose and can be associated with wounding, the middle lamella, or specialised tissue such as pollen tubes. The (1→3,1→4)-β-D-glucans consist of β-D-glucopyranosyl residues linked by (1→3)- and (1→4)-β-glucosidic linkages and are found in the graminaceous monocotyledons, in particular the Poaceae (Dey and Brinson, 1984).

1.3.3.2 Cellulose

Cellulose is an unbranched (1→4)-linked β-D-glucan. Each glucose residue is rotated 180°C with respect to the one before and the structural repeating unit of the polysaccharide is in fact cellobiose. Extended, linear chains of cellulose aggregate to form microfibrils of 5-15 μm in diameter (McCann *et al.*, 1990), stabilised by intermolecular hydrogen bonding between individual cellulose molecules. Each chain starts and finishes in a different place within the microfibril. It is therefore very difficult to determine the exact length of the chains but this is known to be highly variable (Darvill *et al.*, 1980). Cellulose is always found in higher plant cell walls, although its abundance also varies (McNeil *et al.*, 1984, Bacic *et al.*, 1988, Brett and Waldron, 1996).

1.3.4 Cell wall proteins

Plant cell walls may contain up to 10% protein (Bacic *et al.*, 1988). The five most abundant and well-studied classes of wall proteins are extensins, glycine-rich proteins (GRPs), proline-rich proteins (PRPs), lectins of the Solanaceae and arabinogalactan-proteins (AGPs) (Showalter, 1993; Cassab, 1998; Sommer-Knudsen *et al.*, 1998).

Enzymes and less abundant proteins, such as expansins, are also present in the wall or interact intimately with the walls.

1.3.4.1 Structural proteins

Extensins are one family of hydroxyproline-rich glycoproteins. They are made up of about 50% carbohydrate and 50% protein (Tucker and Mitchell, 1993), although these values do vary significantly depending on the plant and tissue type. The protein moiety is rich in hydroxyproline residues, with values of 33-42 mol% found in many tissues (Cassab and Varner, 1988). Other abundant amino acid residues are serine, lysine, tyrosine, valine, proline and histidine. The abundance of basic amino acids results in the protein having a high pI and it has been suggested that extensins interact with acidic pectic polysaccharides in the cell wall (Keller, 1993). A common repeating motif in the protein is Ser-Hyp_4 (Showalter, 1993). Other repeats include Tyr-Lys-Tyr-Lys and Thr-Pro-Val (Bacic *et al.*, 1988). The carbohydrate moiety of extensins is comprised of approximately 96% arabinose and 4% galactose residues (Stuart and Varner, 1980). Most of the Hyp residues are glycosylated with chains of one to four arabinosyl residues, whilst serine residues may be glycosylated with single galactosyl residues (Showalter, 1993). The arabinosyl residues of extensin tend to wrap around the protein backbone and contribute to the helical polyproline II structure adopted by soluble extensins. When viewed under high power electron microscopes, extensin is seen as a rod-like structure (Showalter, 1993).

The amount and location of extensin vary according to plant, tissue and cell type (Showalter, 1993). Extensins are distributed uniformly through the primary cell walls of carrot storage roots, but are not found in the middle lamella (Stafstrom and Staehelin, 1988). Extensins may be cross-linked to each other (Fry, 1982), and cross-links between extensin and polysaccharides have been shown (Qi *et al.*, 1995). These linkages will be discussed in Section 1.3.5.

The functions of extensins are still unclear. Even though they have been given the name extensin, they do not induce extension of plant cell walls and indeed their levels increase after cell extension has ceased (Showalter, 1993). Extensins in plant cell walls are believed to form a fibrillar network that is independent of the cellulosic network, and this second, hydroxyproline-rich glycoprotein network is likely to considerably strengthen the cell wall (Varner and Lin, 1989; Carpita and Gibeaut, 1993). Extensin might regulate or restrict cell expansion and be important in the final structure of the cell wall, thus contributing to the overall shape of the cell (Fry, 1982; Carpita and Gibeaut, 1993). Extensins are also thought to be involved in the protection of plant cells against pathogen invasion, because they are induced in response to microbial attack (Benhamou *et al.*, 1991; O'Connell *et al.*, 1990). Their mode of action could either involve the formation of tight cross-links that make the cell wall more difficult to penetrate, or they might agglutinate bacteria (reviewed by McNeil *et al.*, 1984), although not all extensins show agglutination activity (Dey *et al.*, 1997).

Glycine-rich proteins (GRPs) are comprised of up to 70% glycine (Showalter, 1993). GRPs are generally classed as structural wall proteins. However, there are at least two classes of GRPs. One class, such as the GRPs from petunia (Condit *et al.*, 1990) and bean (Keller *et al.*, 1988; Ryser and Keller, 1992), is associated with the cell wall. The other class is located in the cytoplasm (Showalter, 1993). GRPs are generally found in cells that eventually become lignified (Condit *et al.*, 1990; Ryser *et al.*, 1997; Ye and Varner, 1991). The biological function of GRPs is not understood but wall-bound GRPs are developmentally regulated and may be structurally important for the vascular system and for wound healing (Showalter, 1993).

Proline-rich proteins (PRPs) are characterized by the abundance of Pro-Pro units within other larger repeat units (Showalter, 1993). They are found in a variety of plant types (Josè and Puigdomènech, 1993) but are again associated with lignified tissue (Ye *et al.*, 1991). PRPs are developmentally regulated and are induced by external factors

such as wounding, fungal attack and light (Showalter, 1993). The precise function of PRPs remains unclear.

1.3.4.2 Other proteins

Lectins of the Solanaceae are glycoproteins composed of approximately 40-50% carbohydrate, of which the majority consists of arabinosyl and galactosyl residues, and 50-60% protein, which is rich in hydroxyproline and serine. (Fincher *et al.*, 1983). Suggested functions include cell-cell interactions, wound healing and plant defence (Showalter, 1993).

Arabinogalactan proteins (AGPs) are present in most higher plants, and are found in plasma membranes and in extracellular secretions (Fincher *et al.*, 1983; Bacic *et al.*, 1996). AGPs are proteoglycans; the protein core usually accounts for less than 10% by weight and is characterized by high proportions of hydroxyproline, serine, threonine, alanine and glycine. The carbohydrate component is composed mainly of (1→3,1→6)-β-galactosyl and terminal arabinosyl residues. AGPs do not have a structural function in walls and although their functions have not always been determined unequivocally, they may be involved in defence against pathogens, in cell proliferation, and/or somatic embryogenesis (Bacic *et al.*, 1996; Kreuger and van Holst, 1996).

Expansins are proteins that were recently identified in plant cell walls (McQueen *et al.*, 1992). Two proteins with molecular weights of 29 kD and 30 kD were isolated from walls of growing cucumber seedlings (McQueen *et al.*, 1992) and a number of cDNAs have been cloned from different plants (Shcherban *et al.*, 1995). The protein has a cysteine-rich region, a basic region and a cellulose-binding domain-like region, as well as a hydrophobic signal peptide (Cosgrove, 1997). The role and action of expansins in elongating and expanding tissue is unclear, but they have been implicated in cell wall-loosening and may act by disrupting noncovalent interactions between cell wall polymers (McQueen-Mason and Cosgrove, 1995; Rose *et al.*, 1997). Expansins have also been found in ripening fruit (Rose *et al.*, 1997).

Certain *enzymes* are found either within or closely associated with plant cell walls. Examples include peroxidases, phosphatases, invertases, malate dehydrogenase, proteases and ascorbic acid oxidase (Cassab and Varner, 1988; Varner and Lin, 1989). Cell wall-modifying enzymes are also present; these will be discussed in Section 1.5.

1.3.5 Interactions between cell wall components

Chemical cross-links, which may be covalent or non-covalent in nature, are important for stabilising the overall structure of the cell wall, and are also important in cell extensibility, digestibility and adherence (Fry, 1986). Interactions such as polysaccharide-polysaccharide, polysaccharide-protein, protein-protein and the involvement of phenolics will all occur in plant cell walls (Fry, 1986), and modifications to these cross-links will change the properties of the cell walls (see Section 1.4). A model representing possible cross-links of wall polymers is shown in Figure 1.3 (Fry, 1986).

1.3.5.1 Covalent cross-links

There are a number of covalent cross-links between polysaccharides in plant cell walls. The attachment of galactan and arabinan (in the form of AG-I) *via* glycosidic linkages to the RG-I backbone is one example of a covalent polysaccharide-polysaccharide cross-link (Dey and Brinson, 1984). Another example is the cross-linking of RG-II chains by borate-diol ester bonds (Kobayashi *et al.*, 1996; O'Neill *et al.*, 1996). Pectins and other noncellulosic polysaccharides are cross-linked *via* hydroxycinnamic acids (Fry, 1986; Bacic *et al.*, 1988; Iiyama *et al.*, 1994). Other covalent cross-links between pectins and noncellulosic polysaccharides occur. For example, in cell walls of suspension-cultured sycamore cells, a covalent connection was found between XG and pectic polysaccharides (Keegstra *et al.*, 1973).

Covalent cross-links between cell wall proteins can also be found in walls of higher plants. Extensins may be attached to each other *via* Tyr-Tyr dimers known as isodityrosine (Fry, 1982), and a novel tetrameric derivative of tyrosine, di-isodityrosine,

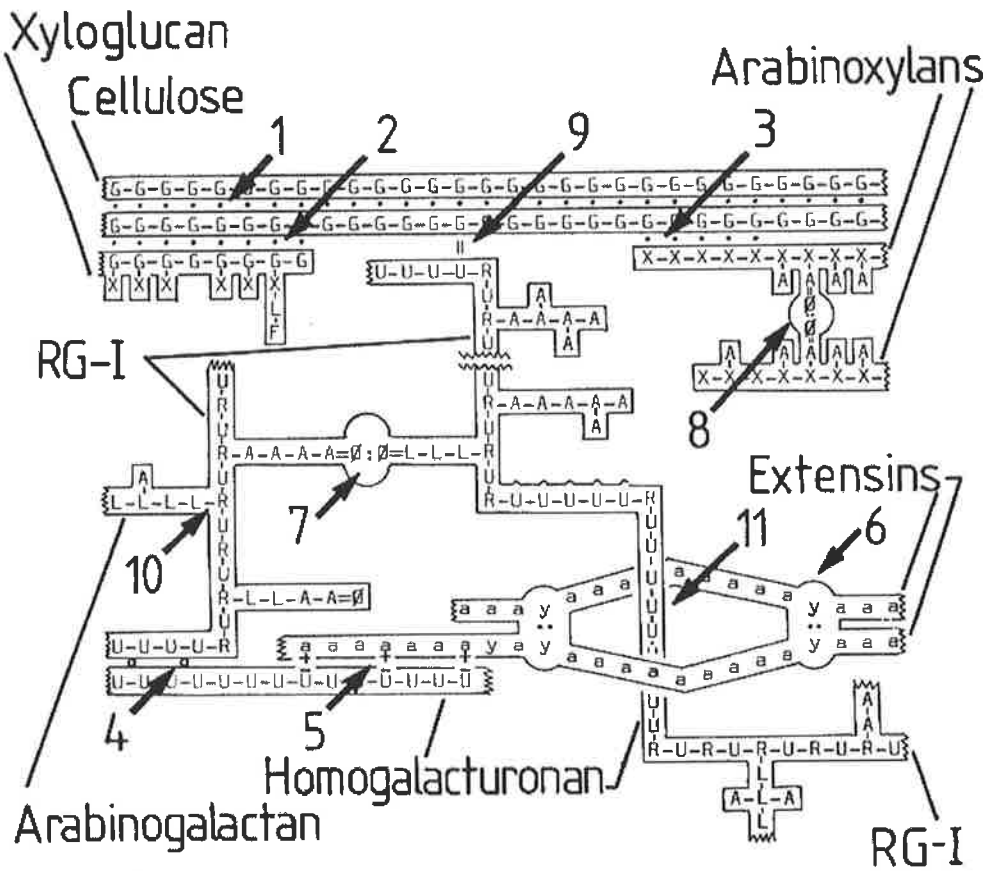

Figure 1.3 Possible cross-links of wall polymers. The diagram illustrates:

Hydrogen bonds (·)	1. Cellulose-cellulose
	2. Xyloglucan-cellulose
	3. Xylan-cellulose
Calcium bridges (°)	4. Homogalacturonan-homogalacturonan
Other ionic bonds (±)	5. Extensin-pectin
Coupled phenols (:)	6. Extensin-extensin
	7. Pectin-pectin
	8. Arabinoxylan-arabinoxylan
Ester bonds (+)	9. Pectin-cellulose
Glycosidic bonds (-)	10. Arabinogalactan-rhamnogalacturonan
Entanglement	11. Pectin and extensin

Abbreviations used: A = Arabinose, F = Fucose, G = Glucose, L = Galactose, R = Rhamnose, U = Galacturonic acid, Û = Galacturonic acid methyl ester, a = Amino acid other than tyrosine, y = tyrosine, y:y = isodityrosine, Ø = Ferulic acid, Ø:Ø = Diferulic acid. (From Fry, 1986)

has been isolated and may also be a molecular cross-link between protein molecules (Brady *et al.*, 1996).

An example of a covalent linkage between a protein and a polysaccharide is afforded by the interaction between extensin and pectin. This interaction has been demonstrated in cotton suspension cultures, where most, if not all, the extensin that had been incorporated into the cell walls is covalently attached to pectin (Qi *et al.*, 1995).

1.3.5.2 Non-covalent cross-links and interactions

One of the major non-covalent interactions between polysaccharides in walls is the hydrogen bonding between cellulose molecules within cellulosic microfibrils (Section 1.2.3). In addition, these microfibrils may be coated with xyloglucan and other non-cellulosic polysaccharides *via* hydrogen bonds (Dey and Brinson, 1984, McCann *et al.*, 1990). Non-cellulosic polysaccharides can also be attached to each other *via* H-bonding (Brett and Waldron, 1996).

Another important example of non-covalent interactions is provided by the "junction zones" that occur in pectin gels (Rees, 1977). Regions of contiguous unsubstituted, unesterified GalA*p* residues in the PG backbone can interact *via* ionic calcium bridges (Fig. 1.4). Antiparallel chains of polygalacturonic acids form a stable junction zone if about 14 contiguous GalA*p* residues are present (Jarvis, 1984). These constitute the so-called "egg-box" junction zones of pectin gels (Rees, 1977). As expected, chelating agents solubilise PG gels by disrupting the calcium bridges (Jarvis, 1982; Redgwell *et al.*, 1988).

Proteins and polysaccharides might also interact non-covalently. Ionic bonding between positively-charged extensins and negatively-charged galacturonans may occur (Showalter, 1993). AGPs and pectins can interact. In grapes, Saulnier *et al.* (1988) found AGP structures associated with the branched regions of water-soluble pectins, although the exact nature of the association is unknown.

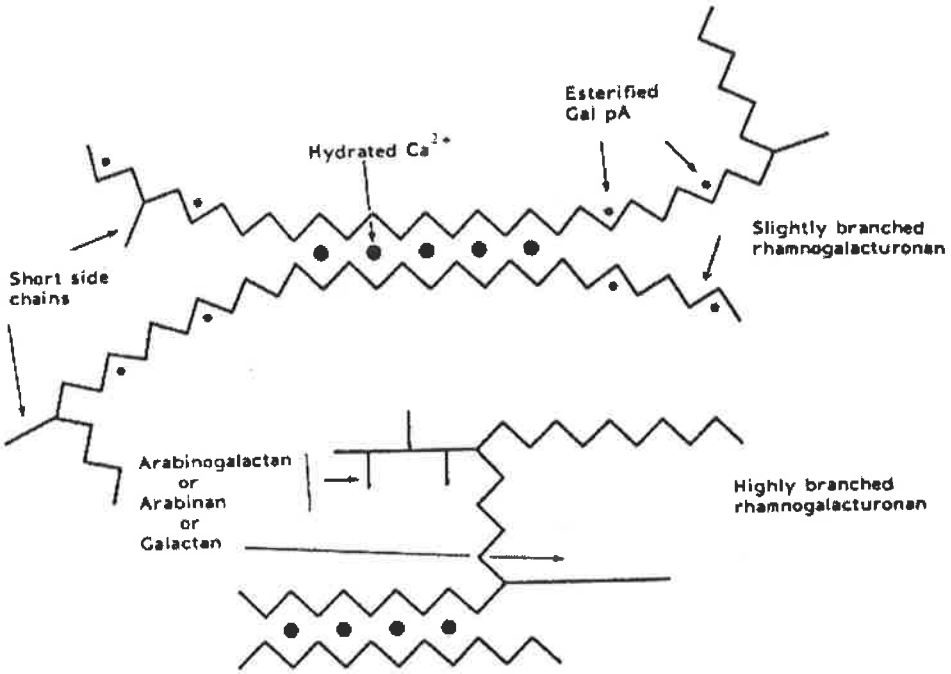

Figure 1.4 Structural features of pectin. Non-covalent interactions between pectic polysaccharides involving calcium ions result in the formation of "junction zones". (From John and Dey, 1986)

1.3.5.3 Physical entanglement

One other important interaction within a cell wall that involves neither covalent bonds nor non-covalent forces is physical entanglement (Fry, 1986; Brett and Waldron, 1996). Polysaccharides with irregular, extended conformations, such as highly-branched pectins or polysaccharides composed of long stretches of β-linked sugar residues (Burchard, 1993), could become intertwined, which is likely to affect the nature of the local cell wall structure.

1.3.6 Models of plant cell walls

The arrangement of components of the primary cell wall of plants has been extensively debated for many years. The structures of major polysaccharides and proteins are known, and some of the cross-links between wall components have been characterized. This has resulted in an evolving variety of models for the primary cell wall. Early models were suggested by Albersheim's group (Keegstra *et al.*, 1973) and by Monro *et al.* (1976). Later models included mechanisms for cell wall extension (Roland and Vian, 1979; Cleland, 1981; Lamport, 1986).

One of the more recent models of primary cell walls, which encompasses many features of the earlier models depicts the primary cell wall as an arrangement of three structurally independent but interacting domains (Carpita and Gibeaut, 1993). The first domain consists of a fibrillar cellulose-xyloglucan framework, which is embedded in a second domain, or matrix, of pectic polysaccharides. The third domain is composed of a fibrillar network of structural proteins (Mort and Lamport, 1977; Lamport and Epstein, 1983). The model proposed by Carpita and Gibeaut (1993) is shown in Figure 1.5. Cellulose and xyloglucan are closely associated (Bauer *et al.*, 1973; Hayashi *et al.*, 1987) and this structural network can be observed using electron microscopy (McCann *et al.*, 1990). Pectic polysaccharides can also form a "network". Thus, when the formation of a cellulose-xyloglucan network in suspension-cultured tomato cells is

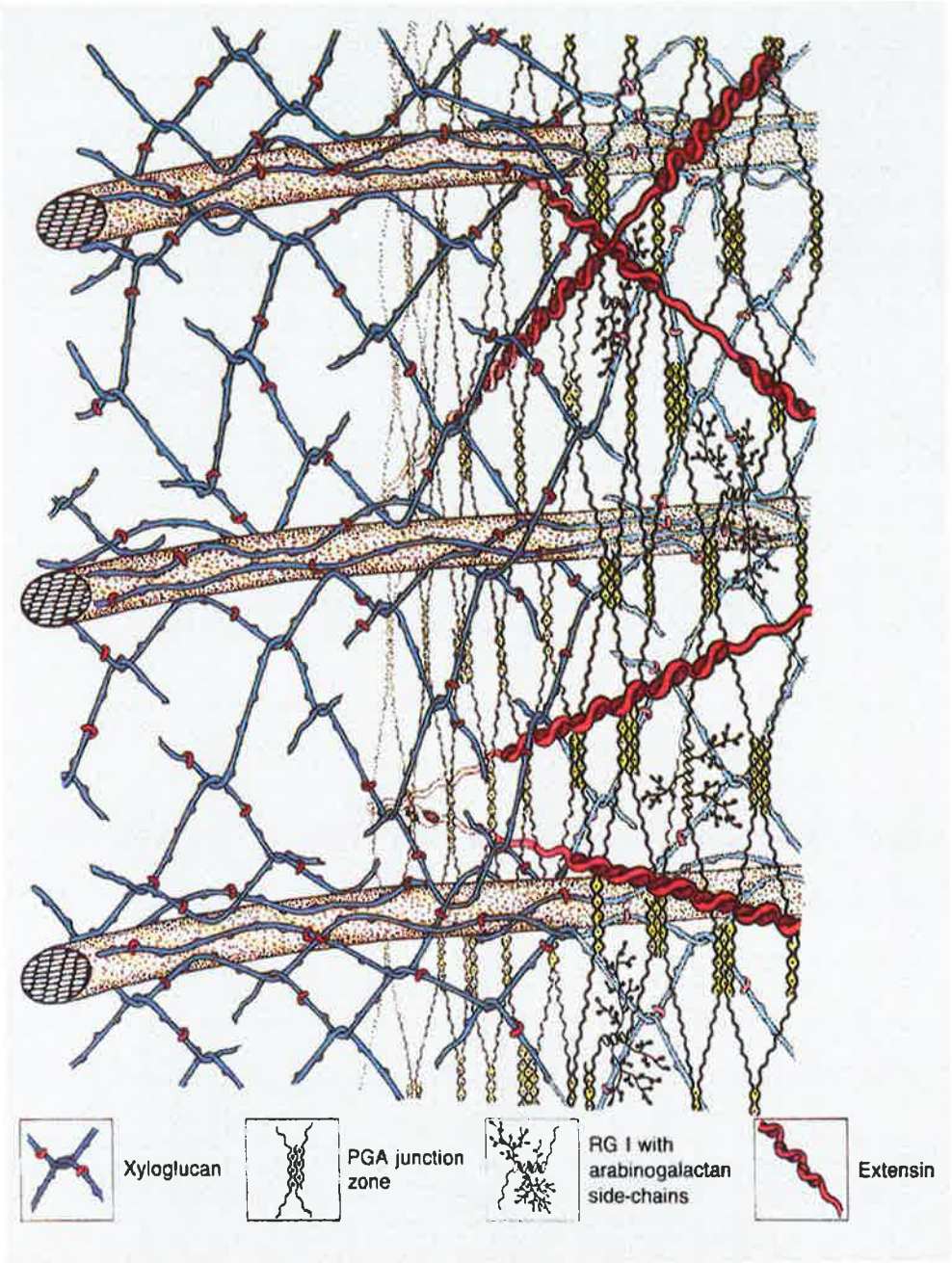

Figure 1.5 Model of the cell wall of higher plants. This model illustrates a possible mechanism for cell wall expansion in plants. It is proposed that the cleavage of xyloglucans permits the microfibrils to separate, resulting in the loosening of the overall cell wall structure. Extensin molecules are then inserted into the cell wall to interlock the microfibrils and thereby to prevent further stretching after cell wall expansion ceases. (From Carpita and Gibeaut, 1993)

inhibited, the cells survive by tightly cross-linking the pectin matrix (Shedletzky *et al.*, 1990).

1.4 Fruit Softening

Against this background of cell wall composition and structure, the processes that occur during softening of ripening fruit will now be considered. Softening is thought to be a result of cell wall modification. Modifications to pectin, other non-cellulosic polysaccharides, cellulose, proteins, or crosslinks within the wall will affect both covalent and non-covalent interactions between these components and may change the overall physicochemical properties of the wall (Seymour and Gross, 1996). Attempts have been made to identify a single event in cell walls of softening fruit that might be considered as the "ripening event", but it appears that major structural changes are not needed to produce significant softening. Relatively minor modifications to cell wall components can result in major alterations of their properties (Seymour and Gross, 1996).

1.4.1 Modification of pectic polysaccharides

The majority of wall modifications in ripening fruit is probably associated with the pectic polysaccharides. The most significant change is often an increase in water-soluble pectins as the fruit ripens and softens. Apple (Knee, 1973), tomato (Gross and Wallner, 1979), pear (Ahmed and Labavitch, 1980a), kiwifruit (Redgwell *et al.*, 1990), cherry (Batisse *et al.*, 1994) and avocado (Sakurai and Nevins, 1997) all show an increase in water-soluble pectic polysaccharides as ripening proceeds. The cause for the increase in pectin solubility is probably complex. Depolymerisation of the RG backbone, the removal of side chains, and the disruption of cross-links can all increase the solubility of specific polysaccharide components of the wall (De Veau *et al.*, 1993). Such modifications will clearly disrupt or diminish polysaccharide associations within the cell wall, which could, in turn, result in an overall softening of the tissue.

Deesterification of pectin can have several effects on the properties of the cell wall. The removal of esterified groups from GalA*p* residues allows the binding of calcium, which results in increased non-covalent cross-linking through increased junction zone formation (Jarvis, 1984). Ricard and Noat (1986) suggested that deesterification also causes a localised pH change, which in turn might activate cell wall-modifying enzymes that cause softening. Changes in esterification of pectins have been observed during the ripening of many fruit. Thus, a decrease in the degree of esterification (DE) of PG during ripening is found in tomatoes (Koch and Nevins, 1989), kiwifruit (Redgwell *et al.*, 1990), apples (Yoshioka *et al.*, 1992) and pears (Martin-Cabrejas *et al.*, 1994).

Although increased junction zone formation following deesterification would be expected to decrease the solubility of pectin, a secondary effect of removing ester groups would be an enhanced susceptibility of the unesterified polyuronide to polygalacturonase (PGase) activity. Thus, the widely reported increase in PG solubility during fruit ripening is thought to be mainly due to depolymerisation of PG by PGase (Huber, 1983a; Seymour and Gross, 1996). Not surprisingly, the polyuronides that become more soluble with softening have lower apparent molecular weights. There are decreases in the molecular weights of pectic polysaccharides in the tomato locule as the tomato softens (Cheng and Huber, 1996) and similar effects are observed in other ripening fruit such as pear and avocado (Yoshioka *et al.*, 1992; Huber and O'Donoghue, 1993).

Modifications of polysaccharide side chains in cell walls have also been reported in softening fruit and are believed to increase the solubility of the wall polysaccharides (Gross and Wallner, 1979). The most common modification is the loss of neutral sugar side chains from the RG-I backbone. Removing these side chains is thought to 'open up' the gel and render the polysaccharide backbone more susceptible to the action of endo-acting enzymes (Ahmed and Labavitch, 1980a). The loss of galactose and/or arabinose is one of the most well-documented changes that occur in ripening fruit. In a comprehensive study, Gross and Sams (1984) analysed cell walls from 17 different

fruit, at three stages of ripeness. Of these samples, 15 showed a net decrease in non-cellulosic neutral sugars, in particular galactose and/or arabinose. Cell walls isolated from ripening tomatoes show significant decreases (40 to 71%) in galactose, depending on the cultivar and on the way in which walls were isolated (Wallner and Bloom, 1977; Gross and Wallner, 1979; Gross, 1984). In tomatoes, the water-soluble polyuronides contained fewer neutral sugars than other pectins (Gross and Wallner, 1979), and the CDTA-soluble polysaccharides contained fewer neutral sugars than the Na_2CO_3-soluble polysaccharides (Seymour *et al.*, 1990). This suggests that a reduction in the number of sidechains or the shortening of sidechains attached to the polysaccharide backbone, indicated by the amount of neutral sugars, influences the solubility of the overall polysaccharide.

1.4.2 Modifications of other noncellulosic polysaccharides and cellulose

Limited changes to other noncellulosic polysaccharides and to cellulose have been found in fruit during ripening. A decrease in the molecular weight of both avocado and melon XG has been observed (O'Donoghue and Huber, 1992; Rose *et al.*, 1998) and a decrease has been found in the molecular weight of alkali-soluble polysaccharides in tomatoes (Huber, 1983a). XG may be hydrolysed and rejoined by the enzyme xyloglucan endotransglycosylase (XET) during ripening (Fry, 1993). Absolute amounts of cell wall XG amounts remain relatively constant throughout the ripening of fruit such as avocado (Sakurai and Nevins, 1997) and kiwifruit (Redgwell *et al.*, 1990).

1.4.3 Changes in protein composition

As fruit develop and soften, changes in the protein component of the walls occur. The insertion of proteins, such as extensins, increases the tensile strength of the wall (Iraki *et al.*, 1989) and might control the rate of expansion in growing tissues. Extensins are thought to be involved in the cessation of expansion rather than in the softening process (Carpita and Gibeaut, 1993). Expansins from tomato, melon and strawberry have also been shown to be expressed during ripening; the expansins may participate in

cell wall disassembly during fruit ripening (Rose *et al.*, 1997). Enzymes, such as PGase, are normally associated with cell walls in fruit. PGase is tightly associated with a specific pectic fraction in the cell walls of tomatoes (Steele *et al.*, 1997).

1.4.4 Cell wall turnover

The softening process during fruit ripening is complicated by the fact that breakdown or modification of existing wall components is usually accompanied by the continuous incorporation of newly-synthesized components into the wall (Lackey *et al.*, 1980; Gibeaut and Carpita, 1994; Seymour and Gross, 1996). Turnover of wall polymers has been reported in fruit such as strawberries, tomatoes and kiwifruit, (Knee *et al.*, 1977; Lackey *et al.*, 1980; Mitcham *et al.*, 1989; Redgwell, 1996). Incorporation of newly-synthesized polymers into the wall and the removal of other polymers produces a less rigid wall, and thus a loss of tissue integrity during fruit ripening (Mitcham *et al.*, 1989). The turnover of cell wall components allows the modification of wall properties while the overall structural framework of the wall is maintained (Labavitch, 1981).

1.4.5 Morphological changes to cell walls

The morphology of the cell walls of many fruit changes as the fruit softens. Redgwell *et al.* (1997) showed that walls in 'soft' fruit undergo pronounced swelling, while in 'crisp' fruit, no swelling occurs. Swelling in kiwifruit cell walls is pronounced; in some tissues a three- to four-fold increase in diameter between unripe fruit and ripe fruit is seen. The degree of swelling of cell walls varies not only between cells but also at different regions around a single cell (Hallett *et al.*, 1992). Dissolution of the middle lamella occurs in some fruit (Crookes and Grierson, 1983; Hallett *et al.*, 1992) and this may contribute to softening.

1.5 Aims

With this background information in mind, the overall objective of work described in this thesis was to gain an understanding of changes that occur in cell walls of grapes as the berry matures and softens.

Within this overall aim, more specific aims of the project were:

- To develop a method to isolate cell walls from grapes (Chapter 2),
- To investigate how the cell wall composition changes during ripening (Chapter 3),
- To study the enzymes involved in softening of grapes during ripening (Chapter 4),
- To isolate cDNAs encoding for some of these cell wall-modifying enzymes (Chapter 5), and
- To study the transcriptional activities of genes encoding these enzymes (Chapter 6).

Chapter 2

Isolation and Characterization of Cell Walls from the Mesocarp of Mature Grape Berries

2.1 Introduction

2.1.1 Plant cell wall isolation

Analysis of cell walls from plant tissue is the first step towards understanding the composition and structure of the wall, but is dependent on the development of a good procedure for wall isolation. Analysis of wall preparations allows individual components to be identified, provides clues as to how the components are linked together, and can indicate how components are distributed within the wall (Darvill *et al.*, 1980). In addressing these questions, it is not only important to have a good method for wall isolation, it is also important to understand how components might be affected during the isolation procedure.

The aim of cell wall isolation procedures in general is to obtain relatively pure walls, usually from a single tissue type, free of contamination from other parts of the cell or plant. The integrity of the wall needs to be preserved to ensure accurate interpretation of results. If possible, no integral components of walls should be lost during isolation. Thus, the isolation of cell walls is a crucially important and fundamental procedure that is a prerequisite for cell wall structural analysis.

Obtaining a pure cell wall preparation from a single tissue type can be difficult to achieve. A major problem is contamination that can occur as a result of co-precipitation of cytoplasmic components onto the cell walls, especially when organic solvents are used in attempts to ensure water-soluble components are not lost during isolation. Precipitation of intracellular proteins, nucleic acids, starch and some polyphenols onto the walls interferes with subsequent analyses (Selvendran and Ryden, 1990). Loss of molecules that are an integral part of the cell wall *via* solubilization has also been problematic. Endogenous cell wall-modifying enzymes might cause autolysis of the walls if the enzymes are not removed or completely inactivated (Gallego, 1996) and modifications that would not normally occur *in vivo* at that time or position in the wall

may occur during isolation. Each of these problems needs to be addressed when designing a cell wall isolation procedure.

Potential problems associated with isolating walls from the mesocarp of grape berries are numerous. Firstly, to isolate mesocarp tissue from grapes, the skin, seeds and vascular tissue need to be removed. Removal of the skin and seeds from the grapes is a slow and laborious process, and damage to underlying mesocarp cells may occur, especially if the grapes have been frozen. Contamination with vascular tissue, which runs through the centre of the grape berry and under the skin surface (Coombe, 1987; Figure 1.3) should be avoided if possible. Cell walls of grape mesocarp tissue are also very thin (Hardie *et al.*, 1996) and therefore fragile, and this could lead to excessive fragmentation of the cell walls during tissue homogenization.

In the few published studies on grape cell walls, grapes are homogenized in alcohol, such as ethanol, and the alcohol-insoluble residue (AIR) is collected (Robertson, 1979; Saulnier and Thibault, 1987a,b). In preparations from grape skin, the AIR contains only 50% polysaccharide, with the remainder consisting of proteins, phenolics and "unknown matter" (Lecas and Brillouet, 1994). Given that most higher plant walls consist of 80% or more of polysaccharides, a large proportion of this AIR preparation is likely to be of cytoplasmic origin. Again, this needs to be considered in the design of a new procedure for isolation of walls from grape berries. Complexing of phenolics to protein is a major cause of enzyme inactivation and protein precipitation (Loomis and Battaile, 1966). Hawker (1969) found that invertase in grapes became insoluble as a result of phenolics binding to the enzyme; such complexes could contaminate cell wall preparations.

2.1.2 Analytical procedures

Many different procedures are used to analyse the polysaccharide composition of plant cell walls. A common procedure involves hydrolyzing the polysaccharides using acid and analysing the released monosaccharides by gas chromatography (GC) once the sugars are converted into volatile alditol acetates (Selvendran and O'Neill, 1987). One

shortcoming of this method is that it provides no information on linkages types present in the polysaccharides. One way to obtain this additional information is by methylation analyses. Methylation of all free hydroxyl groups in the polysaccharide is performed before hydrolysis, which results in the release of partially methylated sugars. Once these sugars are converted to their alditol acetates, the glycosyl linkage positions can be determined by gas chromatography-mass spectrometry (GC-MS), although no information on the relative order of the sugar residues within the polysaccharide is gained (Björndal *et al.*, 1970; Lindberg, 1972). However, from previous studies on individual polysaccharides isolated from plant cell walls, predictions of polysaccharide types and abundance can be made from the linkage data (Bacic *et al.*, 1988; Shea *et al.*, 1989).

2.1.3 Cell walls in fruit

Cell walls of fruit have received considerable attention over many years, mainly because the textural changes, in particular fruit softening, that accompany ripening are believed to result primarily from changes in cell wall polysaccharides (Brady, 1987; Fischer and Bennett, 1991). However, despite the importance of grapes (*Vitis vinifera*) as an edible fruit and in the production of wine and fruit juices, there is very little information available on the chemical composition of their cell walls, and the changes which occur during ripening have not been defined. Pectic polysaccharides from the pulp of mature grapes have been characterized (Saulnier and Thibault, 1987b; Saulnier *et al.*, 1988) and the composition of walls from berry skins has been determined (Lecas and Brillouet, 1994).

In the work reported here, a method has been developed for the isolation of cell walls from the mesocarp of grape berries, and the compositions of walls isolated from mature berries of two *Vitis vinifera* L. cultivars have been determined.

2.2 *Materials and Methods*

2.2.1 Plant material

Ripe grape (*Vitis vinifera* L.) berries cvs. Ohanez and Muscat Gordo Blanco (hereafter referred to as Gordo) were collected at the South Australian Advisory Centre, Nuriootpa, South Australia, Australia. The Gordo berries were collected 114 days post-anthesis (dpa) in late March 1994 and the Ohanez berries 138 dpa in late April 1994. Measurements on 50 berries were made for total soluble solids (°Brix), which gives an indication of % soluble sugar, by measuring the refractive index of juice squeezed from berries, using a hand-held refractometer (Lieca Inc., Buffalo, NY, USA). An average °Brix value of 18 for Gordo and 21.5 for Ohanez berries was obtained. Deformability (or degree of softness) of the 50 berries was also measured according to the method of Coombe and Bishop (1980), using skin-fold calipers that applied a force of 3.4 N at the caliper face; Gordo had an average deformability of 1.8 mm and Ohanez had an average of 1.0 mm. The grapes were rinsed with deionized water and stored at -80°C prior to use.

2.2.2 Preparation of cell walls

The skin and seeds of frozen berries were removed with a scalpel and the remaining tissue, which was predominantly mesocarp but included vascular tissue, was used for the preparation of cell walls. Partially thawed tissue (100 g) was gently homogenized at 4°C in 400 ml absolute ethanol (giving a final concentration of 80% (v/v) ethanol) using a household blender (7 speed Futura, Breville). The homogenate was sieved sequentially through nylon mesh (Swiss Screens, Moorabbin, Victoria, Australia) with pore sizes of 350 μm, 280 μm and 71 μm using 80% (v/v) ethanol at 4°C as the washing solution. Throughout the wet-sieving steps, berry homogenates were suspended and washed through the nylon mesh in 80% (v/v) ethanol and a spatula was used to stir material on the top of the mesh.

The material on the 350 μm and 280 μm mesh was discarded. The material retained on the 71 μm mesh was stirred for 45 min in phenol that had been saturated with 500 mM Tris-HCl buffer, pH 7.0 (Huber, 1991). The suspension was filtered through a single layer of Miracloth and washed with 80% (v/v) ethanol and acetone to remove phenol. The retained material was suspended in 150 ml chloroform-methanol (1:1 v/v), stirred for 1 h and the suspension was filtered under aspiration on a sintered-glass funnel. The wall preparation so obtained was resuspended in chloroform:methanol and the stirring and filtration procedures were repeated. The final wall preparation was suspended in 150 ml acetone, stirred for 1 h at room temperature, filtered on the sintered-glass funnel and dried in a vacuum oven at 40°C. The wall preparation was stored over silica gel in a vacuum desiccator at room temperature.

2.2.3 Microscopy

During the isolation procedures, cell walls were examined by light microscopy using a Zeiss Axiophot Pol photomicroscope (Oberkochem, West Germany). The walls were stained with either 0.25% (w/v) Coomassie Blue, iodine [containing 0.33% (w/v) iodine and 0.66% (w/v) potassium iodine in water], or 0.05% Toluidine Blue O (TBO) in 10 mM sodium benzoate buffer, pH 4.4. The final preparation was also examined by scanning electron microscopy, for which walls were mounted on metal stubs using double-sided adhesive tape and coated with gold to a thickness of approximately 30 nm. Specimens were examined in a S250 Mark 3 scanning electron microscope (Cambridge Scientific Instruments Ltd, Cambridge, UK) at the CSIRO Division of Soils (Urrbrae, SA), using an accelerating voltage of 20 kV.

2.2.4 Protein, amino acid and mineral analyses

Protein in wall preparations was estimated using a modified procedure of Peterson (1977), that was based on the original Folin-Ciocalteu reagent method of Lowry *et al.*, (1951). The initial trichloroacetic acid (TCA) precipitation step was omitted, and subsequently the walls were placed immediately into Reagent A. The cell walls were

incubated in Reagent A overnight instead of 30 min to ensure complete solubilization of associated proteins, and the extraction continued as specified in the original procedure. The protein standard (bovine serum albumin, Sigma) was prepared as described by Peterson (1977).

Gordo and Ohanez cell walls (approximately 1 mg) were hydrolyzed in 100 µl 6 M HCl at 110°C for 24 h in the presence of a crystal of phenol. Amino acids in the hydrolysate were quantitated using a Beckman System 6300 amino acid analyser (Beckman Instruments, Palo Alto, CA, USA). Hydroxyproline in hydrolysates was measured colorimetrically by the procedure of Kivirikko and Liesmaa (1959).

Total nitrogen and ash contents of walls were determined by the National Analytical Laboratories, Notting Hill, Victoria. Total nitrogen values were converted to protein content by the equation: % Protein (w/w) = % Nitrogen (w/w) x 5. The conversion factor of 5.0 was based on amino acid compositions of wall-associated proteins, which are particularly rich in arginine residues (Bacic and Stone, 1981).

2.2.5 Polysaccharide linkage analyses

2.2.5.1 Carboxyl reduction

Reduction of uronic acid and esterified uronic acid residues was by the method of Kim and Carpita (1992), as modified by Sims and Bacic (1995). Cell wall preparations (2 mg) were suspended in 5 ml 500 mM imidazole-HCl buffer, pH 8.0 and esterified uronic acid residues were reduced at 0°C by three additions, at 5 min intervals, of 1 ml 100 mg/ml $NaBD_4$ (Sigma Chemical Company, St. Louis, MO, USA) in 500 mM imidazole-HCl buffer, pH 8.0. After 30 min, excess $NaBD_4$ was destroyed with glacial acetic acid, samples were dialysed for 20 h against water and freeze dried. Samples were redissolved in 1 ml H_2O and 200 µl 0.2 M MES-KOH buffer, pH 4.75 was added. Free uronic acid residues were derivatized with 400 µl 500 mg/ml 1-cyclo-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulphonate (Aldrich, Millwaulkee, WI,

U.S.A.) for 3 h at 30°C; wall suspensions were sonicated on ice for 30 min immediately after the addition of the carbodiimide reagent. Following carbodiimide treatment, 1 ml 2 M Tris-HCl buffer, pH 8.0 was added and samples were reduced for 18 h at 4°C either with 1 ml 70 mg/ml $NaBD_4$ in 0.05 M NaOH for the determination of total uronic acids, or with $NaBH_4$ under the same conditions to determine the proportion of esterified uronic acids (Sims and Bacic 1995). Excess reducing agent was destroyed with glacial acetic acid, samples were dialyzed against H_2O and freeze-dried.

2.2.5.2 Methylation analysis

Carboxyl-reduced cell walls (approximately 1 mg) were swollen in 500 µl DMSO for 18 h at 50°C. Methylation analyses were subsequently performed by the $NaOH/CH_3I$ method of Ciucanu and Kerek (1984) as described by McConville *et al.*, (1990), except that samples were sonicated after each addition of NaOH and CH_3I to enhance reagent penetration to wall polysaccharides. The methylated polysaccharides were hydrolyzed with 2.5 M trifluoroacetic acid for 4 h at 100°C, reduced with 1 M $NaBD_4$ in 2 M NH_4OH for 18 h at room temperature and acetylated using perchloric acid as a catalyst (Harris *et al.*, 1984). Partially methylated alditol acetates were separated on a bonded phase BPX70 (SGE, Australia) capillary column in a Finnigan MAT 1020B GC-MS. Neutral sugar and uronic acid derivatives were identified and quantitated as described by Lau and Bacic (1993) and Sims and Bacic (1995).

2.3 Results

2.3.1 Cell wall isolation

The final procedure developed for the isolation of walls from the mesocarp cells of mature grape berries is shown in Figure 2.1. It should be emphasized that skin and seeds were removed from the berries before homogenization. Mesocarp cells and vascular tissues would therefore be expected to be the major constituents of the initial berry homogenate. Vascular tissue was a problem in initial attempts to isolate cell walls from grapes, but this was overcome by filtering the wall preparations through nylon mesh of progressively smaller pore-sizes. Table 2.1 shows the reduction in the number of vascular pieces with each filtration step. Provided the homogenization process was carefully controlled, vascular tissue survived as relatively large fragments, most of which were retained on nylon mesh with a 350 μm linear pore size. Vascular tissue was easily recognizable by light and scanning electron microscopy, by its characteristic tubular shape and multicellular structure (Figs. 2.2A and 2.3C). The initial removal of the large pieces of vascular tissue on the 350 μm nylon mesh facilitated subsequent sieving on smaller pore size nylon filters. More vascular tissue fragments, together with some of the larger fragments of mesocarp cell walls, were retained on the 280 μm nylon mesh, but most of mesocarp cell wall fragments and a few vascular fragments passed through this mesh size during the wet-sieving procedure. Finally, the material which passed through the 280 μm nylon mesh was sieved exhaustively on 71 μm mesh. The cell wall fragments were retained on this mesh, while soluble cytoplasmic components and membrane fragments passed through. From the results shown in Table 2.1, it was decided that filtering through 280 μm mesh resulted in the optimum reduction in vascular tissue, without excessively compromising the final yield of walls.

Phenol buffered to neutral pH was subsequently used to remove adventitious protein of cytoplasmic origin that precipitated onto the walls during the ethanol extraction procedure, and to inactivate any wall-bound enzymes that might alter constituent

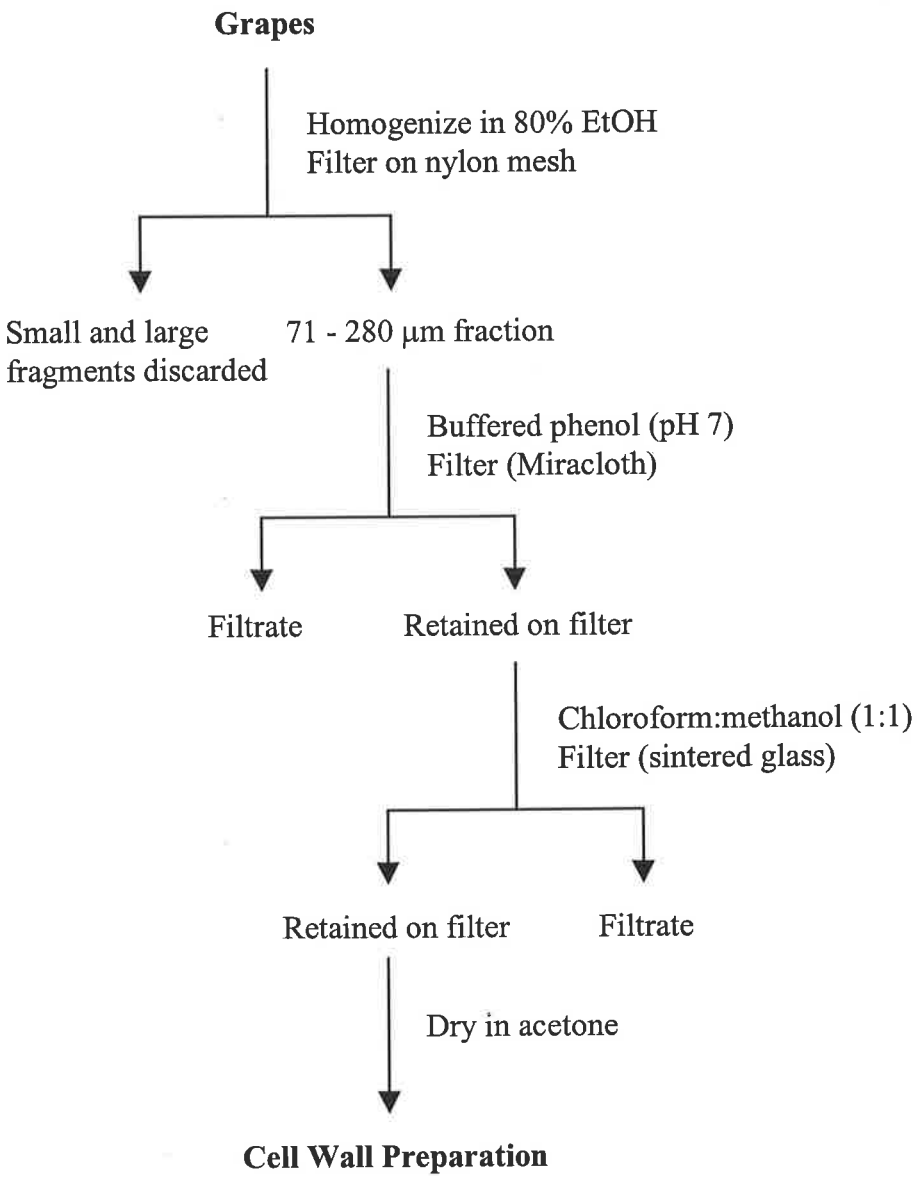

Figure 2.1 Procedure for the isolation of cell walls from the mesocarp of ripe grape berries. The skins of the grapes were removed prior to homogenization.

Table 2.1 Protein content, yields and vascular tissue content of cell walls isolated from Ohanez and Gordo berries after filtering through mesh of different pore-sizes

Grape Cultivar	Pore-Size of Nylon Mesh	Protein Content (% w/w ± se)	CW Yield (mg/100g)	Vascular pieces (%)
Ohanez	no initial filtering	9.6 ± 0.65	370	> 15
	355μm	8.6 ± 0.73	293	approx. 5
	280μm[a]	8.1 ± 0.69	238	approx. 1
	200μm	6.2 ± 0.48	150	< 1
Gordo	280μm[a]	11.4 ± 1.70	177	approx. 1

[a] Final cell wall preparations used for subsequent analyses

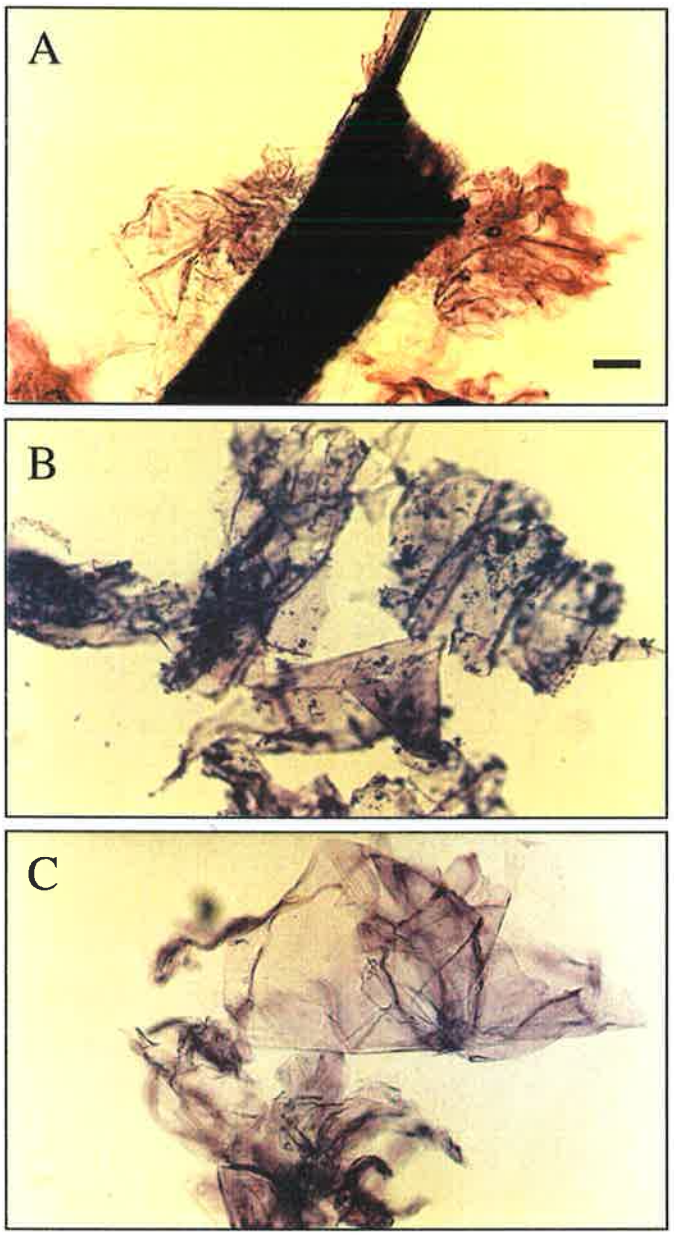

Figure 2.2 Light microscopy of cell walls isolated from the mesocarp of grape berries. **A.** A piece of vascular tissue present in a cell wall preparation, stained with TBO. **B.** Cell walls not treated with buffered phenol and stained with Coomassie Blue. Adventitious proteins, as indicated by the darkly stained specks, are attached to the cell wall fragments. **C.** Cell walls, prepared using the final cell wall isolation procedure, and stained with Coomassie Blue. The wall fragments are free of adventitious proteins. The bar represents 50 μm.

polysaccharides. In initial attempts to remove adventitious proteins, the wall preparations were treated with a number of detergents and reducing agents, but these reagents proved ineffective (results not shown). If the phenol treatment was omitted from the isolation protocol, cell wall preparations with protein contents of 20-30% by weight were obtained, and light microscopy confirmed the presence of numerous pieces of cytoplasmic material adhering to the surfaces of the walls in these preparations (Fig. 2.2B). After phenol treatment, the wall fragments contained little detectable adventitious protein (Fig. 2.2C).

The last step in the purification procedure involved treatment of the wall preparation with chloroform:methanol and was designed to remove lipids and to dislodge membrane-bound proteins that might be associated with the wall fragments (Fig. 2.1). To facilitate storage, the final wall preparations were dried by solvent exchange through acetone (Fig. 2.1). Yields of the dried cell wall material from Ohanez and Gordo berries were 2.4 and 1.8 mg/g fresh weight, respectively (Table 2.1). Iodine staining showed that the wall preparations were essentially free of adherent starch (data not shown).

2.3.2 Protein analysis

Although some variation was observed between cell wall preparations, protein contents estimated by the modified Lowry procedure were approximately 8.1% and 11.4% (w/w) for Ohanez and Gordo, respectively (Table 2.1). The total nitrogen values, were 1.6% and 1.8% (w/w), respectively, and these correspond to protein contents of 8% (w/w) for the Ohanez preparations and 9% (w/w) for the Gordo preparations. The conversion factor of 5.0 was based on amino acid compositions of wall-associated proteins, which are reportedly rich in arginine residues (Bacic and Stone, 1981). We conclude therefore that the wall preparations contained approximately 10% by weight protein, but that the Gordo walls may contain slightly higher levels.

The amino acid compositions of proteins associated with the two cell wall preparations are shown in Table 2.2. The most abundant amino acids were Arg and

Table 2.2. Amino acid composition (mol%)[a] of proteins associated with cell walls of grape berries

Amino acid (Mol%)	Ohanez	Gordo
Asx	4.4	6.5
Glx	9.6	9.0
Ser	5.4	5.8
His	2.3	2.3
Gly	7.4	9.1
Thr	4.8	4.9
Cys	1.2	1.2
Ala	5.7	5.9
Arg	15.1	14.5
Tyr	2.9	2.9
Val	4.7	4.9
Met	0.9	1.1
Phe	2.4	2.7
Ile	3.2	3.6
Leu	4.9	5.5
Lys	5.3	5.9
Pro	5.8	4.9
Hyp[b]	14.2	9.3

[a] excludes Tryptophan
[b] determined colorimetrically as by Kivirikko and Liesmaa (1959)

Hyp, and although Arg contents were similar in both varieties, proteins associated with Ohanez cell walls had much higher Hyp contents than those associated with the Gordo walls. Other abundant amino acids included Gly, Ala, Lys and Pro, but no large differences between varieties were apparent (Table 2.2).

The ash contents for the Ohanez and Gordo preparations were 0.1% and 0.2% (w/w), respectively.

2.3.3 Scanning electron microscopy

Scanning electron microscopy of mesocarp cell wall preparations from the Gordo cultivar are shown in Figure 2.3, but no obvious differences in appearance between the two cultivars could be seen (data not shown). In Figure 2.3A, fragments of the cell wall preparation are shown. The fragments were generally uniform in size, although they were often clumped together and extensively folded (Fig. 2.3B), and in many cases walls from adjoining cells remained attached. Pieces of vascular tissue could occasionally be detected (Fig. 2.3C), but fragment counts at low magnifications indicated that they numbered less than 1% of total wall fragments in the preparation.

Closer examination of the walls revealed relatively smooth, amorphous surfaces (Fig. 2.3D) that were often striated or rippled in appearance (Fig. 2.3E) and adherent cytoplasmic material could sometimes be seen (Fig. 2.3F). There was some suggestion that one side of the walls was relatively smooth in appearance, while the other was rougher and had small deposits on its surface (Fig. 2.3E). These presumably correspond to the outer and inner surfaces of the wall, respectively. Higher magnifications of the fragments suggested that the walls were generally less than 100 nm in thickness (Fig. 2.3E).

2.3.4 Linkage analysis of cell wall polysaccharides

Positions of glycosidic linkages between monosaccharide residues in polysaccharides of the grape berry cell walls were determined by methylation analysis and are shown in

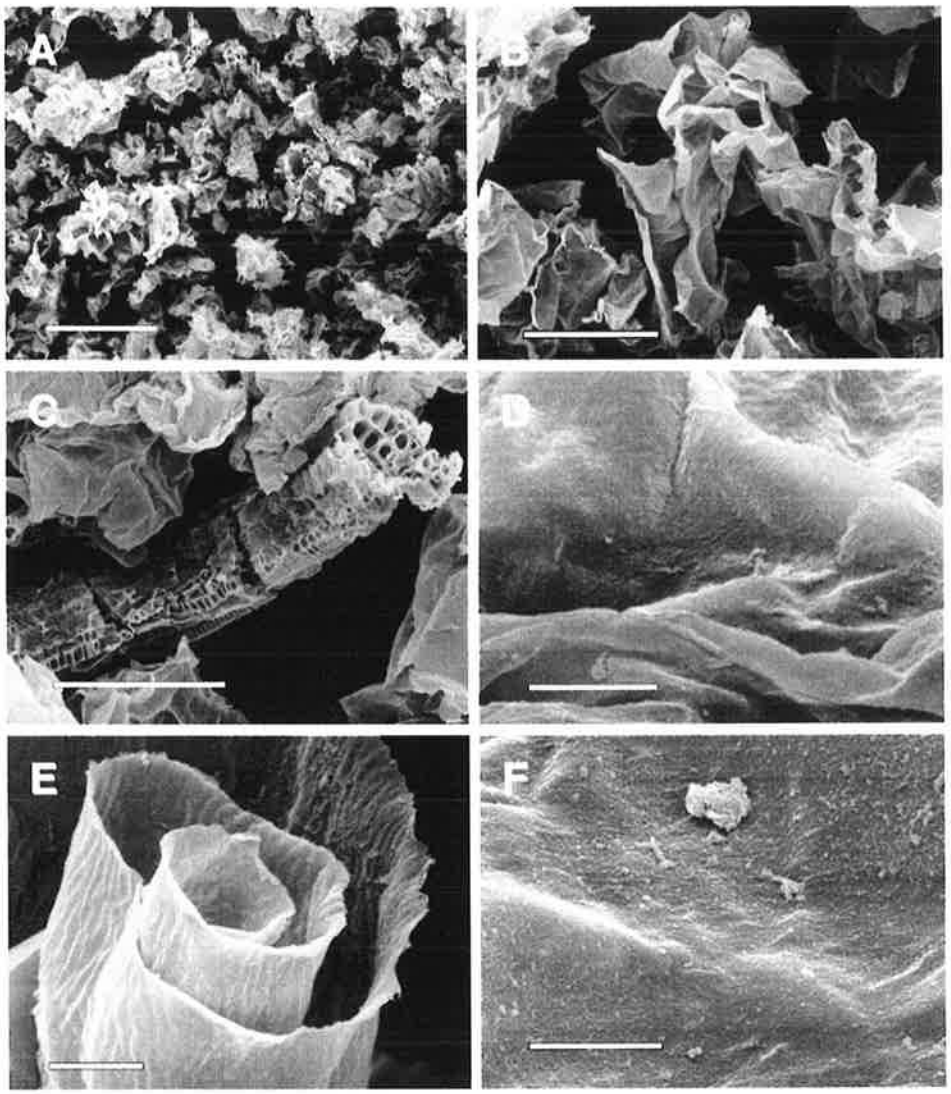

Figure 2.3 Scanning electron microscopy of mesocarp cell wall preparations from Gordo grape berries. **A.** Low magnification micrograph of the final wall preparation. The bar represents 500 μm. **B.** Higher magnification of wall fragments, showing their thin nature and tendency to buckle and fold. The bar represents 100 μm. **C.** A large, tubular fragment of vascular tissue (centre). The bar represents 100 μm. **D.** Smooth, amorphous surface of a mesocarp wall fragment. The bar represents 2 μm. **E.** A mesocarp wall fragment showing the smooth, but rippled outer surface and the rougher inner surface (top right). Bar represents 4 μm. **F.** Higher magnification of the surface of a mesocarp wall fragment and a piece of adhering material, which is presumably of cytoplasmic origin. The bar represents 2 μm.

Table 2.3. The major linkage types in both cell wall preparations are 4-linked D-glucopyranose (Glc*p*) residues, 4-linked D-galacturonic acid (GalA*p*) residues and 4-linked GalA*p* esters. Approximately 48% of total 4-linked GalA*p* residues were esterified (Table 2.3). Ohanez walls contained more (41 mol%) 4-Glc*p* than Gordo walls (32%), but contained less 4-GalA*p* and 4-GalA*p* ester (26% total) than the Gordo walls (38% total). All other linkage types were present in amounts of 5% or less, and only minor differences between varieties were observed (Table 2.3).

The monosaccharide compositions of the wall preparations can be estimated from the sum of all linkage types for a particular monosaccharide. The monosaccharide compositions of the grape berry cell walls are compared with re-calculated data from walls of grape skin and other fruit in Table 2.4. The data shown in Table 2.4 were checked by alditol acetate analysis of monosaccharide composition, where similar results were obtained (data not shown). The major monosaccharides in the grape mesocarp cell walls are glucose and uronic acids and, while the Ohanez walls have higher glucose contents (46.6% cf. 35.6%), uronic acids are higher in the Gordo preparation (40.0% cf. 26.8%). Galactose, arabinose, xylose and rhamnose are also present at lower, but significant levels; no large differences between varieties are apparent. Only very small amounts of fucose and mannose are detected (Table 2.4). These values may be compared with those of cell wall preparations from other fruits, where glucose contents exhibit a broad range from 25% to over 70%, uronic acids range from 10% to over 30% and xylose varies in the range 3.9-32.5% (Table 2.4).

The polysaccharide compositions of grape berry cell walls deduced from the linkage analyses are shown in Table 2.5. These compositions were estimated from the total mol percentages of individual glycosyl residues that are characteristic of well-defined wall polysaccharides (Bacic *et al.*, 1988; Shea *et al.*, 1989). The mol% values for polysaccharides referred to below and in Table 2.5 are calculated by addition of mol% values of the appropriate methylated alditol acetates, and are expressed as mol% of total polysaccharide content of the wall; protein and other wall components are not included.

Table 2.3 Linkage composition of polysaccharides in cell walls isolated from mature grape berries

Deduced Glycosidic Linkage[a]		Linkage Composition (Mol%)	
		Ohanez	Gordo
Rha*p*	terminal	tr	1
	2-	2	2
	2,4-	1	1
Fuc*f*	terminal	1	1
Ara*f*	terminal	5	3
	3-	tr	-
	5-	3	2
Xyl*p*	terminal	3	2
	2-	1	1
	4-	3	4
	2,4-	tr	1
Man*p*	4-	1	1
	4,6-	1	1
Gal*p*	terminal	2	2
	2-	tr	tr
	3-	2	2
	4-	1	2
	6-	tr	tr
	2,4-	tr	tr
	3,4-	1	1
	3,6-	2	1
	3,4,6-	tr	-
Glc*p*	4-	41	32
	2,4-	1	tr
	3,4-	tr	tr
	4,6-	5	3
GalA*p*	4-	13	20
GalA*p* ester	4-	13	18
Glc/GlcA*p*	terminal	1	1
Glc/GlcA*p* ester	4-	1	1

[a] Uronic acids and their esters were reduced to 6,6'-dideuterio neutral sugars before methylation

Table 2.4 Comparison of the monosaccharide composition (mol%) of cell walls from grapes and other fruit

Sugar	Grape Flesh		Grape Skin[a]		Tomato[b]	Kiwifruit[c]	Pear[d]
	Ohanez	Gordo	Chardonnay	Carignan		OP	
Arabinose	8.3	5.1	5.1	6.4	2.7	1.3	9.1
Xylose	6.4	7.0	4.4	3.4	3.9	8.1	32.5
Fucose	0.7	0.6	0.4	0.3	ND	0.5	1.1
Rhamnose	2.6	3.0	1.9	7.7	1.2	0.7	1.3
Mannose	1.5	1.4	4.1	3.7	2.6	1.5	1.7
Galactose	7.3	8.0	5.9	3.7	4.4	6.9	3.1
Glucose	46.6	35.6	36.0	33.0	52.6	71.1	25.7
Uronic acids	26.8	40.0	42.2	41.9	32.6	10.0	25.5

[a] Recalculated from Lecas and Brillouett (1994)
[b] Recalculated from Gross (1984) data on Rutgers RR
[c] Redgwell *et al.* (1990) data on outer pericarp (OP) 7 days after harvest (7D)
[d] Martin-Cabrejas *et al.* (1994)
ND - Not Determined

Table 2.5 Polysaccharide composition of grape cell walls deduced from the linkage composition

Polysaccharide	Polysaccharide Composition (Mol%)	
	Ohanez	Gordo
Polygalacturonan[a]	29	41
Arabinan	3	2
AG-I	1	4
AG-II	6	4
Xyloglucan	12	8
Heteromannan	2	3
Heteroxylan	3	6
Cellulose	39	31
Other[b]	5	1

[a] Includes rhamnogalacturonan and homogalcturonan components of the pectic polysaccharide backbone

[b] Linkage types that cannot be readily assigned to well-characterized polysaccharides from plant cell walls

These calculations embody a number of structural assumptions, but are widely used as a good indicator of trends in contents of specific polysaccharide types (Bacic *et al.*, 1988; Shea *et al.*, 1989; Gorshkova *et al.*, 1996). Thus, XG content was estimated from the sum of 4,6-Glc*p*, 4-Glc*p* equivalent to one-third of 4,6-Glc*p*, terminal and 2-linked D-xylopyranose (Xyl*p*), 2-linked D-galactopyranose (Gal*p*) and terminal L-fucopyranose (Fuc*p*). The remaining 4-Glc*p* was assumed to be cellulose. Xylan was estimated from 4-Xyl*p* and 2,4-Xyl*p*, and terminal L-arabinofuranose (Ara*f*) or terminal D-glucuronic acid (GlcA*p*) equal to the 2,4-Xyl*p*. AG-II was estimated from the sum of 3,6-Gal*p*, 3-Gal*p*, 6-Gal*p* and terminal Ara*f* equivalent to the value for 3,6-Gal*p*. AG-I was from the sum of 4-Gal*p*, 3,4-Gal*p* and terminal Gal*p* equivalent to 3,4-Gal*p*. Arabinan was estimated from 5-Ara*f*, and mannan from the sum of 4-Man*p*, 4,6-Man*p* and terminal Gal*p* equivalent to 4,6-Gal*p*. PG, which includes both RGs and homogalacturonan, was the sum of 4-GalA*p*, 4-GalA*p* ester, 2-Rha*p* and 2,4-Rha*p* (Bacic *et al.*, 1988; Shea *et al.*, 1989).

Cellulose and PGs are the major components of the walls and significant differences can be observed between the two varieties. Thus, the Gordo walls have a higher PG content while the Ohanez walls have relatively more cellulose (Table 2.5). Arabinan and AG-I, which constitute side chains of RGs, are also detected, together with XGs, heteroxylans and small amounts of heteromannans (Table 2.5).

2.4 Discussion

Numerous procedures have been described for the isolation of cell walls from plants, but relatively few methods enable the preparation of walls from a specific tissue type (Selvendran and Ryden, 1990). The initial goal for the preparation of walls from the mesocarp cells of mature grape berries was to develop a procedure that was rapid, simple, involved a small number of steps, was useful for small or large scale preparations and resulted in a cell wall preparation, obtained in good yield, with an acceptably low level of contamination with cytoplasmic material or with cell walls from other tissues of the berry (Fig. 2.1). The grape berries were homogenized into 80% ethanol to ensure that water-soluble components of the walls were not lost, but to ensure that mono- and oligosaccharides in the tissue lysate were removed. The 80% ethanol washes were performed at 4°C to further minimize the activity of polysaccharide-modifying enzymes which are commonly associated with plant cell walls (Fry, 1995) and which are not always completely inactivated in ethanol solutions (Huber and Lee, 1988). Hawker (1969) reported that soluble grape berry invertase becomes tightly associated with cell walls during extraction unless precautions are taken to prevent protein/tannin interactions. Organic solvents used in the extraction procedure might also lead to the co-precipitation of protein, starch, nucleic acids and other cytoplasmic components onto the cell wall fragments (Selvendran and Ryden, 1990), and our observations on cell wall material after the initial ethanol wet-sieving steps confirmed the presence of relatively high levels of adventitious protein. These were subsequently removed by treatment with phenol buffered to pH 7.0 (Fig. 2.1), which would also be expected to completely inactivate any wall-bound enzymes (Jarvis, 1982; Huber, 1991), but would not dissolve polysaccharides (Selvendran and Ryden, 1990).

The yields of walls obtained were 2.4 and 1.8 mg dry wall per g fresh tissue for the Ohanez and Gordo preparations, respectively. Although it cannot be claimed that the isolation procedure is quantitative, the yields may be compared with values of 5-6 mg/g for tomato fruit walls (Huber, 1991) and 6.7 mg/g fresh weight for walls from the outer

pericarp of kiwifruit (Redgwell *et al.*, 1990). It appears that the amount of cell wall material isolated from grapes is cultivar-dependent, consistent with a previous finding (Jona *et al.*, 1983).

Scanning electron microscopy showed that the final mesocarp cell wall preparations contained approximately 1% contaminating vascular tissue, based on fragment counts of the final preparation, and that adherent cytoplasmic material on the walls also appeared relatively infrequently (Fig. 2.3). Final wall preparations contained approximately 11% or less by weight protein and the abundance of hydroxyproline residues suggested that at least part of this protein represents integral, structural protein; hydroxyproline-rich glycoproteins such as extensin are widely distributed in plant cell walls (Cassab and Varner, 1988).

Electron microscopy also indicated that the isolated walls were 100 nm or less in thickness. This value is typical of parenchyma cell walls in higher plants (Carpita, 1985; Bacic *et al.*, 1988). In histochemical studies of the dermal system of mature grape berries, Considine and Knox (1979) noted that the walls of mesocarp cells were relatively thin and unlignified. Nevertheless, thin unlignified walls of plant parenchyma cells usually have sufficient tensile strength to withstand high turgor pressures (Carpita, 1985; Bacic *et al.*, 1988) and the grape berry walls were clearly strong enough to resist extensive fragmentation under the homogenization conditions used in the isolation procedure here.

Methylation analyses of cell wall polysaccharides allowed the monosaccharide compositions of the grape berry mesocarp walls to be determined (Table 2.4). GalA*p*, approximately 50% of which is esterified, and glucose are the predominant monosaccharide constituents. The values obtained for the berry mesocarp cells are similar to values re-calculated from analyses of cell wall material from skins of Chardonnay and Carignan berries (Lecas and Brillouet, 1994), although considerable variation is observed in the minor polysaccharides of the mesocarp walls and skin (Table 2.4). This similarity is somewhat surprising, given the morphological and

functional differences between walls in the two tissues of the berry (Hardie *et al.*, 1996). Larger differences are observed with cell walls of other fruit, particularly in the relative abundance of xylose and glucose residues and in uronic acid content (Table 2.4).

Perhaps the most useful information that can be obtained from detailed methylation analyses of plant cell walls is an estimate of the amounts of different polysaccharides that are present. These estimates have been obtained using linkage structures characteristic of individual, well-characterized polysaccharides from cell walls of many higher plants (Bacic *et al.*, 1988; Gane *et al.*, 1994; Shea *et al.*, 1989), and are shown here in Table 2.5. The most abundant polysaccharides in the grape berry walls are cellulose (39% and 31% by weight in Ohanez and Gordo, respectively) and PG (29% and 41%, respectively). Arabinans and AG-I, which are likely to be water-soluble polymers of the overall pectic complex, contribute an additional 4-6% by weight to the pectic polysaccharides (Table 2.5). The AG-II material, which corresponds to arabino-(1→3,1→6)-galactans, may not be an integral component of the wall but, rather, may represent the AGPs found in the extracellular space or associated with the plasma membrane (Fincher *et al.*, 1983; Saulnier *et al.*, 1988). Previous detailed studies on pectins in grape berries showed regions of homogalacturonan and RG-I with complex sidechains, including arabinan-like structures associated with minor proportions of AG-I and AG-II (Saulnier and Thibault, 1987b; Saulnier *et al.*, 1988). Although comparisons between compositions of whole walls and wall sub-fractions must be viewed with caution, the overall features of the pulp pectic polysaccharides seem to be similar to those of the mesocarp walls isolated here. XGs account for 12% by weight of the walls from Ohanez and 8% of walls from Gordo, and smaller amounts of xylans and mannans are also present.

The most striking difference between the mesocarp walls of the Ohanez and Gordo grape varieties lies in the relative proportions of cellulose and pectic polysaccharides, and in the hydroxyproline content of wall-associated proteins. Ohanez is a firm table grape and its walls have significantly more cellulose than walls from the softer Gordo

berries. XGs, which are probably closely associated with cellulose microfibrils (Bacic *et al.*, 1988; Carpita and Gibeaut, 1993), are also higher in the Ohanez grapes. Conversely, the softer berries of the Gordo cultivar have walls that are enriched in PGs (Table 2.5) and their walls would therefore appear to have a more extensive pectic matrix phase than the firmer Ohanez grapes. The proteins in walls of Ohanez grapes also have a higher hydroxyproline content than proteins in Gordo walls. This might indicate that the hydroxyproline-rich glycoprotein (extensin) network in Ohanez walls is more extensive, consistent again with the firmer texture of this cultivar. Whether or not these differences in proportions of pectic polysaccharides, cellulose and hydroxyproline in mesocarp cell walls of grape berries are actually responsible for the textural differences between the varieties remains to be demonstrated unequivocally, but the results provide circumstantial evidence to support earlier suggestions that cell wall structure can influence berry texture (Huber, 1983a; Brady, 1987).

Wine has been found to contain a complex mixture of polysaccharides, many of which have originated from the pectic fraction of grape cell walls. These include AG-I, AG-II, arabinan, RG-I and RG-II (Brillouet *et al.*, 1989), and explain the emphasis on the characterization of pectic polysaccharides from wine in previous studies (Brillouet *et al.*, 1990; Belleville *et al.*, 1993; Doco and Brillouet, 1993; Pellerin *et al.*, 1995). The pectic polysaccharides, together with others of cell wall origin, are highly likely to affect physicochemical properties of the wine, its behaviour during processing and the stability of the final product, especially with respect to haze formation (Robertson *et al.*, 1980; Brillouet *et al.*, 1989; Belleville *et al.*, 1991; Waters *et al.*, 1994). The detailed analyses of mature grape cell walls reported here may enhance our understanding of the winemaking processes.

The development of a procedure for isolating mesocarp cell walls from grape berries and the success of using methylation analyses to determine monosaccharide and polysaccharide composition of cell walls opens the way for detailed studies on the

changes that occur in walls during berry development. These changes are examined in Chapter 3.

Chapter 3

Changes in Cell Wall Composition during Ripening of Grape Berries

3.1 Introduction

Fruit softening is of commercial importance, not only because it is correlated with flavour and colour development, but also because excessive softening can lead to mechanical damage during handling and can render the fruit susceptible to microbial attack in a process that can severely limit its postharvest storage life (Fischer and Bennett 1991).

It is widely recognized that changes in cell walls accompany fruit softening. Modification of cell wall polysaccharides is one particular change and these alterations are generally attributed to enzymic processes (Fischer and Bennett, 1991; Fry, 1995). However, as mentioned in Chapter 1, gross changes in wall composition may not always occur and indeed more subtle structural modifications of constituent polysaccharides are often observed during softening (Brady, 1987; Fischer and Bennett, 1991). Deesterification, depolymerisation and alteration to side chains of individual polysaccharides can alter cell wall properties without any large change in the total amount of that polysaccharide (De Veau *et al.*, 1993). The softening process is complicated by the fact that breakdown or modifications of different components are usually accompanied by the incorporation of newly-synthesized components into the wall (Gibeaut and Carpita, 1994; Seymour and Gross, 1996). The synthesis of cell wall polymers is probably continuous throughout ripening and a change in the turnover rate of a particular component will affect the overall wall composition (Lackey *et al.*, 1980).

As described in Chapter 1, the most obvious changes in cell walls of ripening fruit are an increase in water-soluble pectins and a loss of galactose and/or arabinose (Huber, 1983b; Gross and Sams, 1984; Redgwell *et al.*, 1992). Modifications of wall components might also be expected in ripening grape berries but little is known about cell wall composition in grapes during ripening, or of the mechanism of softening in this fruit. Many fruit reach their mature size before softening begins. The grape berry is unusual in that it softens and expands concurrently during the ripening phase.

In experiments described in this Chapter, cell walls were isolated from berries at various stages of development using the isolation procedure outlined in Chapter 2. The monosaccharide and polysaccharide compositions of the walls were monitored, as were the protein content and amino acid compositions. Changes in solubility of specific polysaccharides were also analysed. The appearance of the walls during ripening was examined by light and fluorescence microscopy.

3.2 Materials and Methods

3.2.1 Plant material

Grape berries (Gordo) were harvested every two weeks from December to March, 1993/1994 season at the South Australian Advisory Centre, Nuriootpa, South Australia. The time of anthesis was taken to be the point at which anthers were observed on 50% of flowers (28 November, 1993). At this time, bunches that were at mid-flowering were tagged for subsequent sampling. For each sample time, several tagged bunches were picked and all berries were removed. A representative sample of 50 berries was used for measurements described below. The remaining grapes were rinsed thoroughly with water and stored at -80°C until required for wall isolation.

3.2.2 Berry measurements

The lengths and widths of the 50 berries were recorded for the calculation of berry volume, and berry weight was also recorded. Deformability was measured using skin-fold calipers (Coombe and Bishop, 1980) and °Brix was measured as described in Section 2.2.1.

3.2.3 Microscopy

Berries collected at each stage of development were prepared for microscopy, essentially as described by O'Brien and McCully (1981). Freshly picked berries were cut into small pieces and fixed in 3% (v/v) glutaraldehyde, 0.05% (w/v) caffeine and 25 mM sodium phosphate buffer, pH 7, at 4°C for 16 h. The berry pieces were dehydrated for 2 h in each of two changes of methoxyethanol, ethanol, propanol and butanol. The dehydrated berries were infiltrated with a solution of glycol methacrylate (GMA), containing 7% (w/v) PEG 400 and 0.6% (w/v) benzoyl peroxide, and butanol (1:1, v/v) for 2 h at 4°C. The solution was removed and the berry pieces infiltrated with the GMA mix for a further 2 d at 4°C. The berry pieces were resuspended in fresh GMA mix and stored at 4°C prior to embedding. Each infiltrated berry piece was placed in a small

plastic capsule containing GMA mix and the GMA was polymerized at 60°C for 2 d. Berries were sectioned to a thickness of approximately 3 μm using a Reichert-Jung Microtome 2050 Supercut (NuBloch, Germany). The sections were stained with 0.05% TBO in 10 mM sodium benzoate buffer, pH 4.4, or 0.01% (w/v) Calcofluor in water for 3 min and rinsed with water. The sections were viewed using a Zeiss Axiophot Pol photomicroscope.

3.2.4 Preparation of cell walls

Cell walls were isolated from the mesocarp of grape berries at various stages of development using the final procedure described in Section 2.2.2 and shown in Figure 2.1. The stages of berry development were 44, 58, 72, 86, 100 and 114 dpa. The isolated cell wall preparations were stored over silica gel in a vacuum desiccator until required for analyses.

3.2.5 Fractionation of cell walls

Wall preparations were fractionated essentially as described by Redgwell *et al.* (1988), with the major difference being the use of 40°C during the water and *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) solubilization steps. The walls (200 mg) were extracted twice in 20 ml water at 40°C for 3 h with continuous stirring. After each solvent extraction, the wall suspension was centrifuged for 10 min at 8000g. The supernatants were combined, dialysed exhaustively against water at 4°C, reduced in volume by rotor evaporation at 45°C, and freeze dried. The water-insoluble pellet was suspended in 20 ml 0.05 M CDTA and extracted twice, as described above. The combined supernatant was dialysed against two changes of 0.1 M NaCl, pH 6.5, before dialysing against water. The remaining walls were extracted with 0.05 M Na_2CO_3 containing 20 mM sodium borohydride at 4°C for 16 h with continuous stirring. The supernatant was neutralized with glacial acetic acid before dialysis. The pellet was extracted with 4 M KOH containing 20 mM sodium borohydride at room temperature for 24 h with continuous stirring. Insoluble material was washed with water and freeze-

dried. Freeze-dried cell wall fractions were stored over silica gel in a vacuum desiccator.

3.2.6 Protein and amino acid estimations

Protein in the wall preparations and amino acid compositions were determined as described in Section 2.2.6. Hydroxyproline in hydrolysates was measured colorimetrically by the procedure of Kivirikko and Liesmaa (1959).

3.2.7 Polysaccharide linkage analysis

Linkage analyses of the total cell walls isolated from the six stages of berry development and each fraction of cell walls isolated at three stages of berry development (44, 72 and 114 dpa) were performed as described in Section 2.2.5. All methylation analyses were performed in duplicate and where significant variation was observed, the analyses were repeated, again in duplicate.

3.3 Results

3.3.1 Berry development

Berry weight and volume, deformability and the accumulation of sugars during berry development are shown in Figure 3.1. There was a steady increase in berry weight and volume throughout the developmental period. Berry deformability and sugar content remained low until 58 dpa, when both parameters began to increase markedly (Fig. 3.1B). Total soluble solids remained at 5-6 °Brix until 58 dpa, but thereafter increased markedly and reached a value of 18 °Brix when the last sample was taken at 114 dpa (Fig. 3.1B). Deformability, which is a measure of berry softening, was only 0.1-0.2 mm until 58 dpa but increased rapidly to a value of 1.8 mm at 114 dpa (Fig. 3.1B). Based on the rapid increase in soluble solids and deformability from 58 dpa, it was concluded that the inception of ripening (veraison) occurred at that time.

3.3.2 Microscopy

The appearance of cell walls in intact berries was monitored throughout development by fluorescence and light microscopy (Fig. 3.2). Examination of the walls of the skin and hypodermis, and the outer mesocarp (Figs. 3.2A and 3.2B) reveal few obvious differences between berries at veraison (58 dpa) with a deformability of 0.2 mm, and those at 86 dpa with a deformability of 1.3 mm. It should be noted that the outermost layers of cells, which make up the skin and hypodermis, were removed before cell walls were prepared. It is also noteworthy that TBO, which stains polysaccharides purple and polyphenolics green, reveals a high content of phenolics in the skin cells (Fig. 3.2B).

Comparison of the mesocarp tissue of a grape at 114 dpa, with a deformability of 2 mm (Fig. 3.2D), with the mesocarp tissue of a grape at 58 dpa and a deformability of 0.2 mm (Fig. 3.2C) shows that cells are generally larger in the berry at 114 dpa compared with the 58 dpa berry. No apparent change in cell wall thickness could be detected during ripening (Figs. 3.2C and 3.2D).

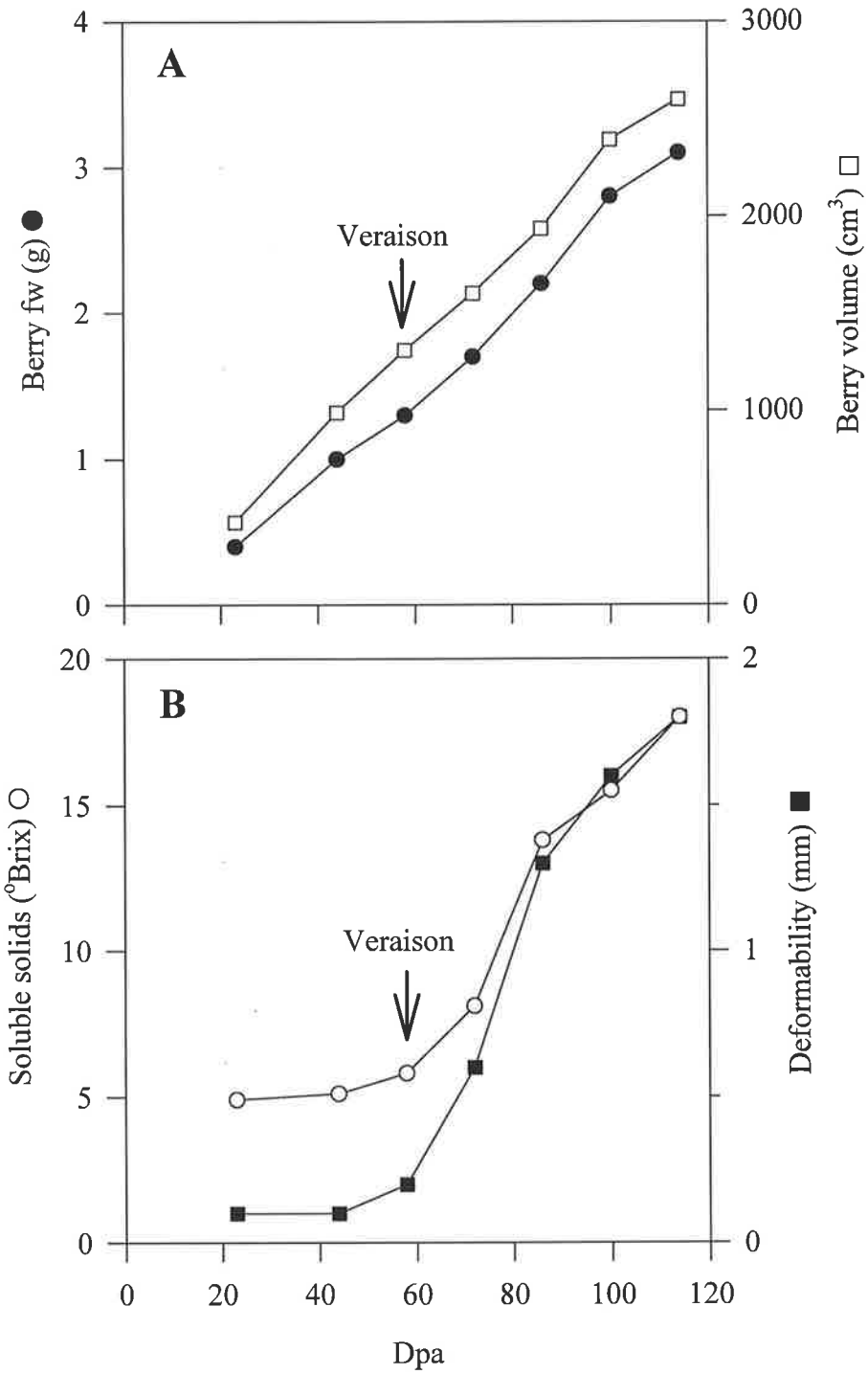

Figure 3.1 Growth curves for Gordo grapes showing **(A)** change in berry fresh weight (●) and volume (□) during development, and **(B)** change in soluble solids (°Brix) (○) and deformability (■).

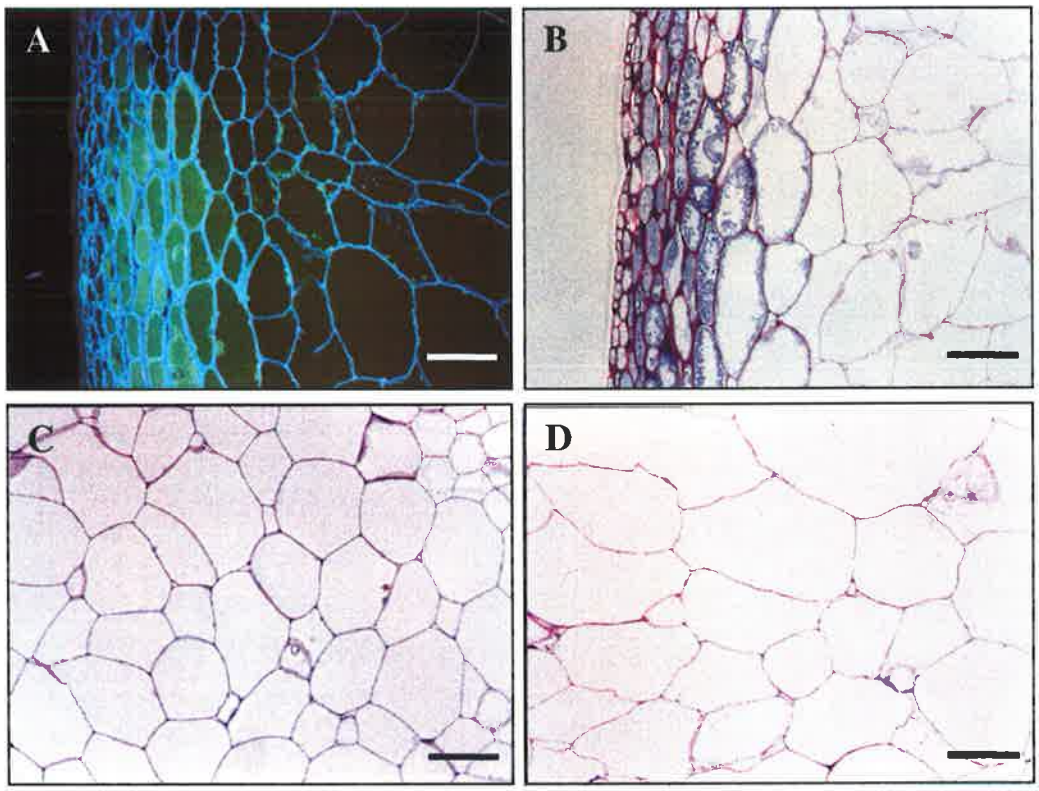

Figure 3.2 Microscopy of the ripening grape berry. Sections of the outermost region of the grape were prepared at 58 dpa **(A)** and 86 dpa **(B)**. The sections were stained with Calcofluor and TBO, respectively, and show the epidermis to the left and the mesocarp to the right (Bar = 100 μm). The sections in **(C)** and **(D)** are from approximately the same location in the central mesocarp at 58 dpa and 114 dpa, respectively, and are stained with TBO (Bar = 200 μm).

3.3.3 Cell wall isolation

Based on the developmental patterns observed in Figure 3.1, six specific times of berry development were chosen for the isolation of cell walls. These were preveraison (44 dpa), veraison (58 dpa) and 4 time points after veraison where berry softening was occurring (72, 86, 100 and 114 dpa). Because of the limited amount of available plant material, the low yields of cell walls from grapes generally (Chapter 2) and the time required for wall preparation, all of the grapes at each developmental stage were pooled and a single cell wall preparation was made. Nevertheless, yields for replicated wall isolations with different samples of ripe grapes varied by less than 7% (data not shown). The yields of cell walls from Gordo berries at each stage of development are shown in Figure 3.3. On a fresh weight per gram basis, the amount of cell wall isolated decreased steadily throughout development. However, on a per berry basis, there was an initial increase in the cell wall yield until 72 dpa, after which yields decreased to preveraison levels. A similar decrease in cell wall yield was observed when walls were isolated during the development of Ohanez (data not shown).

3.3.4 Protein and amino acid content

The changes in protein content of the isolated cell walls during development are shown in Figure 3.4. Phenol treatment has been shown to remove most adventitious proteins and the remaining protein is assumed to be an integral component of the cell walls (Chapter 2). The protein content of walls from young berries was less than 8% but as the berries matured the amount of protein associated with the cell walls increased to almost 12% by weight.

The amino acid composition of isolated cell walls is shown in Table 3.1. The major amino acids were Glx, Arg, Hyp and Gly. The amino acids that showed the largest changes were Arg, which decreased from 23.8 mol% to 14.5 mol% of wall protein and Hyp, which increased from 3.6 mol% preveraison to 9.9 mol% at 100 dpa. Over the same period the amount of Hyp increased from 1.7 to 7.4 μg per mg cell wall (Fig. 3.4).

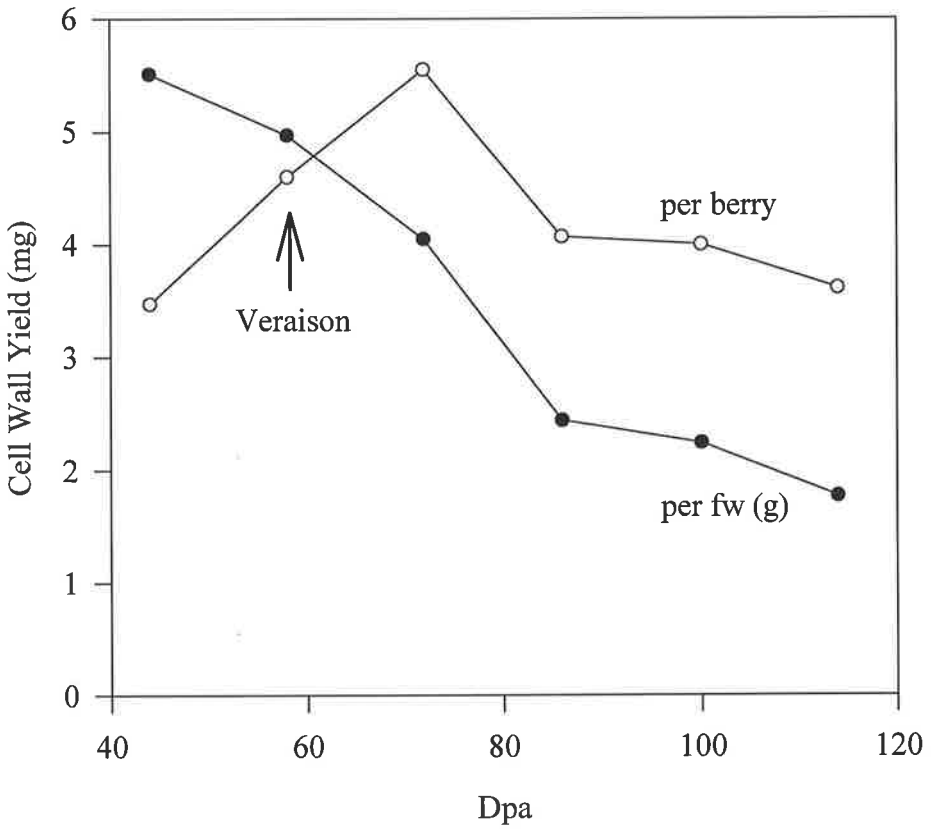

Figure 3.3 Cell wall yield from Gordo berries during ripening, expressed on a per gram of fresh weight basis (●) and on a per berry basis (O). The walls were isolated at six stages of development from pre-veraison (44 dpa), through veraison (58 dpa) to the fully ripe berry (114 dpa).

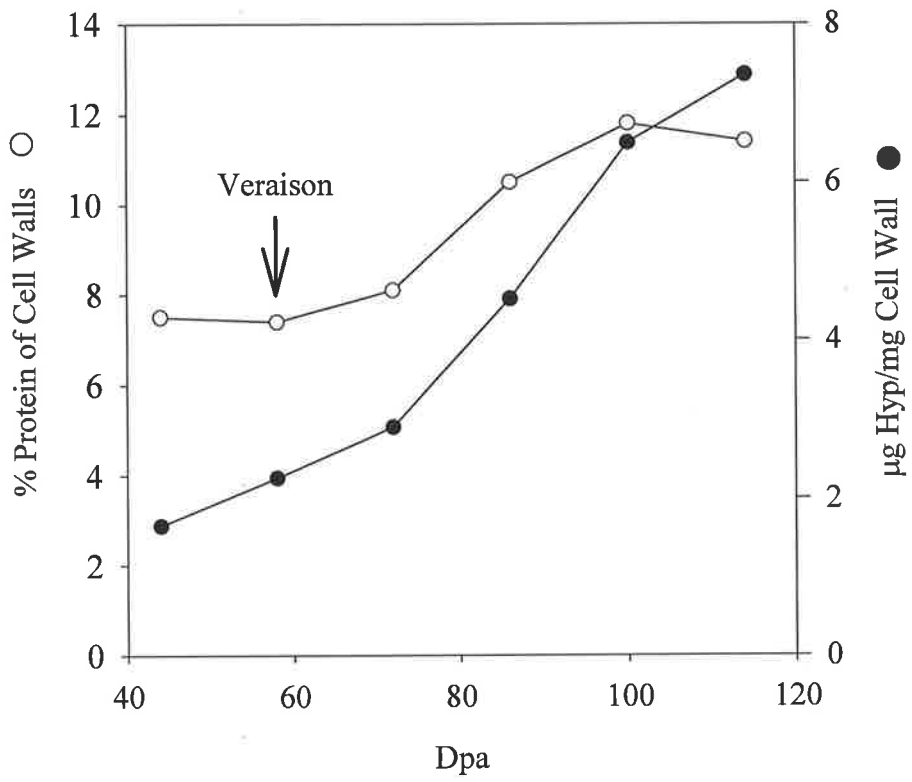

Figure 3.4 Protein (O) content (% by weight) and hydroxyproline (●) content (µg/mg cell wall) of purified cell walls isolated from developing Gordo berries.

Table 3.1 Amino acid composition (Mol %) of the proteins associated with cell walls isolated from developing berries

Amino	Stage of berry development (dpa)					
Acid	44	56	72	86	100	114
Asx	3.4	6.2	5.9	6.6	6.3	6.5
Glx	11.9	9.1	10.3	9.1	8.9	9.0
Ser	5.9	5.8	6.1	5.7	5.4	5.8
His	1.7	1.5	1.4	2.0	2.1	2.3
Gly	7.6	7.6	8.1	8.0	8.0	9.1
Thr	4.4	4.4	4.7	4.6	4.6	4.9
Cys	1.0	0.9	0.8	1.1	1.0	1.2
Ala	7.0	6.8	7.5	6.5	6.2	5.9
Arg	23.8	23.1	16.6	18.4	17.2	14.5
Tyr	2.4	2.3	2.6	2.7	2.6	2.9
Val	4.9	4.9	5.5	5.0	4.7	4.9
Met	1.4	1.2	1.2	1.3	1.0	1.1
Phe	3.1	3.1	3.3	3.0	2.7	2.7
Ile	3.9	3.9	4.1	3.8	3.5	3.6
Leu	6.3	6.5	6.7	5.9	5.4	5.5
Lys	4.0	4.2	4.5	5.0	5.1	5.9
Pro	3.9	4.1	4.6	4.7	5.4	4.9
Hyp	3.6	4.5	6.4	6.6	9.9	9.3

3.3.5 Wall fractionation

The yields of various fractions of walls isolated from different stages of development are shown in Figure 3.5. The largest change was the two-fold increase in the water-soluble component after veraison. A temporary decrease in the CDTA-soluble fraction at 58 dpa was observed in two separate fractionations but otherwise the amounts stayed relatively constant throughout ripening. The Na_2CO_3-soluble fraction increased somewhat before veraison but decreased as the berries softened. Similarly, the KOH-soluble fraction also showed a small increase before veraison and thereafter decreased steadily. The final alkali-insoluble residue decreased throughout development, although the overall decrease in this fraction was relatively small.

3.3.6 Linkage analysis and major polysaccharides in the cell walls

The monosaccharide compositions and linkage types of mesocarp cell wall polysaccharides isolated at the six stages of development were analysed by standard methylation procedures. Linkage analyses were performed in duplicate for each of the six wall preparations, and values averaged. The monosaccharide compositions are shown in Table 3.2. The major change was the decrease in 4-Gal*p* residues from 19 mol% before veraison to 1 mol% in fully ripe berries. The unesterified 4-GalA*p* increased during this ripening period from 10 to 20 mol% and the esterified 4-GalA*p* increased from 14 to 18 mol%. The 4-Glc*p* showed an initial small increase, followed by a larger decrease later in development. The other derivatives constituted 5% or less of the total polysaccharide in the cell wall and did not vary significantly during ripening (Table 3.2).

The most abundant polysaccharide types found in the developing grape berry cell walls were subsequently deduced from the linkage composition (Table 3.3), based on the totals of individual glycosyl residues that are characteristic of well-defined wall polysaccharides (described in Chapter 2). On a mol% basis, AG-I decreased from 21% of total polysaccharides in preveraison walls to 1% in ripe, 100 dpa berries. PG

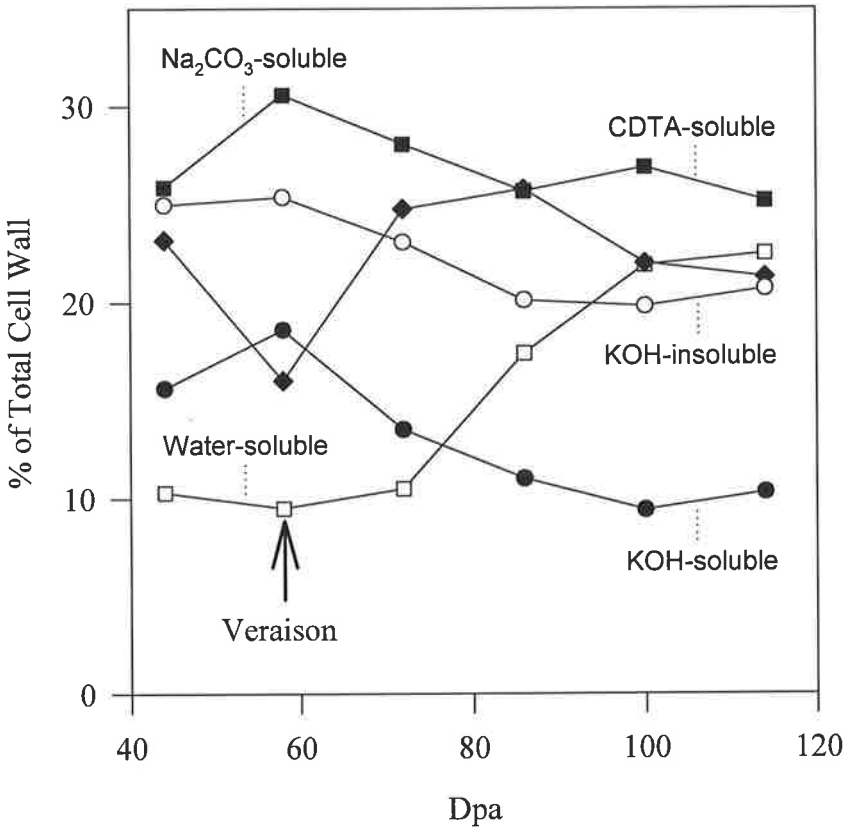

Figure 3.5 Changes in solubility of cell wall material isolated from developing Gordo berries, expressed on a dry weight basis. The cell walls were fractionated into water-soluble (□), 0.05 M CDTA-soluble (◆), 0.05 M Na_2CO_3-soluble (■) and 4 M KOH-soluble (●) components, and a KOH-insoluble residue (O).

Table 3.2 Change in linkage composition of cell walls during the ripening of Gordo berries

Monosaccharide	Deduced Linkage[a]	Linkage Composition (Mol%)[b,c]					
		44 dpa	58 dpa	72 dpa	86 dpa	100 dpa	114 dpa
Rha*p*	terminal	tr	tr	tr	tr	tr	1
	2-	1	1	1	1	1	2
	2,4-	1	tr	tr	1	1	1
Fuc*f*	terminal	1	1	1	1	1	1
Ara*f*	terminal	2	2	2	3	3	3
	5-	1	2	2	2	2	2
Xyl*p*	terminal	2	2	2	2	3	2
	2-	tr	-	-	tr	1	1
	4-	3	3	3	4	4	4
	2,4-	tr	-	tr	tr	1	1
Man*p*	4-	1	tr	tr	1	1	1
	4,6-	tr	tr	-	tr	1	1
Gal*p*	terminal	1	1	1	1	2	2
	2-	tr	tr	tr	tr	tr	tr
	3-	2	2	2	2	2	2
	4-	19	12	9	3	1	2
	6-	tr	tr	-	-	tr	tr
	2,4-	tr	-	-	-	tr	tr
	3,4-	1	tr	1	1	tr	1
	3,6-	1	1	tr	1	1	1
Glc*p*	4-	34	37	37	38	35	32
	2,4-	tr	-	-	tr	tr	tr
	3,4-	1	1	1	1	1	tr
	4,6-	5	5	5	3	4	3
GalA*p*	4-	10	16	17	19	18	20
GalA*p*OMe	4-	14	15	17	16	17	18
Glc/GlcA*p*	terminal	1	1	1	1	1	1
Glc/GlcA*p*OMe	4-	1	-	tr	1	1	1

[a] Terminal Rha*p* is deduced from 1,2,3,4,5-penta-O-acetyl 6-deoxyhexitol etc. [b] tr, < 0.5%.
[c] -, not detected.

Table 3.3 Changes in polysaccharides (Mol%) present in cell walls during the development of Gordo grapes

Polysaccharide	Stage of berry development (dpa)[a]					
	44	58	72	86	100	114
Polygalacturonan[b]	26 (58)	32 (49)	35 (49)	37 (47)	37 (47)	41 (48)
Arabinan	1	2	2	2	2	2
AG-I	21	12	11	5	1	4
AG-II	3	4	2	4	4	4
Xyloglucan	10	10	10	7	10	8
Heteromannan	1	tr	tr	1	2	3
Heteroxylan	3	3	3	4	6	7
α-Cellulose	32	35	35	37	34	31
Other[c]	3	2	2	3	4	-

[a] tr, < 0.5 %. [b] Numbers in parenthesis is the percentage of esterification. [c] Linkage types that cannot be readily assigned to well-characterized polysaccharides from plant cell walls.

increased from 26% preveraison to 41% in ripe berries; the degree of esterification decreased from 58% to 48% in the preveraison period and thereafter remained approximately constant. The cellulose content increased slightly early in development and decreased later in development. XG was present but did not change significantly during ripening, while the smaller amounts of arabinan and mannan that were present remained approximately constant. Heteroxylan levels were low generally, but increased during ripening. Small amounts of material could not be readily assigned to well-characterized polysaccharides from plant cell walls and are therefore shown in Table 3.3 as 'other'.

Because comparisons of mol% data are complicated by the possibility that a change in one component actually reflects a change in another, attempts were made to express the contents of specific polysaccharides on an absolute basis. From the relative proportion (mol% of total polysaccharides) of each polysaccharide in Table 3.3 and the cell wall yields of the same samples (Fig. 3.3), we were able to estimate the amounts of each polysaccharide on a per g fresh weight basis and on a per berry basis. These data are presented in Fig. 3.6, but should be viewed with some caution because changes in wall yields may be influenced not only by the absolute amount of wall material present, but also by factors such as changes in wall fragility.

All polysaccharides decreased on a fresh weight basis (Fig. 3.6A), but on a per berry basis (Fig. 3.6B), PG and cellulose contents increased up to and just after veraison before showing a slight decrease. AG-I content decreased during ripening but XG content per berry was relatively constant throughout development.

3.3.7 Fractionated polysaccharides

Linkage analyses of the fractionated cell walls from three stages of development (44, 72 and 114 dpa) were performed (results not shown). The major components of the water- and CDTA-soluble fractions were esterified and unesterified 4-linked GalA*p*, and in the Na_2CO_3-soluble fraction high levels of 4-GalA*p* were present. XGs and

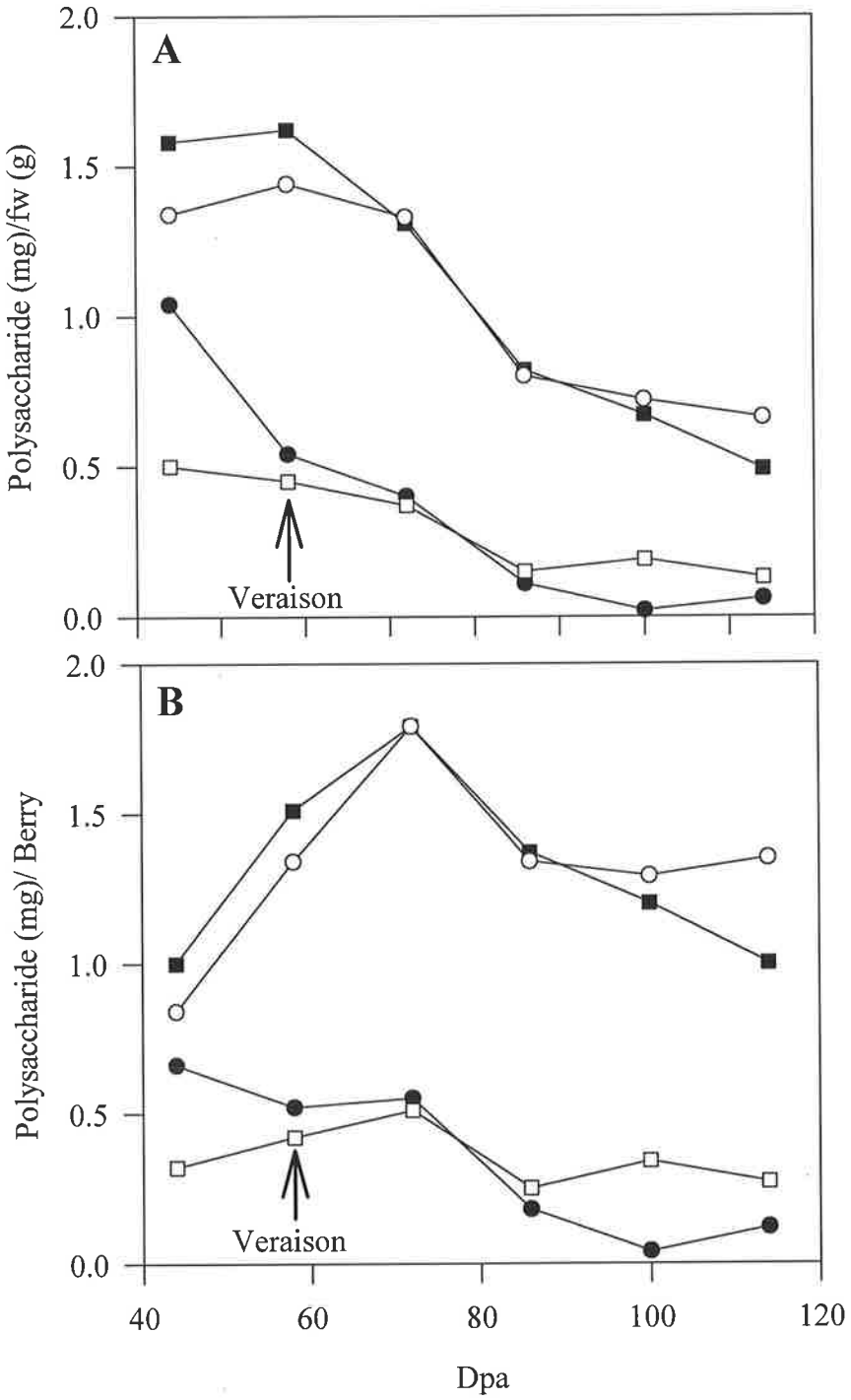

Figure 3.6 Changes in levels of specific cell wall polysaccharides (dry weights) during grape berry development, expressed on a fresh weight of tissue basis **(A)** and a per berry basis **(B)**. The four most abundant polysaccharides found in the cell walls are shown; these account for 85-90% of total wall polysaccharides. Cellulose is shown as ■, PG as ○, AG-I as ●, and XG as □.

heteroxylans were the major polysaccharides in the KOH-soluble fraction and the residual KOH-insoluble cell wall material was mainly 4-linked Glc*p* (data not shown).

Changing solubilities of polysaccharides, as indicated by their movement between fractions, are shown in Table 3.4. The increase in the water-soluble fraction was due to the shift of PG from the CDTA- and Na_2CO_3-soluble fractions to the water-soluble fraction. The AG-II became completely water-soluble as ripening progressed. The increase in water-soluble arabinan came from the KOH-soluble and residue fractions. The remaining AG-I in the cell walls at 114 dpa was associated with the Na_2CO_3-soluble fraction, whereas it was found in the CDTA-, Na_2CO_3-, KOH-soluble and KOH-insoluble residue fractions when the berries were young. Heteroxylans became slightly more soluble as the berry ripened; there was a decrease in the amount associated with the KOH-insoluble residue fraction and a corresponding increase in the Na_2CO_3-soluble fraction. XG and cellulose solubilities changed very little during ripening.

Table 3.4 Distribution of major polysaccharides in cell wall fractions at different stages of grape berry development

Polysac. Type	dpa	Polysac. Total (Mol%)	Percentage polysac. in each fraction[a]				
			H_2O	CDTA	Na_2CO_3	KOH	Residue
PG	44	26	7	49	42	1	1
	72	35	11	45	42	1	1
	114	41	26	41	32	1	0
Arabinan	44	1	tr	13	53	13	20
	72	2	4	22	48	4	22
	114	2	18	15	64	3	tr
AG-I	44	21	0	15	59	8	19
	72	11	0	0	92	8	0
	114	4	0	0	100	0	0
AG-II	44	3	30	20	30	20	0
	72	2	73	tr	23	4	0
	114	4	100	0	tr	tr	0
Xyloglucan	44	10	0	0	0	80	20
	72	10	3	0	0	72	25
	114	8	0	0	0	70	30
Heteromannan	44	1	0	38	38	23	0
	72	tr	5	26	29	8	32
	114	3	0	19	42	4	35
Heteroxylan	44	3	27	10	3	34	26
	72	3	27	16	10	44	3
	114	7	22	11	24	36	7
α-Cellulose	44	32	2	3	5	4	86
	72	35	3	0	0	1	97
	114	31	9	3	3	1	85

[a] tr, < 0.5%.

3.4 Discussion

The onset of ripening in grape berries varies between bunches and even between individual berries in the same bunch (Coombe and Bishop, 1980; Coombe, 1992). In order to minimize the effects of this variation on physical and chemical analyses, bunches which flowered at the same time were tagged and used to provide samples at different stages of berry development. At each time point, a subsample of 50 berries was taken to measure berry characteristics; the remainder was used to isolate cell walls. The double sigmoidal pattern normally seen during the development of individual berries was not obvious here (Fig. 3.1A) and was probably masked not only by the averaging of values for 50 berries, but also because berries were sampled only once every two weeks (Coombe and Bishop, 1980; Coombe, 1992). Nevertheless, the onset of ripening, or veraison, was clearly indicated by the relatively sudden increase in soluble solids and by the concomitant increase in berry deformability which occurred at 58 dpa (Fig. 3.1B).

Following veraison, the size of central mesocarp cells appeared to increase (Fig. 3.2). The cell walls of many fruit swell as the fruit ripens (Redgwell *et al.*, 1997). Swelling in kiwifruit cell walls during ripening is pronounced and in some tissues a three- to four-fold increase in cell wall thickness has been observed (Redgwell *et al.*, 1997). No swelling of the grape cell walls was visible by light microscopy during development (Fig. 3.2), in agreement with a previous study by Hardie *et al.* (1996).

The yield of isolated cell walls on a per berry basis appeared to increase before veraison and into the start of the second growth phase, but decreased throughout the remainder of berry development (Fig. 3.3). It should be emphasized, however, that the procedure for wall isolation is not quantitative (Chapter 2) and that the decrease in yield could be explained in several ways. If there were a net decrease in cell wall synthesis, an increase in hydrolysis of wall polysaccharides, or an increase in solute accumulation relative to wall deposition, then yields would be expected to fall. Alternatively, if the

walls become more fragile during ripening, they may be fragmented into smaller pieces during homogenization of the berry and be lost through the 71 μm nylon mesh.

Interpretation of variable cell wall yields during fruit development is also complicated by the simultaneous degradation and synthesis of polysaccharides. Saito and Kasai (1978) have studied incorporation of newly synthesized polymers into the cell walls of grapes. They found that incorporation of labelled substrate into cellulose and pectic polysaccharides in grapes was continuous throughout development, although the rate of incorporation did decline as the berries ripened. Additionally, in other fruit such as strawberries, tomatoes and kiwifruit, there is a continuous turnover of cell wall polymers during ripening (Knee *et al.*, 1977; Lackey *et al.*, 1980; Mitcham *et al.*, 1989; Redgwell, 1996). It is therefore likely that the sum of these two processes, synthesis and degradation, will influence the yield of cell wall during grape development.

The most significant change in the composition of grape cell walls during ripening was the dramatic decrease in the amount of (1→4)-linked Gal*p* residues (Tables 3.2 and 3.3). AG-I is a major component of the neutral side chains of pectic polysaccharides and the loss of these side chains has been observed in other ripening fruit (Huber, 1983b; Gross and Sams, 1984). In tomatoes, Lackey *et al.* (1980) found that galactan was continuously synthesized but the rate of synthesis progressively declined, and this resulted in a decrease of galactan in the cell walls. A corresponding increase in free galactose was found in tomatoes (Gross, 1983), but in kiwifruit no free galactose was detected even though galactan was lost from the cell walls (Redgwell *et al.*, 1990).

The loss of AG-I, which constituted 21 mol% of total polysaccharides in grape cell walls before veraison (Table 3.3), began well before softening was detected (Table 3.3 compared with Fig. 3.1B), suggesting that loss of the AG-I could be a crucial step that is associated with the initiation of softening. However, Redgwell and Harker (1995) suggested that cell wall-associated galactose loss and softening are separate processes because inhibiting galactose loss in kiwifruit discs did not inhibit fruit softening; neither did inhibiting the softening process prevent galactose loss. The enzyme β-galactosidase

has been shown to degrade galactans in fruit such as apples and pears (Ross *et al.*, 1993; Kitagawa *et al.*, 1995) but additional enzymic or non-enzymic processes are also thought to be involved in AG-I degradation (Ross *et al.*, 1993; Kitagawa *et al.*, 1995).

The other significant changes in the cell walls of developing grapes were increases in PG and heteroxylan content. Proportions (mol% of total wall polysaccharides) of the other major polysaccharides, cellulose and XG, remained at similar levels throughout the ripening process (Table 3.3). Changes in the amounts of these polysaccharides in grapes have not previously been reported, and studies on other fruit have been concentrated on changes in solubilities of polysaccharides rather than changes in their relative abundance (see discussion below).

The cell wall preparations were subsequently extracted with a series of increasingly harsh solvents, in the expectation that polysaccharides recovered at each stage would provide an indication as to the nature of intermolecular interactions and as to how tightly individual polysaccharides were associated with one another and with other wall components. The increase in the water-soluble fraction was attributed to an increased solubility of PG that had required CDTA and Na_2CO_3 to extract it early in development, together with increases in AG-II and arabinan that had been found in all fractions before the grapes began to soften (Table 3.4). An increase in water-soluble polysaccharides has also been reported in other fruit (Gross and Wallner, 1979; Ahmed and Labavitch, 1980a), including grapes (Silacci and Morrison, 1990). The movement of particular polysaccharides from one fraction to another presumably results from modifications to the fine structure of polysaccharides which affect the way they aggregate with each other or which simply increase the inherent solubilities of the polysaccharides. Other explanations for the increased solubility include the new synthesis of more soluble forms of the polysaccharides, or the partial hydrolysis of existing wall components.

In general, polyuronides that become more soluble during ripening have lower apparent molecular weights (Yoshioka *et al.*, 1992; Huber and O'Donoghue, 1993). This may result from the removal of neutral side chains, as discussed above, or it may

result from other modifications. Enzymes that have been implicated in these modifications, and therefore in softening, include pectin methylesterase (PME) which removes the methyl groups from esterified PGs, and PGase which is an endo-hydrolase that cleaves unesterified PGs (Seymour and Gross, 1996). The removal of methyl groups by PME allows increased interactions of the polysaccharide with calcium and therefore increases the number of non-covalent cross-links between polysaccharide chains (Jarvis, 1984). The removal of some methyl groups may be required before PGase can act. Hydrolysis of long PGs by PGase would decrease the molecular weight of pectic polysaccharides and presumably increase their solubility.

In contrast to the relatively large decreases in the degree of methyl esterification (DE) observed during ripening in tomatoes (Koch and Nevins, 1989) and pears (Martin-Cabrejas *et al.*, 1994), it was found here that there was a smaller decrease from an initial DE of 58% in the grape PGs before veraison to 48% at veraison, and thereafter there was no further decrease (Table 3.3). Indeed, the level of esterified 4-linked galacturonic acid residues increased slightly during ripening (Table 3.2). This result suggests that the increased solubility of pectic polysaccharides as the grape softens is not due to changes in their overall DE, although the initial decrease in DE may be important to allow other ripening-related processes to occur.

The large increase in the activity of PGase observed in many ripening fruit has led to the suggestion that PGase plays an important role in pectin solubilization and softening of fruit (Tucker *et al.*, 1980). 'Never ripe' (*Nr*) and 'ripening inhibitor' (*rin*) mutant tomatoes have very low or no PG activity and show very slow or very little softening (Tucker and Grierson, 1982). Conversely, apples, in which no PGase activity is detected, and transgenic tomatoes, which show only 1% of wildtype PGase activity, both undergo 'normal' softening but no pectin solubilization occurs (Smith *et al.*, 1988; Yoshioka *et al.*, 1992). These experiments therefore suggest that PGase is not the primary determinant of softening in certain fruit, although PGase is likely to be involved in pectin degradation. Because linkage analysis gives little indication as to the

molecular weights or degrees of polymerization of polysaccharides, no conclusions can be made about the role of PGase in grape berry softening from this information.

As mentioned above, cellulose and XG levels remained approximately the same during ripening of grapes (Table 3.3) and there was no apparent increase in their solubility (Table 3.4). This may be compared with the decrease observed in the molecular weight of avocado XG (O'Donoghue and Huber, 1992) and a decrease found in the molecular weight of alkali-soluble polysaccharides in tomatoes (Huber, 1983a). It should be noted that XGs might also be modified by XETs (Fry, 1993), which hydrolyse and transglycosylate XGs. Modification of grape cell wall XG in this way by XET might not be detected in the analyses of overall polysaccharide composition performed here.

In addition to the changes seen in polysaccharide composition and solubility in walls of ripening grape berries, levels of wall-associated proteins were found to increase from approximately 7% (w/w) at veraison to almost 12% later in ripening (Fig. 3.4). Although it might be argued that the increase could be attributed simply to increasing contamination of wall preparations with protein of cytoplasmic origin (Chapter 2), the concomitant increase in hydroxyproline content from 2 to 7 μg/mg cell wall (Fig. 3.4) and from 2.6 to 3.6 μg/berry (data not shown) suggests that wall proteins such as the hydroxyproline-rich glycoproteins, extensins, are being deposited in the walls as the berries develop (Fig. 3.4). Hydroxyproline-rich glycoproteins are believed to form a fibrillar network in plant cell walls that is independent of the cellulosic network, and this second, hydroxyproline-rich glycoprotein network is likely to considerably strengthen the cell wall (Varner and Lin, 1989; Carpita and Gibeaut, 1993). It is possible that expansion of mesocarp cells in the ripening berry (Figs. 3.2C and 3.2D) necessitates the reinforcement of the walls with hydroxyproline-rich glycoproteins so that cellular integrity can be maintained during the softening process. The changes in amino acid composition during ripening, such as the decrease in Arg content, suggest that other modifications are also occurring (Table 3.1).

In summary, the major changes in cell wall composition during ripening of grape berries include an increase in water-soluble polysaccharides, a decrease in galactan/AG-I and an increase in hydroxyproline. Overall, the changes in cell wall polysaccharides during grape berry ripening suggest that a limited number of enzymes play an important role in the softening process. In particular, these would include endo- or exo-hydrolases capable of depolymerising the (1→4)-β-galactans constituents of pectic polysaccharides. Low, but significant, levels of PGase or PME, which could be responsible for the partial solubilization of PGs (Table 3.4), might also be present, but only low levels of cellulase, xyloglucanase and xylanase would be expected. The activities of enzymes potentially involved with cell wall modification during grape berry ripening were therefore investigated and results are described in the following chapter.

Chapter 4

Activity of Cell Wall-Modifying Enzymes in Grape Berries

4.1 Introduction

As discussed in Chapter 3, the major changes that occur in cell walls during grape berry softening are a decrease in the amount of galactose/galactan, an increase in water-soluble polysaccharides and an increase in the amount of hydroxyproline present in the cell walls. The solubilities of a number of polysaccharides increase as the berry softens; these include PG, AG-II and arabinan. Other non-cellulosic polysaccharides and cellulose show little change during softening of the berries.

The changes in cell wall polysaccharides that occur as fruit soften are thought to reflect the action of enzymes (Seymour and Gross, 1996). *Pectin methylesterase* (PME) removes methyl groups from esterified galacturonans, producing variable effects on the cell wall structure. Firstly, removal of methyl ester groups permits the formation of Ca^{2+}-pectate junction zones, thus increasing the strength of the pectic gel (Jarvis, 1984). Conversely, removal of these groups increases the accessibility of the polysaccharide to the enzyme *polygalacturonase* (PGase), subsequently resulting in breakdown of the pectic gel (Jarvis, 1984; Fischer and Bennett, 1991). PGase catalyses the hydrolysis of (1→4)-α-galacturonyl linkages in unesterified galacturonan. Both exo- and endo-PGase activities are found, but endo-PGases (hereafter referred to as PGase) are relatively more abundant, they have been well-characterized, and are likely to have a greater impact on cell wall structure during ripening (Fischer and Bennett, 1991). *β-Galactosidase* is an exo-acting enzyme and is characterized by its ability to release galactose from *p*-nitrophenyl-β-D-galactopyranoside and to remove β-galactosyl residues from wall polysaccharides. The native substrates are thought to be RG-I and XGs (Fry, 1995). *α-Galactosidase* is also an exo-acting enzyme, but is characterized by its ability to release galactose from *p*-nitrophenyl-α-D-galactopyranoside. There is some debate concerning the native substrate for α-galactosidase. It has been suggested that RG-II (Seymour and Gross, 1996), galactoglucomannan (Fernández-Bolaños *et al.*, 1995) or galactomannans (Fry, 1995) may be substrates. All of these contain α-(1→4)-galactosyl residues. α-

Galactosidase may also act upon galactose residues in extensin (Fry, 1995). *Galactanase* hydrolyses the polysaccharide (1→4)-β-galactan (Buckeridge and Reid, 1994), and again its endo-action is of potentially greater influence on polysaccharide structure and solubility than exo-acting enzymes. *Xyloglucanase* (XGase) cleaves (1→4)-β-D-glucosyl linkages in XG and is therefore important for the XG depolymerisation that is observed in some fruit during ripening (Redgwell *et al.*, 1990; O'Donoghue and Huber, 1992; Sakurai and Nevins, 1997). *Cellulase* is an endo-acting enzyme that hydrolyses (1→4)-β-D-glucosyl linkages in cellulose. It is not particularly active on crystalline cellulose, but acts on soluble cellulose derivatives and XG (Maclachlan and Brady, 1992; Fry, 1993).

A number of assays is available to detect the activity of these enzymes. β-Galactosidase and α-galactosidase activities can be readily measured with the synthetic substrates *p*-nitrophenyl β-D-galactoside and *p*-nitrophenyl α-D-galactoside, respectively. If activity is present, *p*-nitrophenol will be released from the colourless glycoside and the change in absorbance can be measured. PME activity releases methyl groups from pectin, and thus causes a decrease in the pH. The amount of PME activity is determined by the amount of base that is required to maintain the polysaccharide/enzyme solution at a constant pH (Wegrzyn and MacRae, 1992). PGase, XGase and cellulase activities are measured by the decrease in viscosity of solutions of the respective polysaccharide substrates. Galactanase activity cannot be measured using viscometry because of the low viscosity of the galactan substrate (Buckeridge and Reid, 1994). However, potato galactan cross-linked to the dye azurine can be used as a substrate in colorimetric assays. Galactanase activity will cause the release of soluble dyed fragments from the insoluble azurine-potato complex and the corresponding increase in colour is measured spectrophotometrically (Megazyme, Wicklow, Ireland).

To assay enzymes from plant tissues, a number of factors need to be considered, including enzyme extractability and stability. Buffers, reducing agents, chelating agents and protease inhibitors may be required for enzyme stability (Scopes, 1994). Some

enzymes can be very unstable and require additional reagents, such as glycerol, for stability (Scopes, 1994). Furthermore, enzymes such as β-galactosidase and PGase may be associated with cell walls (Kitagawa *et al.*, 1995; Steele *et al.*, 1997) and reagents which can solubilize these enzymes from the wall therefore need to be included in the extraction buffer.

Other factors specific to grapes also should be considered when extracting enzymes. Grapes contain high levels of phenolics, such as tannins, which can cross-link enzymes and inactivate them. The inclusion of reducing agents is necessary to minimise these protein-polyphenol associations (Loomis and Battaile, 1966; Hawker, 1969). Furthermore, grape juice has a low and variable pH, depending on the stage of development of the berries (Coombe, 1975). Thus, it is important to use strong buffering systems.

The development of a suitable procedure for the extraction of active enzymes, and the subsequent extraction of proteins from grapes at different stages of development are described in this Chapter. Thereafter, the activities of a number of cell wall-modifying enzymes are measured for each stage of berry development, and these activities are related back to the changes in wall composition described in Chapter 3.

4.2 Materials and Methods

4.2.1 Preparation of plant material

Vitis vinifera L. (cv. Gordo) berries were collected at the South Australian Advisory Centre, Nuriootpa, South Australia, Australia. The berries were collected 41, 63, 86, 105 and 126 dpa in the 1994/1995 season. The grapes were rinsed with deionized water and stored at -80°C prior to use. Measurements of the dimensions, weight, deformability, and °Brix of the grape berries were as described in Section 3.2.2.

4.2.2 Extraction of crude protein

Protein was extracted from berries at five developmental stages. Grape berry mesocarp was homogenized to a powder under liquid N_2 using a coffee grinder and stored at -80°C. From these stock preparations, proteins were extracted from the tissue using an extraction buffer which consisted of 100 mM sodium acetate buffer, pH 5.0, 5 mM dithiothreitol (DTT), 10 mM ethylenediaminetetraacetic acid (EDTA), 3 mM phenylmethylsulfonyl fluoride (PMSF), 1 M NaCl, 10 mM diethyldithiocarbamate (DIECA), 5% (w/v) polyethylene glycol 4000 (PEG), 2% (w/v) polyvinylpolypyrrolidone (PVPP) and 10% (v/v) glycerol. Samples of powdered grape tissue (2g) from the stock were placed into 4 ml extraction buffer and stirred for 1 h at 4°C. The samples were centrifuged for 20 min at 8000g, the supernatant transferred to a new tube and the pellet discarded. The final volume of each suspension was approximately 4.5 ml. A portion of the protein extract (2.5 ml) was desalted on Sephadex PD-10 columns (Amersham Pharmacia, Uppsala, Sweden) that had been equilibrated with 100 mM sodium acetate buffer, pH 5.0, and 5 mM DTT. The collected fractions were approximately 3.5 ml. The extractions were performed in triplicate for each stage of development. Protein estimations and enzyme assays were performed on each replicate for each stage of development (Sections 4.2.3 and 4.2.4).

Protein was also extracted from ripe tomatoes purchased from a local fruit shop using the same procedure described above.

4.2.3 Protein determination

The protein concentrations of the extracts from grape berries were determined using the Coomassie Protein Assay Reagent (Pierce, Rockford, USA) and measuring the absorbance at 595 nm (Bradford, 1976). Bovine serum albumin was used for the preparation of standards.

4.2.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analysed by SDS-PAGE on 12.0% (w/v) Tris-glycine gels as described by Fling and Gregerson (1986). Proteins were stained with Coomassie Brilliant Blue G-250 (0.1% w/v in 50% v/v methanol, 7% v/v acetic acid) at 65°C for 30 min, and gels were destained with 20% (v/v) methanol, 7% (v/v) acetic acid and 3% (v/v) glycerol. The markers used were low molecular weight (LMW) markers from Pharmacia. The protein samples were prepared as described below.

The protein samples from the developmental series (Section 4.2.2.3) were concentrated by precipitation with TCA. Ice-cold TCA (20 μl, 60% w/v) was added to 100 μl protein sample to give a final concentration of 10% (w/v) TCA. The samples were held on ice for 15 min and centrifuged for 15 min at 12,000g. The pellet was washed twice with ice-cold ethanol:ethyl acetate (2:1, v/v), air dried and resuspended in 20 μl 50 mM Tris-HCl buffer, pH 8.8. After the addition of 5 μl 5x load buffer (300 mM Tris-HCl buffer, pH 6.8, 10% w/v SDS, 40% v/v glycerol, 500 mM DTT and 0.05% w/v bromophenyl blue), the samples were heated at 80°C for 5 min before analysis by SDS-PAGE.

4.2.5 Enzyme assays

4.2.5.1 β-Galactosidase and α-galactosidase assays

β-Galactosidase and α-galactosidase activities were measured colorimetrically using *p*-nitrophenyl-β-galactoside and *p*-nitrophenyl-α-galactoside (Sigma, St Louis, MO, USA), respectively. Each reaction mix contained 25 μl substrate (2% w/v), an aliquot (20-150 μl) of enzyme extract and an aliquot of 100 mM sodium acetate buffer, pH 5.0, to give a final volume of 250 μl. Three tubes were set up for a time course reaction (0, 15 and 30 min) at 37°C, and reactions were stopped by the addition of 500 μl 200 mM Na_2CO_3. After thorough mixing, the absorbance at 410 nm was measured. Standards contained 0 to 100 nM *p*-nitrophenol (total volume 250 μl) and 500 μl 200 mM Na_2CO_3 was added to each. Specific activity is expressed as units per gram fresh weight (g fw), per mg protein or per berry, where one unit equals the amount of enzyme required to release 1 nmole *p*-nitrophenol per min at 37°C.

4.2.5.2 Cellulase, polygalacturonase and xyloglucanase assays

Cellulase, PGase and XGase activities were assayed using viscometric procedures. To assay cellulase activity, the substrate was 0.3% (w/v) CM-cellulose (degree of substitution 0.54) (Sigma). To assay PGase activity, both 0.3% (w/v) pectin (methoxy content 10%) (Sigma) and 2% (w/v) polygalacturonic acid (PGA) (Sigma) were used as substrates. To assay XGase activity, 0.3% (w/v) xyloglucan (Deltagen, Boronia, Victoria, Australia) was used as the substrate. All substrates were dissolved in 100 mM sodium acetate buffer, pH 5.0, and equilibrated at 37°C for 16 h. The viscometer was from Cannon (Model # 100 C194, State College, PA, USA). Substrate (550 μl) was placed in the viscometer at 37°C and 50 μl 100 mM sodium acetate buffer, pH 5.0 (to determine t_b, see the equation below), or 50 μl enzyme diluted in 100 mM sodium acetate buffer, pH 5.0, if required, was added to the substrate. Times taken for the

reaction mixture to pass through the viscometer were recorded for a number of time points over 30 min. The activity was calculated using the following equation:

$$\frac{1}{\Delta\eta sp} = \frac{1}{(t_x / t_b)-1} - \frac{1}{(t_0 / t_b)-1}$$

where $\Delta\eta sp$ = change in specific viscosity,

t_x = flow time of sample at a particular time (sec),

t_0 = flow time at time 0 (sec),

t_b = flow time of buffer (sec).

Specific activity is expressed as units per gram fresh weight, per berry or per mg protein, where one unit equals the change in the reciprocal of specific viscosity per min at 37°C.

4.2.5.3 Pectin methylesterase assay

The activity of PME was determined as described by Wegrzyn and MacRae (1992). Protein extract (2 ml) was added to 10 ml 1% w/v pectin (67% degree of esterification) (Sigma) in 100 mM NaCl, pH 7.0. The solution was maintained at pH 7.0 for approximately 10 min by titrating with 25 mM NaOH. PME activity is expressed as units per gram fresh weight, per berry or per mg protein, where one unit equals the amount of enzyme consuming 1 μmole base per min at room temperature.

4.2.5.4 Galactanase assay

Activity of endo-1,4-β-galactanase was determined using Galactazyme tablets (Megazyme) and following the manufacturer's instructions. Protein extract (500 μl) was equilibrated at 40°C for 5 min. One Galactazyme tablet was added to the extract and, after 10 min, the reaction was stopped with 10 ml 1% w/v Trizma Base solution (Sigma) and mixed thoroughly. After standing for 5 min, the solutions were mixed again and filtered through Whatman No.1 paper. An enzyme blank was prepared by the

addition of the Trizma base to the enzyme sample before adding the tablet. The absorbance at 590 nm was measured for each sample and the activity was determined as described by the manufacturer, where one unit equals the amount of enzyme required to release 1 μmole galactose reducing-sugar equivalents per minute.

4.3 Results

4.3.1 Berry measurements

Gordo berries collected in the 1994/1995 season were measured for weight, volume, soluble solids (expressed as °Brix), and deformability. Figure 4.1 shows the change in °Brix during development. The time of veraison was estimated at 63 dpa.

4.3.2 Extraction of proteins from grape tissue at different stages of development

Proteins were extracted from the mesocarp tissue of grape berries at five stages of development. Initial attempts to extract proteins from grapes using a simple sodium acetate extraction buffer, containing a reducing agent (5 mM DTT), a chelating agent (10 mM EDTA) and a protease inhibitor (3 mM PMSF), resulted in relatively low yields of protein and low activities of many of the enzymes assayed (results not shown). The inclusion of additional reagents, such as DIECA, PEG, PVPP and glycerol, to the extraction buffer as described in Section 4.2.2 improved both protein yield and enzyme activities. The amount of protein extracted at each stage of berry development is shown in Figure 4.2. The amount of protein extracted before and at veraison (41 and 63 dpa) was low, but increased dramatically after veraison. On a fresh weight basis, the level of protein extracted from mature grape berries was approximately 3-fold greater than for preveraison grapes. Analysis of extracted proteins by SDS-PAGE (Fig. 4.3) revealed that a large part of the overall increase in extractable protein was probably attributable to increases in abundance of two major proteins with molecular masses of approximately 30 kDa and 24 kDa.

4.3.3 Measurement of enzyme activities during berry development

The activities of β-galactosidase, α-galactosidase, PME, PGase, cellulase, XGase and galactanase were determined in the protein extracts at the five stages of grape berry development (Table 4.1). In some cases enzyme activities that were expected to be

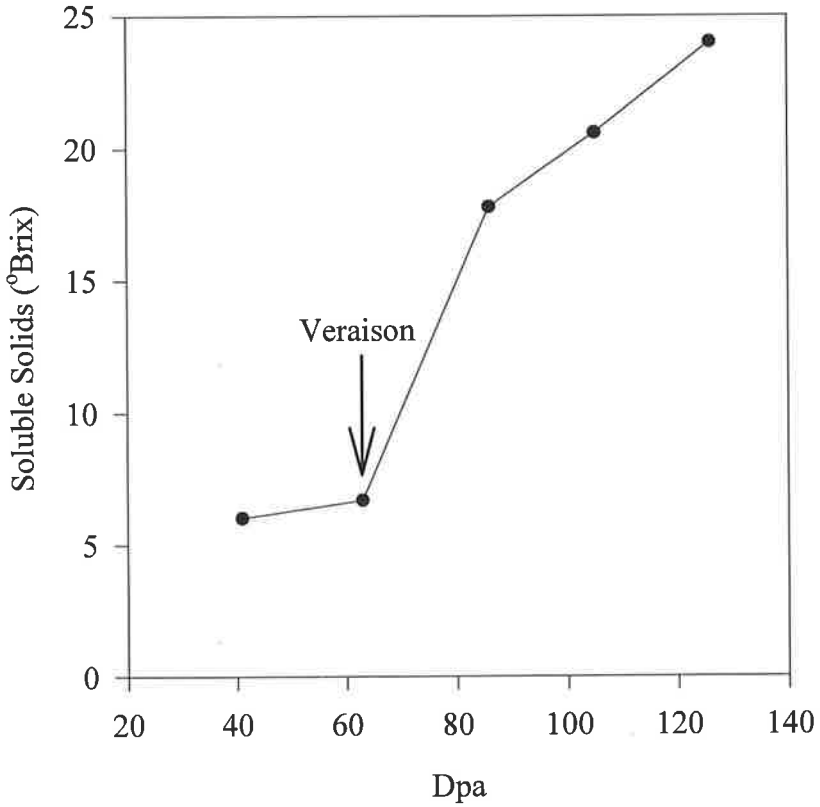

Figure 4.1 Change in °Brix as Gordo grapes developed in the 1994/1995 season. Veraison was predicted to occur at 63 dpa.

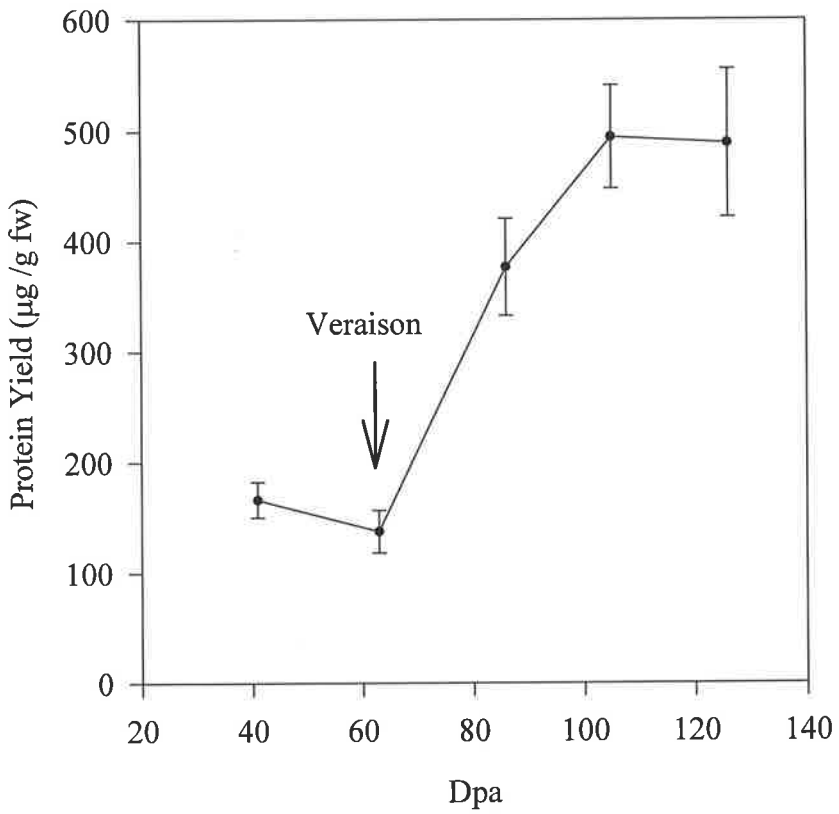

Figure 4.2 The yield of protein (µg/g fw) extracted from grapes at five stages of development. The data plotted are the means of three replicates. Error bars indicate standard deviations.

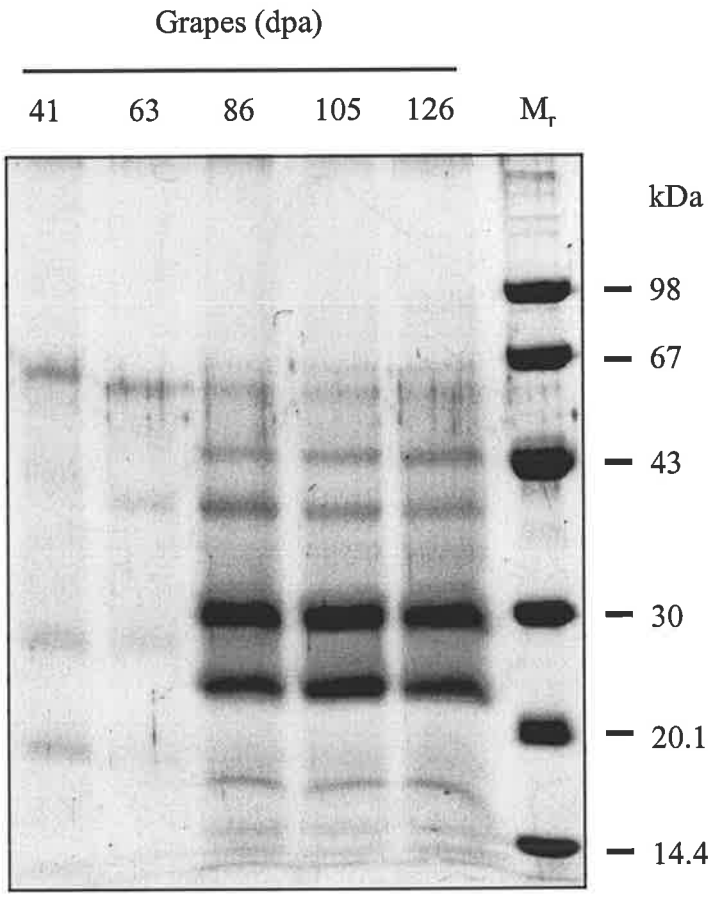

Figure 4.3 SDS-PAGE analysis of proteins extracted at five stages of grape berry development. Each lane represents protein extracted from approximately 42 mg fresh weight of berry tissue. M_r = molecular mass standards.

Table 4.1 Specific activity (U/ mg protein) of enzymes in developing grape berries

Enzyme	Substrate	Grape (dpa)					Tomato (ripe)
		41	63 (veraison)	86	105	126	
β-Galactosidase	NP-β-Gal	7	11	141	90	95	190
α-Galactosidase	NP-α-Gal	24	47	46	35	29	270
PME	Pectin	40	41	8	7	6	2016
Polygalacturonase	Pectin, PGA	0	0	0	0	0	50.9 [a]
Cellulase	CM-Cellulose	0	0	0	0	0	0.02
Xyloglucanase	Xyloglucan	0	0	0.2	0.2	0.1	0
Galactanase	"Galactozyme"	0	0	0	0	0	0

β- and α-Gal 1U = 1 nmole *p*-nitrophenol released per min
PME 1U = amount of enzyme consuming 1 μmole base per min
Viscometry 1U = change in reciprocal of specific viscosity per min
[a] Pectin used as the substrate
NP-β-Gal = *p*-nitrophenyl. β-D-galactoside
NP-α-Gal = *p*-nitophenyl α-D-galactoside

present could not be detected or were lower than anticipated, and there was therefore some concern that either the protein extraction procedure or the enzyme assays were inappropriate. As a result, proteins were also extracted from ripe tomatoes, the same assays performed, and results compared with those of developing grapes to ensure that the enzymes of interest could be successfully extracted and measured.

β-Galactosidase activity was detected in grapes throughout ripening. The activity was initially low, but increased approximately 14-fold after veraison, in 86 dpa berries, and later decreased as the berries matured. A comparable level of β-galactosidase was found in ripe tomatoes, and the level of enzyme activity detected in this study for ripe tomatoes is similar to that reported by Carrington and Pressey (1996).

α-Galactosidase activity was also found throughout ripening, but declined as the berries approached maturity. α-Galactosidase activity in ripe tomato was up to six times higher than that found in grapes. Again, tomato α-galactosidase activity comparable was to that found elsewhere (Wallner and Walker, 1975).

PME activity decreased dramatically in postveraison berries. Compared to grapes, the level of PME activity in tomatoes was much higher, and similar to levels reported by Pressey and Woods (1992). No PGase activity, using either pectin or PGA as a substrate, or cellulase activity was detected in berries at any stage of development. In contrast, high levels of PGase activity and low levels of cellulase activity were found in tomatoes; these were similar to levels reported by Ali and Brady (1982) and Huber (1985). Trace amounts of hydrolytic activity against xyloglucan were found in grapes after veraison, but no activity was detected in tomatoes. Galactanase activity was not detected in either the grape or tomato extracts.

The activities of β-galactosidase, α-galactosidase and PME in grapes and tomato were also expressed as units per gram fresh weight (Table 4.2). In grapes, similar trends in activities on a fresh weight basis were found during development compared with those observed for specific activities (U/mg protein). The β-galactosidase activity

Table 4.2 Enzyme activity (U/ gram of fresh weight) in developing grape berries

Enzyme	Grape (dpa)					Tomato (ripe)
	41	63 (veraison)	86	105	126	
β-Galactosidase	1.1	1.5	53.0	44.6	45.7	44.4
α-Galactosidase	4.2	6.3	17.2	23.1	14.2	59.6
PME	2.7	2.2	1.4	1.3	0.9	494

β- and α-Gal 1U = 1 nmole *p*-nitrophenol released per min
PME 1U = amount of enzyme consuming 1 μmole base per min

expressed on a fresh weight basis was more than 30-fold greater in postveraison berries compared with preveraison berries. On a fresh weight basis, α-galactosidase activity increased during development before declining in ripe berries. PME activity slowly declined throughout ripening.

To provide an indication of enzyme activity in individual berries, the activities were also expressed as units per berry (Table 4.3). Again, the activities show a similar trend to those on a per mg protein (Table 4.1) and a per gram fresh weight basis (Table 4.2). Thus, there was a dramatic increase in the amount of β-galactosidase activity detected after veraison. The highest level of activity was found in ripe berries, and was approximately 150-fold greater than that in preveraison berries. α-Galactosidase activity showed an increase of almost 15-fold compared to preveraison berries. On a per berry basis, PME activity stayed relatively constant throughout berry development.

Table 4.3 Enzyme activity (U/ berry) in developing grape berries

Enzyme	Grape (dpa)				
	41	63 (veraison)	86	105	126
β-Galactosidase	0.6	0.9	63.9	62.9	91.4
α-Galactosidase	2.1	3.7	20.6	32.3	28.4
PME	1.4	1.3	1.7	1.8	1.8

β- and α-Gal 1U = 1 nmole *p*-nitrophenol released per min
PME 1U = amount of enzyme consuming 1 μmole base per min

4.4 Discussion

Work described in this chapter was designed to measure the activity of cell wall-modifying enzymes in grape berries during development. However, the extraction of a complex complement of active enzymes from grapes is a relatively difficult task, because of the high levels of phenolic compounds (Hawker, 1969). It was therefore necessary to develop a suitable method for protein extraction before enzyme assays could be performed.

In order to extract maximum enzyme activity and to maintain enzyme stability, a relatively complex extraction buffer, incorporating PEG, PVPP, DIECA and glycerol, was used in this study. Firstly, PEG and PVPP were included to decrease the formation of complexes between enzymes and either tannins or plant cell walls, thereby increasing protein solubility (Loomis and Battaile, 1966; Hawker, 1969; Linn, 1990). Additionally, DIECA, a copper-chelating reagent that inhibits polyphenol oxidase activity (Mayer and Harel, 1979), was included to prevent the formation of quinones, which, in turn, can inactivate enzymes (Loomis and Battaile, 1966). Finally, the use of glycerol was also been shown to be effective for the stabilization of extracted enzymes (Scopes, 1994; M. Hrmova, personal communication).

When proteins were extracted from grapes at five stages of development, it was evident that yields increased dramatically after veraison, both on a per gram fresh weight basis and on a per berry basis. When the proteins extracted from each stage of development were analysed by SDS-PAGE, it appeared that the increase in protein could be attributed predominantly to the accumulation of proteins with apparent molecular masses of approximately 30 and 24 kD. It is likely that these prominent proteins correspond to chitinases and thaumatin-like proteins, respectively (Waters *et al.*, 1996). The latter are pathogenesis-related proteins, known to be major soluble proteins of berries, and have been shown to increase in abundance during ripening (Robinson *et al.*, 1997; Tattersall *et al.*, 1997).

In addition to the pathogenesis-related proteins, it is possible that some of the increase in protein is a result of the accumulation of enzymes that are involved in cell wall modification. From the changes in cell wall composition observed in Chapter 3, the presence, and the subsequent increase in abundance, of several wall-modifying enzymes was expected at veraison or during softening.

The major change to the cell wall composition during grape berry ripening was a decrease in galactose/galactan content. Endo-galactanase was a likely candidate to cause this decrease. However, no galactanase activity was detected at any of the five stages of berry development (Table 4.1). Alternatively, β-galactosidases might have been responsible for the decline in galactose content of grape cell walls, because β-galactosidases degrade galactans and other cell wall fractions in various plant tissues (Bartley, 1974; De Veau *et al.*, 1993; Kitagawa *et al.*, 1995; Carrington and Pressey, 1996; Ali *et al.*, 1998).

In grape berries, β-galactosidase activity was detected at all stages of berry development, with activity increasing dramatically as the berry began to soften. β-Galactosidase activity has previously been reported in five grape cultivars, including Gordo, and activity has been shown to increase in all cultivars during ripening (Aryan *et al.*, 1987). The levels of β-galactosidase activity detected here for Gordo were approximately three times greater than the levels found by Aryan *et al.* (1987). Other fruit, such as apple, mango and papaya, also show increases in β-galactosidase activity as softening progresses (Bartley, 1974; Ali *et al.*, 1995; Lazan *et al.*, 1995), although in some fruit, such as tomato and kiwifruit, β-galactosidase activity remains relatively constant (Pressey, 1983; Wegrzyn and MacRae, 1992). However, it should be noted that β-galactosidase might not necessarily play a central role in the initiation of softening of some fruit. For example, in tomato, softening occurs before β-galactosidase activity or galactose loss is detected (Carrington and Pressey, 1996).

β-Galactosidases are generally assayed by their ability to hydrolyze *p*-nitrophenyl β-D-galactopyranoside. However, many can also hydrolyse poly- and oligosaccharides progressively from the non-reducing termini. These might be more correctly classified as β-galactan exohydrolases, but might not be easily detected in the endo-galactanase assays. The enzymes therefore have the potential to modify wall polysaccharides containing (1→4)-linked β-D-galactosyl residues (Seymour and Gross, 1996). There are many isoforms of β-galactosidase in fruit, and a variety of substrate specificities and variable levels of activity are shown by individual isoforms throughout fruit development (Pressey, 1983; Edwards *et al.*, 1988; Kitagawa *et al.*, 1995). The ability of isoforms of β-galactosidase isolated from muskmelon to degrade polysaccharides isolated from pre-ripe tissue into sizes similar to those found in ripe fruit (Ranwala *et al*, 1992) supports suggestions that β-galactosidases are important in ripening fruit. In the present study, β-galactosidase activity was only assayed in a crude protein extract, using one type of assay system and a synthetic substrate. As a result, the precise action and role of enzymes detected by the "β-galactosidase" assays in ripening grapes cannot yet be ascertained.

From data obtained from this study, it could be suggested that apart from β-galactosidase, α-galactosidase might also have a role in the softening process. α-Galactosidase activity was detected throughout development of the grape berry, with activity greatest at, or after, veraison (Tables 4.1, 4.2 and 4.3). It is unclear what role α-galactosidase plays in the softening of fruit. In some fruit, such as pears (Ahmed and Labavitch, 1980b) and olives (Fernández-Bolaños *et al.*, 1995), there is an increase in α-galactosidase activity during ripening, but in other fruit, such as tomato and muskmelon (Wallner and Walker, 1975; Ranwala *et al.*, 1992), the level of α-galactosidase activity remains constant throughout ripening. There are a few components in grapes that may be substrates for α-galactosidase (Fernández-Bolaños *et al.*, 1995; Fry, 1995). For example, cell wall analyses in Chapter 3 revealed the presence of heteromannans, and

also proteins rich in hydroxyproline, which in turn suggests the presence of extensin. Furthermore, RG-II has been isolated from wine, and presumably originated from the grape cell walls (Doco and Brillouet, 1993). All have α-linked Gal*p* residues.

Another major change observed in cell walls of ripening grapes was the increase in water-soluble pectic polysaccharides (Chapter 3). Apart from the activities of β-galactosidase and α-galactosidase, PME and PGase may contribute to this change. PME modifies RG-I/homogalacturonan and may affect polysaccharide aggregation (John and Dey, 1986). Low levels of PME were detected in ripening grapes. PME activity decreased on a per gram fresh weight and per mg protein basis but, on a per berry basis, PME activity remained relatively constant throughout berry development. In agreement with the low PME activity is the finding that the DE of PG did not change as the berry softened (Chapter 3). PME activity has been reported in many grape cultivars, including Gordo. However, PME activity was shown to increase during the initial stages of development and then to decrease in the cultivars Gordo, Sultana and Traminer (Luis, 1983). In Gordo berries, the maximum PME activity was detected in berries at 15-18 °Brix, approximately 86 dpa (Luis, 1983). In other grape cultivars, such as Concord, PME activity increases continuously throughout ripening, but in Riesling, activity increases initially and then remains constant until maturity (Lee *et al.*, 1979). An explanation for the apparently contradictory findings in PME activity between this study and the study by Luis (1983) may be related to different berry tissues from which proteins were extracted. Mesocarp tissue was used in this study, whereas Luis (1983) extracted proteins from both the skin and mesocarp of the berry.

No single trend in PME activity occurs in other fruit during ripening. Activities increase in cherry and papaya (Barrett and Gonzalez, 1994; Lazan *et al.*, 1995), decrease in avocado and kiwifruit (Awad and Young, 1979; Wegrzyn and MacRae, 1992), and remain constant throughout ripening in pears (Ahmed and Labavitch, 1980b). Overall, this suggests that PME is not vital for the softening of all fruit. Instead, PME is likely to have an important role in cell wall assembly and/or structure, as newly-synthesized

pectins are highly esterified (Zhang and Staehelin, 1992) and may undergo partial demethylation as a result of PME activity, either before or after insertion into the wall.

Another enzyme that modifies pectic polysaccharides is PGase. It hydrolyses homogalacturonan, but will not hydrolyze the backbone if the carboxyl groups of galacturonosyl residues are esterified or if sidechains are present (Seymour and Gross, 1996). No PGase activity was detected at any stage of development in the Gordo berry. This is in contrast to other findings where PGase activity was detected in grapes (Pansolli and Belli-Donni, 1973; Tyurina, 1977; Luis, 1983; Selvaraj *et al.*, 1995). Luis (1983) detected low PGase activity in Gordo, Traminer and Sultana berries for a period of 2-3 weeks after veraison. The discrepancy may again by accounted for by the exclusive use of mesocarp tissue in the present study.

Increasing PGase activity is found during the development of many fruit, such as pears, kiwifruit and papaya (Bartley *et al.*, 1982; Wegrzyn and MacRae, 1992; Lazan *et al.*, 1995). Additionally, while activity is not detected in green tomatoes (Wallner and Walker, 1975) or preclimacteric avocado (Awad and Young, 1979), it is detected as the fruit softens. Further supporting the role of PGase in ripening fruit was the discovery of tomato PGase isoforms which, when added to green tomato tissue, caused dissolution of the middle lamella in a fashion similar to that seen in ripening tomatoes (Crookes and Grierson, 1983). However, PGase is not the primary or universal determinant of softening in fruit. For example, in persimmon, no endo- or exo-PGase activity is detected (Cutillas-Iturralde *et al.*, 1993) and, in apples, only exo-PGase is found (Bartley, 1978). Thus, there are well-documented examples in which PGase activity cannot be directly linked to wall modification in ripening fruit, and the absence of PGase in ripening grapes (Table 4.1) is consistent with these other examples.

Other enzymes that may be important during fruit development are cellulases and XGases. In ripening avocado, tomato and strawberry, cellulase activities increase (Awad and Young, 1979; Huber, 1985; Abeles and Takeda, 1990) and XGase is responsible for XG depolymerization during ripening of kiwifruit and avocado

(Redgwell *et al.* 1990; O'Donoghue and Huber, 1992; Sakurai and Nevins, 1997). There are several isoforms of cellulase in tomato, and some have activity against both cellulose and XG (Maclachlan and Brady, 1992). In contrast to these previous studies, no cellulase activity was detected in developing grapes and only trace amounts of XGase activity were detected in grapes after veraison (Table 4.1). There has only been one report of cellulase activity in grapes (Hobson, 1968), although activity was assayed in that study by measuring the production of reducing ends, which detects both exo- and endo-activity. The detection of little or no cellulase and XGase activity is consistent with the minimal changes found for the alkali-soluble and cellulosic polysaccharides of grape cell walls during ripening (Chapter 3). Similarly, in apple, levels of cellulase activity decrease as softening proceeds (Abeles and Biles, 1991), suggesting that cellulase is not important for ripening of some fruit.

Many enzymes other than the six enzymes assayed here may be involved in cell wall modification during cell expansion and fruit softening. Candidates include rhamnogalacturonase, for which activity has been detected in grape berries (Gross *et al.*, 1995), pectate lyase, for which cDNAs have been isolated from banana and strawberry (Domínguez-Puigjaner *et al.*, 1997; Medina-Escobar *et al.*, 1997), and XET, which increases in activity in softening kiwifruit (Redgwell and Fry, 1993) and tomato (Maclachlan and Brady, 1994). Rhamnogalacturonase and XET were not assayed in grapes because the requirement for specialized substrates and/or complicated analyses makes them difficult to assay accurately (Fry *et al.*, 1992; Lorences and Fry, 1993; Gross *et al.*, 1995). Further, a function for pectate lyases in fruit ripening remains to be demonstrated and it was therefore considered less important compared with the other enzymes studied here.

As mentioned earlier, it should be stressed that enzyme assays can be complicated by a number of factors, including the presence of inhibitors, pH optima that are outside the pH of the extraction buffer, and variable substrate specificities. Inhibitors of some cell wall-modifying enzymes exist in fruit. A PGase-inhibitor has been detected in

raspberry (Johnston *et al.*, 1994) and a glycoprotein, which inhibits PME, is present in kiwifruit (Balestrieri *et al.*, 1990). These have the potential to reduce or completely inhibit enzyme activities. The presence of multiple isoforms with different pH optima, as found for tomato cellulases (Sobotka and Stelzig, 1974, Maclachlan and Brady, 1994), may mean that less than maximal activities will be detected when a specific pH is used in the assay system. Furthermore, the success of an assay system is dependent on the type of substrate used i.e. native *versus* synthetic or non-native substrates. For example, β-galactosidases have been reported to hydrolyse different substrates at different rates and to varying degrees (Buckeridge and Reid, 1994; Kitagawa *et al.*, 1995).

In summary, enzyme activities in extracts of ripening grapes were relatively low compared with other fruit (Table 4.1), with the possible exception of β-galactosidase. Nevertheless, activities could generally be reconciled with the changes in cell wall polysaccharides described in Chapter 3. In particular, loss of Gal*p* residues from cell wall polysaccharides was correlated with high levels of β-galactosidase activity in ripening grapes. However, the increased solubility of PG (Chapter 3) could not be correlated with increases in PGase activity, and the enzymic mechanisms for the partial solubilization of PG in ripening grapes remains unclear.

Low activities *per se* are not particularly surprising, because high enzyme activities are not necessarily required to have a large impact on the overall properties of wall polysaccharides and hence on the wall itself. In ripening grapes, it appears that β-galactosidase may be a key enzyme in the decrease in galactan found in the cell walls as the berry softens. However, this enzyme is probably a β-galactan exohydrolase and should now be purified and subjected to a detailed analysis of its substrate specificity and action pattern. When this information is available, its potential role in wall modification in ripening fruit might become more apparent. Similarly, the role of α-galactosidase in the ripening berry is unknown but may also be important in the softening/expansion process. Low PME activity was detected throughout berry

development and, because no change was found in the DE of cell wall pectins during berry development, its role in softening appears to be minor. Little or no activity was detected for XGase, PGase, cellulase and galactanase in ripening berries.

In view of the relatively low activities of cell wall-modifying enzymes in ripening grape berries (Table 4.1) and because of the potential difficulties associated with the extraction of active enzymes from grapes, as discussed above, it was decided to further examine the expression of the enzymes through examination of the transcriptional activities of the corresponding genes. Thus, if an enzyme such as PGase was particularly susceptible to inactivation in berry extracts, estimation of the relative abundance of the corresponding mRNA could cast further light on the potential role of the enzyme in berry softening. In pursuing this approach, cDNAs encoding several of the enzymes assayed here have been isolated. The isolation and characterization of the cDNAs is described in Chapter 5. The cDNAs were subsequently used to monitor mRNA levels by Northern hybridization analyses. These are described in Chapter 6.

Chapter 5

Isolation of cDNAs encoding Cell Wall-Modifying Enzymes from Grapes

5.1 Introduction

The measurement of enzyme activity can be a relatively quick and straightforward method to determine the presence and abundance of a particular enzyme in plant tissues. Unfortunately, difficulties are often encountered in reliably measuring enzymes in some tissues and may preclude the use of enzymic assays to monitor the activity of the enzyme in question. Common problems include insufficient plant tissue, low protein levels in the plant tissue extracts, insensitive activity assays, labile enzymes, the presence of inhibitors and high levels of proteases (Scopes, 1994). Grape berry tissue in particular is recalcitrant with respect to protein extraction, because berries contain enzyme inhibitors and interfering compounds such as phenolics (Hawker, 1969).

The isolation of cDNAs encoding the enzymes of interest and the use of these cDNAs as molecular probes can help address these problems. Detection of mRNA by Northern hybridization analysis is a sensitive technique, although no information is gained on translational efficiency and care should be exercised when extrapolating mRNA abundance to protein abundance. However, the presence of mRNA in different tissues at various stages of development can provide some indication of possible enzyme distribution and abundance.

Before commencement of this study little was known about changes in the structure of cell wall carbohydrates during grape berry development or about the presence or changes in the levels of many of the enzymes implicated in cell wall softening in grapes. Modification of the grape berry cell walls during ripening (Chapter 3) suggested the presence of wall-modifying enzymes. Subsequently, assays revealed certain enzymes were active, although not all the expected activities were detected (Chapter 4). It was then decided to isolate cDNAs encoding β-galactosidases, PMEs, PGases, cellulases and XETs, with the intention of monitoring the respective mRNA levels during berry development. From Chapters 3 and 4, β-galactosidase is apparently an important enzyme during grape berry development. In addition to β-galactosidase, it was of

interest to study the transcriptional expression patterns of PME and PGase genes considering that pectin solubility increases substantially during berry ripening (Chapter 3) but no PGase activity and only low PME activity was detected in grapes (Chapter 4). No cellulase activity was detected in Gordo berries (Chapter 4) but it appears to be important in the ripening of other fruit (Awad and Young, 1979; Huber, 1985; Abeles and Takeda, 1990) and as a consequence, studying cellulase transcriptional activity could determine if it has a role in grape berry softening. XET, an enzyme not assayed in Chapter 4 because of the requirement for specially prepared and ^{3}H-labelled XG oligosaccharides (Fry *et al.*, 1992; Lorences and Fry, 1993), was felt to be of greater importance than XGase, for which little activity was detected in ripening berries (Chapter 4). α-Galactosidase plays an uncertain role in fruit softening and it was decided not to attempt to isolate its cDNA.

To increase the chances of cloning the respective grape cDNAs, consideration must be given to the most appropriate stage of berry development from which to extract poly(A^+)-RNA for cDNA synthesis. As discussed in Chapter 4, β-galactosidase and PME activities were detected throughout ripening and the presence of mRNA encoding these two enzymes was therefore anticipated throughout berry maturation. No PGase or cellulase activities were detected in berries in this study, as discussed in Chapter 4. However, PGase activity was previously reported in *Vitis vinifera* cv. Gordo berries after veraison (Luis, 1983) and cellulase activity has also been detected in ripe grape berries (Hobson, 1968). Thus, it was predicted that mRNA for these enzymes may be present after veraison. XET activity has never been reported in grapes, so the timing of any mRNA presence was unknown. However, XET activity has been found in other fruit during ripening (Redgwell and Fry, 1993; Maclachlan and Brady, 1994), suggesting that XET activity may be present in postveraison grapes. It was therefore decided to synthesize cDNA from postveraison berry poly(A^+)-RNA, at 70 dpa.

Comparison of the nucleotide sequences of cDNAs encoding β-galactosidases, PMEs, PGases, XETs and cellulases from other plants revealed a relatively high degree

of amino acid sequence identity within enzyme classes. The availability from other plants of heterologous cDNAs encoding some of the above enzymes enabled the respective cDNAs to be isolated from a *Vitis vinifera* berry cDNA library. To obtain the remaining cDNAs for which no suitable heterologous cDNAs were readily available, polymerase chain reaction (PCR) was employed with the use of degenerate primers designed against the conserved amino acid regions of other plant enzymes.

The cloning of cDNAs encoding the enzymes β-galactosidase, PME, PGase, XET and cellulase is described in this chapter. These cDNAs were sequenced and compared to other plant cDNAs encoding the same enzymes.

5.2 Materials and Methods

Unless otherwise stated, standard molecular biological techniques were performed, and buffers prepared, as described by Sambrook *et al.* (1989).

5.2.1 Materials

5.2.1.1 cDNA library

The 70 dpa grape berry (cv. Shiraz) cDNA library in λZAP II (Stratagene) was prepared by Dr. Chris Davies (CSIRO, Plant Industry, Horticultural Unit, Adelaide, South Australia). cDNA was prepared using a SuperScript Choice cDNA synthesis system (Gibco BRL) according to the manufacturer's instructions, using 5 μg of poly(A^+)-RNA from 70 dpa, deseeded Shiraz berries. *Eco*RI adaptors were ligated to the cDNA and the resultant fragments were cloned into predigested LambdaZAPII/*Eco*RI/CIAP vector and packaged in GigapackII Gold packaging extract (Stratagene). Titering, amplification and screening of the library were carried out as described in the manufacturer's instruction manual.

5.2.1.2 Description of heterologous cDNAs

A number of heterologous cDNAs, cloned into various plasmid vectors, was available for use as probes for screening the grape cDNA library. Descriptions of the cDNAs are given in Table 5.1. The apple PGase cDNA, kiwifruit XET cDNA and the two β-galactosidase cDNAs were a kind gift from Dr. Gavin Ross (Horticulture and Food Research Institute of New Zealand Ltd., Mt Albert Research Centre, Auckland, New Zealand). The peach PME and PGase cDNAs were generously provided by Dr. Jim Speirs (CSIRO, Plant Industry, Horticultural Unit, Adelaide, South Australia).

Table 5.1 Description of heterologous cDNAs

cDNA clone	Origin	Name	Size (bp)	Vector	Accession number
β-Galactosidase	Apple	pABG1	2637	pBluescript SK(+)	L29451
	Kiwifruit	pKBG1	851	pGEMT	Unpublished
PME	Peach	pPPE8B	1687	pBluescript SK(+)	X95991
PGase	Apple	pGDPG1	1790	pSPORT1	L27743
	Peach	pRF5	1486	pBluescript SK(+)	X76735
XET	Kiwifruit	pKFXET	~ 800	pGEMT	Unpublished

5.2.2 Preparation of heterologous cDNAs for probes

5.2.2.1 Transformation using electroporation

The plasmid DNA containing the cDNA inserts (Section 5.2.1.1) were transformed into *E. coli* DH5α (Clontech, Palo Alto, CA, USA) competent cells. Transformation by electroporation was performed essentially as described in the BioRad Gene Pulser instruction manual. Plasmid DNA (1 μl, 10 ng/ml) was added to 50 μl competent cells in a cuvette and pulsed (1.8 volts, capacitance = 25 μFD, resistance =200Ω). Transformants were plated out and, after incubation, a number of colonies were selected from which to isolate plasmid DNA.

5.2.2.2 Minipreparation of plasmid DNA

Plasmid DNA was isolated as described by the method of Holmes and Quigley (1981). Cultures (1-1.5 ml) were centrifuged for 1 min at 12,000g and the pellet resuspended in 350 μl STET buffer (50 mM Tris-HCl buffer, pH 8.0 containing 8% sucrose, 5% Triton X-100 and 50 mM EDTA) and 12.5 ml lysozyme (40 mg/ml in 10 mM Tris-HCl buffer, pH 8.0). The samples were held at 100°C for 1 min, centrifuged for 15-30 min at 12,000g, and the pellets removed and discarded. To the supernatants, 40 μl 3 M sodium acetate buffer, pH 5.5 and 220 μl isopropanol that had been stored at –20°C were added, the contents mixed and the samples centrifuged for 10 min at 12,000g. The pellets were washed with 300 μl 80% (v/v) ethanol and allowed to dry. The dried DNA samples were resuspended in 30 μl of water.

The plasmid DNA was digested with restriction enzymes (Boehringer Mannheim), and the fragments separated on an agarose gel to check insert sizes. If insert sizes were as expected, larger scale plasmid preparations were performed.

5.2.2.3 Template preparation of plasmid DNA for sequencing and probe preparation

High purity plasmid DNA was isolated using a procedure modified from that described by Holmes and Quigley (1981). This material was prepared for use as a template in sequencing reactions or for hybridization probes. Cell cultures (10 ml) were grown for 16 h, centrifuged for 10 min at 2000g, and the supernatants decanted. Pellets were resuspended in 600 μl STET buffer (50 mM Tris-HCl buffer, pH 8.0, 8% w/v sucrose, 5% v/v Triton X-100, 50 mM EDTA) and lysozyme (10 μl, 40 mg/ml) was added. The suspensions were mixed and held at 100°C for 1 min. The samples were centrifuged for 30-45 min at 12,000g, and pellets were removed with sterile toothpicks and discarded. Supernatants were treated with 20 μl RNase A (20 mg/ml) at 37°C for 20 min to remove contaminating RNA. One volume phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v), that was stored under 100 mM Tris-HCl buffer, pH 7.6, was added to each tube and mixed well. The tubes were centrifuged for 3-4 min at 12,000g and the aqueous phase transferred to a new tube, carefully avoiding the interface. The phenol/chloroform/isoamyl alcohol extraction described above was repeated and followed by an extraction with one volume of chloroform/isoamyl alcohol (24:1, v/v) to remove residual phenol. DNA was precipitated from the aqueous phase by adding one volume of isopropanol at -20°C and 0.1 volume of 3 M sodium acetate buffer, pH 5.2. The contents of the tubes were mixed and centrifuged for 10 min at 12,000g. The supernatants were decanted and the pellets washed with 80% (v/v) ethanol. The pellets were resuspended in 200 μl TE (10 mM Tris-HCl buffer, pH 7.6 containing 0.1 mM EDTA), and 50 μl 3 M sodium acetate buffer, pH 5.2, and 500 μl ethanol at -20°C were added. The samples were placed at -20°C for 1 h to precipitate the DNA, before centrifugation for 10 min at 12,000g. The pellets were washed with 80% (v/v) ethanol and dried under vacuum for 5-10 min. The DNA pellets were resuspended in 40 μl water and stored at -20°C.

5.2.2.4 Digestion and isolation of cDNA fragments for use as probes

The cDNA inserts were isolated from plasmid vectors by digesting with the appropriate restriction enzymes. The plasmid containing the apple PGase sequence was cut with *Xba*I and *Sac*I. This yielded two fragments, of which the 1.2 kb fragment was used as a probe. The digests were separated on a 2% (w/v) NuSieve (FMC BioProducts, Rockland, ME, USA) agarose gel and the DNA fragments purified using the QIAquick Gel Extraction Kit (QIAGEN Inc, Chatswood, CA, USA), following the manufacturer's instructions. The DNA fragments were labelled as described below.

5.2.3 Preparation of [^{32}P]-radiolabelled DNA probes

The [^{32}P]-radiolabelled DNA probes were prepared using the Bresatec (Adelaide, Australia) Gigaprime DNA labelling kit, following the protocol provided by the manufacturer. DNA (approximately 100 ng) was labelled with 50 μCi [α-^{32}P]-dATP (Bresatec), and labelled DNA was separated from the unincorporated DNA on a 1 ml Sepharose G-50 column (equilibrated in 10 mM Tris-HCl buffer, pH 7.6, containing 0.1 mM EDTA).

5.2.4 Screening the 70 dpa Shiraz berry cDNA library

The library was screened as described in the Statagene instruction manual (La Jolla, CA, USA). The complete library consisted of approximately 7.5x10^5 recombinant phage; a total of 1.8x10^5 pfu were screened with each probe. The Shiraz library in λZAP phage was plated with XL1-Blue MRF cells (Stratagene) to give a plaque density of approximately 4.5x10^4 pfu/14 cm plate. The DNA was transferred onto Amersham Hybond-N filters and fixed using a UV crosslinker by irradiation at 120,000 μjoules/cm^2 (Stratagene). The membrane was prehybridized in hybridization buffer (0.25 M Na_2HPO_4, 1 mM EDTA and 7% w/v SDS) for 2 h at 65°C and the [^{32}P]-radiolabelled DNA probes (approximately 2-7 x10^4 cpm) were incubated overnight with the filters. The filters were washed in a solution of 1x SSC (20x SSC contains 88 g sodium citrate

and 175 g sodium chloride per L) and 0.1% (w/v) SDS for 15 min at 65°C. The filters were dried and placed in exposure cassettes with Kodak X-ray film. Positive plaques were removed from the plates and phage were eluted from the plug of agar with 2% (v/v) chloroform in SM buffer (50 mM Tris-HCl buffer, pH 7.5 containing 100 mM NaCl, 8 mM magnesium sulphate and 0.01% w/v gelatin). The phage were plated out at a density of 20 to 200 pfu/9cm plate and a secondary screen performed. Plasmids were rescued from the phage of single positive plaques as described below.

5.2.5 Plasmid rescue

The plasmids were rescued from the phage as described in the Stratagene user manual. Phage stocks were added to XL1-Blue MRF cells (Stratagene) and ExAssist helper phage (Stratagene) and incubated in 2xYT media (10 g sodium chloride, 16 g tryptone and 10 g yeast extract per L) for approximately 2 h at 37°C. After heating the cells at 70°C for 20 min, the samples were centrifuged and the supernatants decanted into new tubes. The phage was added to SolR cells (Stratagene) and plated onto LB plates containing 100 μg/ml ampicillin. A number of colonies were selected and plasmid DNA isolated as described in Section 5.2.2.2.

5.2.6 Nucleotide sequence analysis

Plasmids were partially sequenced to establish their identity, before subcloning (described in Section 5.2.7) and complete sequence analysis. The cDNAs were sequenced using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) employing the T3 or T7 primers (Stratagene). Sequencing was performed at the Sequencing Facility, Department of Haematology, Flinders Medical Centre, South Australia. Computer analysis of the sequence data was carried out with programs from the University of Wisconsin (Devereux *et al.*, 1984), provided by the Australian National Genomic Information Service (ANGIS).

5.2.7 Subcloning of cDNA inserts

The longest cDNA matching each enzyme of interest was subcloned so that the cDNA could be fully sequenced. The plasmids were digested with appropriate restriction enzymes and fragments were separated by electrophoresis and purified using a QIAquick Gel Extraction Kit. Fragments were ligated into *Eco*RV-cut pBluescript SK(+) vector (Stratagene) that had been phosphatase treated. The plasmids were transformed into DH5α cells as described in Section 5.2.2.1 and plated out onto LB plates containing 100 μg/ml ampicillin, 40 μg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactoside and 12.5 μg/ml isopropylthio-β-D-galactoside. The plates were incubated at 37°C for 16 h and white colonies were selected for isolation of plasmid DNA using the procedure described in Section 5.2.2.3.

5.2.8 Design of degenerate PCR primers

Degenerate oligonucleotide primers were designed against regions of conserved amino acid sequence of plant cellulases and XETs. The primers designed are shown in Figure 5.1 and were made by Gibco BRL.

5.2.9 Polymerase Chain Reaction

Different combinations of primers were employed in PCR procedures to maximize the chances of obtaining the desired amplified product. Each 25 μl reaction contained 2.5 μl first strand cDNA template, from a total of 520 μl of cDNA synthesized from approximately 1 μg Poly $(A)^+$ -RNA isolated from 70 dpa Shiraz berries (Section 5.2.1), 1 μM forward primer, 1 μM reverse primer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 2.5 μl 10x Taq polymerase buffer and 1U Taq polymerase. The mixture was overlaid with mineral oil and the DNA amplified using a DNA thermal cycler (Perkin Elmer Cetus, Norwalk, CT, USA). The mixture was heated at 94°C for 3 min prior to two initial cycles (94°C for 1 min, 37°C for 2 min and 72°C for 3 min) that allowed heterologous primer binding. Thereafter, 33 cycles of amplification (94°C for 1 min, 55°C for 1 min and

```
CEL1F                    W   E   R   P   E   D   M
      5'    GC GAATTC TGG GAA AGI CCI GAA GAC ATG
                EcoR1       G C         G   T

CEL2F                            Y
                         C   P   F   Y   C
      5'    GC GAATTC TGC CCI TTT TAT TG
                EcoR1   T       AC   C

CEL3F*                   D   E   L   L   W
      5'    GC GAATTC GAT GAA CTT CTA TGG G
               EcoR1        G   G T G

CEL4R            T           E
             K   K   Q   V   D   Y
            AAA AAI CAA GTI GAI TAC
              G  C    G           T

      5'    GC CTGCAG TA ITC IAC TTG ITT TTT
                Pst1             C    G  C

CEL5R                        Y
             Y   M   V   G   F   G
            TAC ATG GTI GGI TTT GGI
              T               AC

      5'    GC CTGCAG CC AAA ICC IAC CAT GTA
                Pst1     GT              A

XET1F                     H   D   E   I   D   F   E   F
                         CAC GAC GAA ATA GAC TTC GAA TT
                           T   T   G   C   T   T   G
                                       T

      5'    GC GAATTC CAC GAC GAA ATI GAC TTC GAA TT
                EcoR1   T   T   G       T   T   G

XET1R                     Y   N   Y   C   T   D
            5'           TAC AAC TAC TGC ACI GAC
                           T   T   T   T       T

      5'    CG GACGTC ATC IGT GCA GTA GTT GTA
                Pst1  G       A   A   A   A
```

Figure 5.1 Degenerate primers designed against conserved regions of plant XETs and cellulases. Each primer has a restriction site at its 5' end, incorporated for cloning of the fragment. CEL = cellulase, F = forward primer and R = reverse primer. Numbers indicate primer number.

72°C for 3 min) were used. Finally, the mixture was heated at 72°C for 7 min. A small volume (2 μl) of each PCR mixture was reserved as template for subsequent nested PCRs while the remainder of each reaction was separated on a 2% (w/v) NuSieve TAE (40 mM Tris-acetate buffer, pH 8.0, containing 1 mM EDTA) buffered gel. The DNA bands of expected sizes were excised and purified using the QIAquick kit. Nested PCR was performed on the purified DNA and the reserved PCR mixtures (diluted 1 in 50 with water) (Section 5.2.9) employing the internal primers. The mixtures were separated on a 2% (w/v) NuSieve gel (TAE buffered), the DNA bands of expected sizes were excised and purified using the QIAquick kit.

5.2.10 Subcloning PCR fragments

The PCR fragments were ligated into *Eco*RV-cut pBluescript SK(+) plasmid which had been T-tailed according to the method of Marchuk *et al.* (1991), the mixture was transformed into *E. coli* DH5α cells (Section 5.2.2.1) and the resulting plasmids isolated as described in Section 5.2.2.2.

5.3 *Results*

The 70 dpa Shiraz berry cDNA library was screened for phage containing cDNA inserts with homologies to β-galactosidase, PME, PGase and XET, using heterologous probes isolated from other plants (Section 5.2.1.1).

5.3.1 Isolation of a β-galactosidase cDNA

The cDNA library was screened using a partial-length kiwifruit β-galactosidase cDNA (851 bp) as a probe. Five strongly hybridizing plaques were obtained (Table 5.2). After plaque purification, plasmids were rescued and insert sizes checked by restriction digestion. The insert sizes were of the expected size for near full-length cDNAs, as judged by the size of other plant β-galactosidases. However, two banding patterns were seen when the plasmids were digested with *Rsa*I. Two clones, representing the two banding patterns, were partially sequenced, but only one clone showed homology to plant β-galactosidases (Table 5.2). This putative grape β-galactosidase was subsequently subcloned and sequenced using the strategy shown in Figure 5.2.

The nucleotide sequence and deduced amino acid sequence of the putative β-galactosidase isolated from grape are shown in Figure 5.3. This clone, named VvBG1 contains a 2855 bp insert. The sequence around the first in-frame Met is similar to the translation start sites of other plant β-galactosidases (Fig. 5.4). The open reading frame (ORF) extends from nucleotides 6 to 2567, and encodes a 94.9 kDa polypeptide of 854 amino acids. A putative NH_2-terminal signal sequence of 27 amino acids was predicted (von Heijne, 1983). The likely cleavage site is between Cys and Ser residues (Fig. 5.4), which matches the cleavage sites predicted for the tomato and asparagus β-galactosidase sequences used in the alignment. Removal of the NH_2-terminal signal sequence would result in a mature protein of 827 amino acids, a putative molecular mass of 92 kDa and a predicted pI of 8.0. There is one potential N-glycosylation site (Smith *et al.*, 1985) in

Table 5.2 Screening of the 70 dpa Shiraz berry cDNA library

Heterologous probe	# of positives plaques	Name of partially sequenced clone[a]	Best match
Kiwifruit β-gal (pKBG1)	5	BG1 (VvBG1)	Asparagus β-gal
		BG2	None
Peach PME (pPPE8B)	17	PME1 (VvPME1)	Peach PME
		PME2	Peach PME
		PME3	Peach PME
		PME4	Peach PME
Peach PGase (pRF5)	28	PG1	None
		PG2	None
		PG3	None
		PG4	None
		PG5	None
Apple PGase (pGDPG1)	19	P1 (VvPG1)	Kiwifruit PGase
		P2	Kiwifruit PGase
		P3	Kiwifruit PGase
		P4	Kiwifruit PGase
		P5	Kiwifruit PGase
		P6	None
Kiwifruit XET (pKFXET)	9	X1	None
		X2	None
		X3	Rye heat-shock protein
		X4	None
		X5	None

[a] Names in parenthesis are the final names of the fully sequenced clones

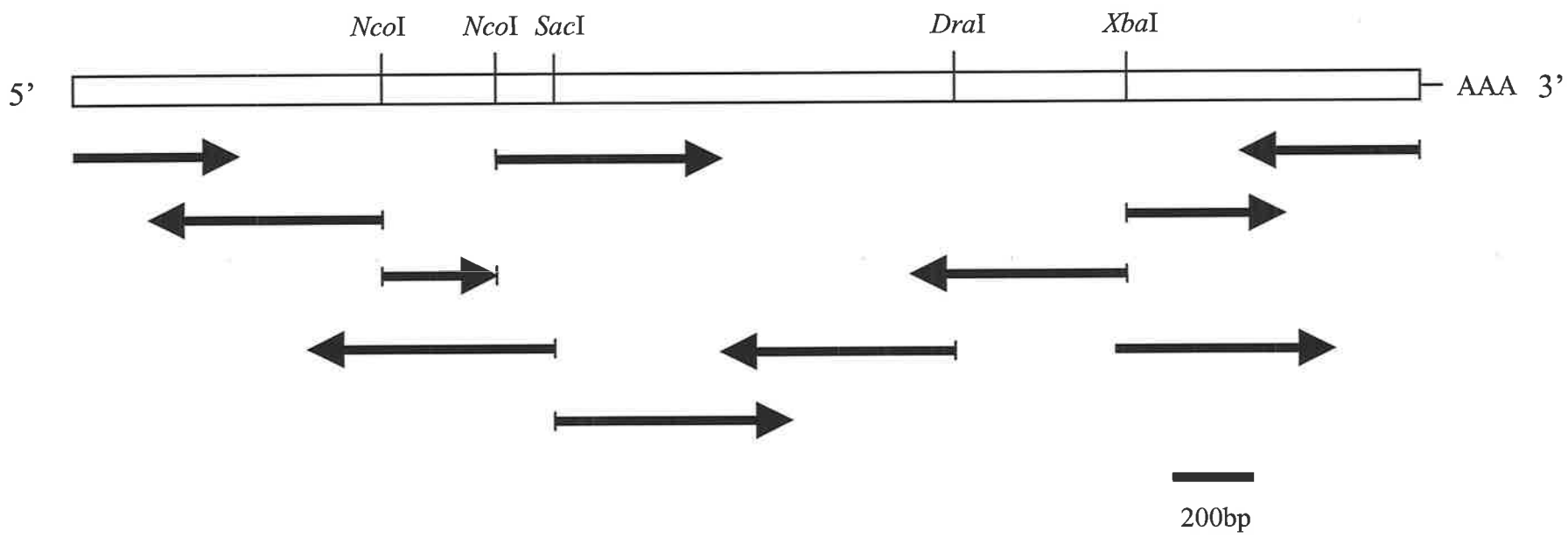

Figure 5.2 Sequencing and subcloning strategy for the VvBG1 clone (2855 bp). The arrows indicate the direction and length of the individual sequences. Arrows starting with a line are sequences obtained from a subcloned fragment. Arrows finishing with a line indicates the end of the subcloned fragment. Arrows without a line are sequences obtained from primers designed to known sequences.

Figure 5.3 Complete nucleotide sequence and deduced amino acid sequence of the cDNA VvBG1. The first in-frame Met is in bold. The putative signal peptide is underlined. A potential glycosylation site is boxed in grey and a putative polyadenylation signal is boxed. The stop codon is marked by an asterisk. Amino acids are numbered on the left relative to the putative first amino acid of the mature protein, and nucleotides are numbered on the right.

```
        GAGAAATGGAGACTAGCTCAGTCTCCAAGCTGTTCATCTTCTTCTTCGTGCCATTGATGTTTCTGCATTCTCAGCTGATCCAATGTAGCG    90
-27         M  E  T  S  S  V  S  K  L  F  I  F  F  F  V  P  L  M  F  L  H  S  Q  L  I  Q  C  S  V
        TTACCTACGATAAGAAGGCCATTGTCATCAATGGGCAAAGGAGAATCCTCATCTCCGGCTCTATACACTACCCCAGAAGCACCCCTGATA   180
2        T  Y  D  K  K  A  I  V  I  N  G  Q  R  R  I  L  I  S  G  S  I  H  Y  P  R  S  T  P  D  M
        TGTGGGAAGATCTTATACGGAAGGCTAAAGATGGAGGCTTAGATGTCATCGACACCTATATTTTCTGGAATGTTCATGAGCCTTCTCCTG   270
32       W  E  D  L  I  R  K  A  K  D  G  G  L  D  V  I  D  T  Y  I  F  W  N  V  H  E  P  S  P  G
        GGAATTATAATTTTGAGGGGAGATACGATCTGGTACGGTTCATTAAGACTGTGCAGAAAGTAGGGCTCTATGTTCATCTCCGGATTGGAC   360
62       N  Y  N  F  E  G  R  Y  D  L  V  R  F  I  K  T  V  Q  K  V  G  L  Y  V  H  L  R  I  G  P
        CCTATGTTTGTGCAGAATGGAACTTCGGGGGATTTCCTGTTTGGTTGAAGTTTGTTCCTGGTATCAGCTTCAGAACAAATAATGAACCTT   450
92       Y  V  C  A  E  W  N  F  G  G  F  P  V  W  L  K  F  V  P  G  I  S  F  R  T  N  N  E  P  F
        TCAAGATGGCAATGCAAGGGTTCACTCAGAAGATTGTCCACATGATGAAGAGTGAAAACCTGTTTGCATCACAGGGTGGGCCCATCATCC   540
122       K  M  A  M  Q  G  F  T  Q  K  I  V  H  M  M  K  S  E  N  L  F  A  S  Q  G  G  P  I  I  L
        TTTCTCAGATTGAGAATGAGTACGGACCAGAGAGTAGGGAATTAGGAGCTGCTGGTCATGCATACATCAACTGGGCTGCAAAAATGGCTG   630
152      S  Q  I  E  N  E  Y  G  P  E  S  R  E  L  G  A  A  G  H  A  Y  I  N  W  A  A  K  M  A  V
        TTGGATTGGACACAGGAGTCCCATGGGTGATGTGCAAAGAAGATGATGCCCCAGATCCTGTGATAAATGCATGTAATGGCTTTTACTGTG   720
182      G  L  D  T  G  V  P  W  V  M  C  K  E  D  D  A  P  D  P  V  I  N  A  C  N  G  F  Y  C  D
        ATGCTTTCTCTCCAAACAAACCTTACAAGCCCAGAATATGGACAGAGGCTTGGAGTGGCTGGTTTACAGAGTTTGGTGGCACAATTCACC   810
212      A  F  S  P  N  K  P  Y  K  P  R  I  W  T  E  A  W  S  G  W  F  T  E  F  G  G  T  I  H  R
        GGCGACCAGTTCAAGATTTGGCATTTGGGGTTGCTCGTTTCATACAAAATGGTGGCTCATTTGTTAATTACTATATGTACCATGGAGGAA   900
242      R  P  V  Q  D  L  A  F  G  V  A  R  F  I  Q  N  G  G  S  F  V  N  Y  Y  M  Y  H  G  G  T
        CCAATTTTGGACGCTCTGCTGGAGGCCCTTTCATTACAACCAGTTATGATTATGATGCTCCAATTGATGAATATGGTTTGATCAGACAAC   990
272      N  F  G  R  S  A  G  G  P  F  I  T  T  S  Y  D  Y  D  A  P  I  D  E  Y  G  L  I  R  Q  P
        CAAAATATGGTCATTTGAAGGAGCTCCATAAAGCAATTAAGTTATGTGAACATGCTGTAGTTTCTGCGGACCCTACTGTTATCTCATTGG  1080
302      K  Y  G  H  L  K  E  L  H  K  A  I  K  L  C  E  H  A  V  V  S  A  D  P  T  V  I  S  L  G
        GAAGCTATCAACAGGCCCATGTATTCTCTTCAGGGCGAGGCAACTGTGCAGCTTTTCTCTCCAACTACAATCCCAAGTCATCTGCAAGGG  1170
332      S  Y  Q  Q  A  H  V  F  S  S  G  R  G  N  C  A  A  F  L  S  N  Y  N  P  K  S  S  A  R  V
        TGATTTTCAATAATGTGCACTATGATTTGCCGGCTTGGTCCATTAGCATCCTTCCTGATTGCAGAACCGTAGTCTTTAATACTGCAAGAG  1260
362      I  F  N  N  V  H  Y  D  L  P  A  W  S  I  S  I  L  P  D  C  R  T  V  V  F  N  T  A  R  V
        TTGGAGTTCAAACTTCACATATGCGAATGTTTCCAACCAACTCTAAGTTGCACTCTTGGGAAACTTATGGTGAAGACATTTCTTCTCTGG  1350
392      G  V  Q  T  S  H  M  R  M  F  P  T  N  S  K  L  H  S  W  E  T  Y  G  E  D  I  S  S  L  G
        GGAGCAGTGGAACAATGACTGCTGGCGGACTCTTGGAGCAGATAAATATCACCAGAGATTCCACTGACTATCTGTGGTACATGACCAGTG  1440
422      S  S  G  T  M  T  A  G  G  L  L  E  Q  I  N  I  T  R  D  S  T  D  Y  L  W  Y  M  T  S  V
        TCAACATTGACTCATCGGAATCATTTCTACGAAGAGGTCAAACTCCAACCCTCACTGTGCAATCTAAAGGCCACGCTGTTCATGTCTTTA  1530
452      N  I  D  S  S  E  S  F  L  R  R  G  Q  T  P  T  L  T  V  Q  S  K  G  H  A  V  H  V  F  I
        TCAATGGACAGTATTCAGGATCAGCATACGGAACCAGGGAGAACAGGAAATTCACATATACCGGAGCAGCCAATCTACATGCAGGAACAA  1620
482      N  G  Q  Y  S  G  S  A  Y  G  T  R  E  N  R  K  F  T  Y  T  G  A  A  N  L  H  A  G  T  N
        ATAGAATTGCCTTGCTTAGCATAGCTGTTGGACTACCGAATGTTGGATTGCATTTTGAGACATGGAAGACAGGAATCCTAGGTCCAGTTT  1710
512      R  I  A  L  L  S  I  A  V  G  L  P  N  V  G  L  H  F  E  T  W  K  T  G  I  L  G  P  V  L
        TGCTTCATGGTATTGACCAAGGAAAGAGGGATTTATCATGGCAGAAGTGGTCTTACCAGGTTGGCCTGAAAGGAGAGGCAATGAATCTGG  1800
542      L  H  G  I  D  Q  G  K  R  D  L  S  W  Q  K  W  S  Y  Q  V  G  L  K  G  E  A  M  N  L  V
        TCTCCCCAAATGGAGTCTCAGCAGTTGAGTGGGTTCGAGGGTCACTTGCTGCCCAAGGTCAGCAGCCTTTAAAATGGTATAAGGCTTATT  1890
572      S  P  N  G  V  S  A  V  E  W  V  R  G  S  L  A  A  Q  G  Q  Q  P  L  K  W  Y  K  A  Y  F
        TCAATGCACCTGAAGGAGATGAGCCATTGGCTTTGGACATGCGGAGCATGGGAAAGGGTCAAGTTTGGATCAATGGGCAGAGCATTGGAA  1980
602      N  A  P  E  G  D  E  P  L  A  L  D  M  R  S  M  G  K  G  Q  V  W  I  N  G  Q  S  I  G  R
        GATATTGGATGGCTTACGCAAAAGGTGACTGCAATGTTTGTAGCTACTCTGGAACATACCGACCCCCAAAGTGTCAGCATGGTTGCGGTC  2070
632      Y  W  M  A  Y  A  K  G  D  C  N  V  C  S  Y  S  G  T  Y  R  P  P  K  C  Q  H  G  C  G  H
        ATCCAACCCAAAGATGGTATCATGTTCCAAGGTCTTGGTTGAAGCCAACACAAAATTTGTTGATAATTTTTGAAGAACTTGGTGGTGATG  2160
662      P  T  Q  R  W  Y  H  V  P  R  S  W  L  K  P  T  Q  N  L  L  I  I  F  E  E  L  G  G  D  A
        CATCAAAGATAGCTCTTATGAAGAGAGCAATGAAGAGTGTTTGTGCCGATGCTAATGAACACCACCCAACTCTAGAGAATTGGCATACTG  2250
692      S  K  I  A  L  M  K  R  A  M  K  S  V  C  A  D  A  N  E  H  H  P  T  L  E  N  W  H  T  E
        AAAGCCCTAGCGAATCAGAAGAACTTCATCAGGCCAGTGTGCACCTGCAATGTGCCCCAGGGCAGTCCATATCAACCATTATGTTTGCAA  2340
722      S  P  S  E  S  E  E  L  H  Q  A  S  V  H  L  Q  C  A  P  G  Q  S  I  S  T  I  M  F  A  S
        GTTTTGGAACTCCTTCTGGAACCTGTGGAAGTTTCCAGAAAGGAACCTGCCATGCACCAAACTCTCAAGCCATCTTAGAGAAGAATTGCA  2430
752      F  G  T  P  S  G  T  C  G  S  F  Q  K  G  T  C  H  A  P  N  S  Q  A  I  L  E  K  N  C  I
        TAGGGCAAGAGAAATGCTCGGTCCCCATATCCAACAGTTACTTTGGGGCTGACCCATGTCCTAATGTACTAAAACGGTTATCTGTCGAAG  2520
782      G  Q  E  K  C  S  V  P  I  S  N  S  Y  F  G  A  D  P  C  P  N  V  L  K  R  L  S  V  E  A
        CTGCTTGTTCCCCTACGGTAACAACAACCACTCAACCTGATTCAAGATGAGATGTCTGCAAAGAAGGATTCAAGCAACTTCCTGGTGTAA  2610
812      A  C  S  P  T  V  T  T  T  T  Q  P  D  S  R  *
        TTATGGTTATTTATACACTACAGCAAGCCGTGGAGCAAGAAGCACATGGAATAAAGGGGGTGAAATTACAGGCAAAGGTCCTTCAACTAC  2700
        ATTGGTGTGATTCATATTCATATTTACAAGATAGGTCAGTAAATTATTAGTTTTGACTAGAAATGGAGCCCCGAGAATAATGTGTATTGA  2790
        AGATAATGCGAATAATTTGAGTGAAATATTCTTTGGGCGCAATGAAGGTGGGGTCTAAAAAAAAA                           2855
```

Figure 5.4 Alignment of the deduced amino acid sequence of the grape clone VvBG1 with the amino acid sequences of other plant β-galactosidases using the PILEUP and PRETTYBOX programs (Genetics Computer Group, Madison, WI). Residues that are identical in at least three of the five sequences are highlighted in black and residues that are similar are highlighted in grey. The EMBL accession numbers of the amino acid sequences are L29451 (apple), X83854 (tomato), X77319 (asparagus), and O65736 (chickpea).

PRETTYBOX of: /home/cspi03/chrisd/kylien/Bgal/bg/bg-4.msf{*} May 6, 1999 16:41:50.62

```
chickpea   METNSVSKIL SLFFF.LFFV CSQLIHCSVT YDRKAIIING QRRILISGSI HYPRSTPEMW 59
vvbgl      METSSVSKIF IFFEVP.MFL HSQLIQCSVT YDKKAIVING QRRILISGSI HYPRSTPDMW 60
apple      MGVGIQTMWS ILLLFSCIF. .SAAS.ASVS YDHKAIIING QKRILISGSI HYPRSTPEMW 57
tomato     ..MGFWMAML LMLLLCIWV. .SCGI.ASVS YDHKAIVNG QRKILISGSI HYPRSTPEMW 55
asparagus  MALKLVLMIM VALLAAVWS. .PPAVTASVT YDHKSVIING QRRILISGSI HYPRSTPEMW 58

chickpea   EDLIQKAKVG GLDVIDTYVF WNVHEPSPSN YNFEGRYDLV RFIKTVQKVG LYVHLRIGPY 119
vvbgl      EDLIRKAKDG GLDVIDTYIF WNVHEPSPGN YNFEGRYDLV RFIKTVQKVG LYVHLRIGPY 120
apple      FDLIQKAKDG GLDVIQTYVF WNGHEPSPGN YYFEERYDLV KFIKLVQQEG LEVNLRIGPY 117
tomato     PDLIQKAKEG GVDVIQTYVF WNGHEPEEGK YYFEERYDLV KFIKVVQEAG LYVHLRIGPY 115
asparagus  PDLIQKAKDG GLDVIQTYVF WNGHEPEEGQ YYFGGRYDLV RFLKLVKQAG LYAHLRIGPY 118

chickpea   VCAEWNFGGF PVWLKYVPGI SFRTDNGPFK AAMQGFTQKI VQMMKNEKLF QSQGGPIILS 179
vvbgl      VCAEWNFGGF PVWLKEVPGI SFRTNNEPFK MAMQGFTQKI VHMMKSENLF ASQGGPIILS 180
apple      VCAEWNFGGF PVWLKYVPGI AFRTDNEPFK AAMQKFTEKI VSMMKAEKLF QTQGGPIILS 177
tomato     ACAEWNFGGF PVWLKYVPGI SFRTNNEPFK AAMQKFTTKI VDMMKAEKLY ETQGGPIILS 175
asparagus  VCAEWNFGGF PVWLKYVPGI HFRTDNGPFK AAMGKFTEKI VSMMKAEGLY ETQGGPIILS 178

chickpea   QIENEYGFQG RALGAVGHAY SHWAAKMAVG LGTGVPWVMC KEDDAPDPVI NSCNGFYGDD 239
vvbgl      QIENEYGFES RELGAAGHAY INWAAKMAVG LDTGVPWVMC KEDDAPDPVI NACNGFYGDA 240
apple      QIENEFGPVE WEIGAPGKAY TKWAAQMAVG LDTGVPWIMC KQEDAPDPVI DTCNGFYGEN 237
tomato     QIENEYGPME WELGEPGKVY SEWAAKMAVD LGTGVPWIMC KQDDVPDPII NTCNGFYGDY 235
asparagus  QIENEYGPVE YYDGAAGKSY TNWAAKMAVG LNTGVPWVMC KQDDAPDPVI NTCNGFYGDY 238

chickpea   FSPNKPYKPK LWTESWSGWF SLFGGPVLQR FAQDLAFAVA RFIQKGGSFF NYYMYHGGTN 299
vvbgl      FSPNKPYKPR IWTEAWSGWF TEFGGTIHRR FVQDLAFGVA RFIQNGGSFV NYYMYHGGTN 300
apple      FKPNKDYKPK MWTEVWTGWY TEFGGAVFTR PAEDVAFSVA RFIQSGGSFL NYYMYHGGTN 297
tomato     FTPNKANKPK MWTEAWTAWF TEFGGPVFYR PAEDMAFAVA RFIQTGGSFI NYYMYHGGTN 295
asparagus  FSPNKDNKPK MWTEAWTGWF TGFGGAVLQR PAEDMAFAVA RFIQKGGSFI NYYMYHGGTN 298

chickpea   FGRSAGGPFI TTSYDYDAPL DEYGLLKEPK YGHLKDLHKA IKQGEHALVS SDPTVTSLGA 359
vvbgl      FGRSAGGPFI TTSYDYDAPL DEYGLIKQPK YGHLKELHKA IKLGEHAVVS ADPTVISLGS 360
apple      FGRTAGGPFM ATSYDYDAPL DEYGLPREPK WGHLRDLHKA IKSGKSALVS VDPSVTKLGS 357
tomato     FGRTSGGPFI ATSYDYDAPL DEFGSLRQPK WGHLKDLHRA IKLGEPALVS VDPTVTSLGN 355
asparagus  FGRTAGGPFI STSYDYDAPL DEYGLLRQPK WGHLRDLHKA IKLGEPALVS GEPTITSLGQ 358

chickpea   YEQAHVFSJG TQTCAAFLAN YHSNSAARVT FNNRHYDLPP WSISILPDGK TDVFNTARVR 419
vvbgl      YQQAHVFSSG RGNCAAFLSN YNPKSSARVI FNNVHYDLPA WSISILPDGR TVVFNTARVG 420
apple      NQEAHVFKSE SD.CAAFLAN YDAKYSVKVS FGGGQYDLPT WSISILPDGK TEVYNTAKVG 416
tomato     YQEARVFKSE SGACAAFLAN YNQHFFAKVA FGGMHYNLPP WSISILPDGK NTVYNTARVG 415
asparagus  NQESYVYRSK SS.CAAFLAN FNSRYYATVT FNGMHYNLPP WSVSILPDGK TTVFNTARVG 417

chickpea   FQNSKIQMLP SNSKLLSWET YDEDVSSLAH SSRITASGLL EQINATRDTS DYLWYITSVD 479
vvbgl      VQTSHMRMFF TNSKLHSWET YGEDISSLGS SGTMTAGGLL EQINITRDST DYLWYMTSVN 480
apple      SQSAQVQMTP VHSGP.PWQS FIPETTSSDE TDTTTLDGLY EQINITRDTT DYLWYMTDIT 475
tomato     AQSAQMKMTE VSRGP.SWES P.NEDAASHE DDTFTVVGLL EQINITRDVS DYLWYMTDIE 473
asparagus  AQTTTMKMQY LG.GP.SWKA YTEDTDAIND .NIFTKDGLV EQLSTTWDRS DYLWYTTYVD 474

chickpea   ISPSESFLRG GNKPSISVHS SGDAVHVFIN GKPSGSAFGT REQRSCTFNG PINLHAGTNK 539
vvbgl      IDSSRSFLRR GQTPTLTVQS KGHAVHVFIN GQYSGSAYGT RENRKPTYTG AANLHAGTNR 540
apple      IGSDEAFLKN GKSPLLTIFS AGHALNVFIN GQLSGTVYGS LENPKLSFSQ NVNLRSGINK 535
tomato     IDPTEGFLNS GNWPWLTVFS AGHALHVFVN GQLAGTVYGS LENPKLTFSN GINLRAGVNK 533
asparagus  IAKNEEFLKT GKYPYLTVMS AGHAVHVFIN GQLSGTAYGS LDNPKLTYSG SAKLWAGSNK 534

chickpea   IALLSVAVGL PNGGIHFESW KTGITGFILL HGIDHGQKDL TWQKWSYQVG LKGEAMNLVS 599
vvbgl      IALLSIAVGL PNVGLHFETW KTGILGPVLL HGIDQGKRDL SWQKWSYQVG LKGEAMNLVS 600
apple      LALLSISVGL PNVGTHFETW NAGVLGPITL KGLNSGTWDM SGWKWTYKTG LKGEALGLHT 595
tomato     ISLLSIAVGL PNVGPHFETW NAGVLGPVSL NGLNEGTRDL TWQKWFYKVG LKGEALSLHS 593
asparagus  ISILSVSVGL PNVGNHFETW NTGVLGPVTL TGLNEGKRDL SLQKWTYQIG LHGETLSLHS 594

chickpea   PNGVSSVDWV RESLASQNQP QLKWHKAYFH APDGNEALAL DMSGMGKGQV WINGQSIGRY 659
vvbgl      PNGVSAVEWV RGSLAAQGQQ PLKWYKAYFN APEGDEPLAL DMRSMGKGQV WINGQSIGRY 660
apple      VTGSSSVEWV KGPSMAEKQ. PLTWYKATFN APPGDAPLAL DMGSMGKGQI WINGQSVGRH 654
tomato     LSGSPSVEWV EGSLVAQKQ. PLSWYKTTFH APDGNEPLAL DMNTMGKGQV WINGQSLGRH 652
asparagus  LTGSSNVEWG EASL...QKQ. PITWYKTFFH APPGNEPLAL DMNTMGKGQI WINGQSIGRY 650

chickpea   WLVY.AKGNC NSGNYAGTYR QAKCQLGCGQ PTQKWYHVPK SWIKPTNNLM VVFEELGGNF 718
vvbgl      WMAY.AKGDC NVGSYSGTYR PPKCQHGCGH FTQRWYHVPR SWLKPTQNLI IIFEELGGDA 719
apple      WPGYIARGSC GDGSYAGTYD DKKCRTHCGE PSQRWYHIPK SWITPTGNLI VVFEEWGGDF 714
tomato     WPAYKSSGSC SVGNYTGWFD EKKCLTNCGE GSQRWYHVPR SWIYPTGNLL VVFEEWGGDP 712
asparagus  WPAYKASGPC GSGDYRGTYN EKKCLSNCGE ASQRWYHVPR SWIIPTGNFL VVLEEWGGDP 710

chickpea   WKISLVKPTI ........... ........HFP ASSEP.NLRT NTTQV..... ........... 745
vvbgl      SKIALMKRAM KSVCADANPH HITLENWHTE SPSESEELHQ ASVIIQCAFG ASIITIMFAS 779
apple      SRISLVERGT ALDAKKL.... ........... ........... ........... ........... 731
tomato     YGITLVKREI GSVCADIYEW QPQLLNWQRL VSGKFDRPLR PKAIIKCAFG QKISSIKFAS 772
asparagus  TGISMVKRSV ASVCAEVEEL QPTMDNWRTK AYG......R PKVHISCDFG QKMSKIKFAS 764

chickpea   ........... ........... ........... ........... ........... ........... 745
vvbgl      FGTESGTGGS FQKGTGHAPN SQAILE.... .KNGIGQMKG SVPISNSYFG ADPGPNVLKR 834
apple      ........... ........... ........... ........... ........... ........... 731
tomato     FGTPEGVGGN FQQGSGHAPR SYDAFK.... .KNTVGKESG SVQVTPENFG GDPGRNVLKK 827
asparagus  FGTPQGTGGS FSEGSGHAHK SYDAFEQEGL MQNGVGQEFG SVNVAPEVFG GDPGPGTMKK 824

chickpea   ........... ........... 745
vvbgl      ISVEAAGSPT VTTTSQPDSR 854
apple      ........... ........... 731
tomato     ISVEAIGS.. ........... 835
asparagus  IAVEAIGE.. ........... 832
```

the VvBG1 sequence. The 3' untranslated region of the cDNA contains a putative polyadenylation sequence of AATAAA (Wahle and Keller, 1992) and a polyadenylic acid tail of 9 units in length.

Alignment of the deduced amino acid sequence of VvBG1 with other plant β-galactosidases shows a high degree of similarity through most of the sequence (Fig. 5.4). In addition, it appears that the VvBG1 cDNA is likely to be nearly full-length. VvBG1 is most similar to the tomato β-galactosidase, sharing 65.5% positional identity and 77.1% similarity at the amino acid level, although all sequences aligned in Figure 5.4 also share a high degree of similarity with VvBG1.

5.3.2 Isolation of a PME cDNA

The cDNA library was also screened with a peach PME cDNA (1687 bp). Seventeen strongly hybridizing clones were found (Table 5.2) but a high background of hybridizing plaques was observed on all the filters. Six clones were taken through a second screening and plasmids were rescued from the resulting positive plaques. When digested with restriction enzymes, all clones but one had the same banding pattern. Four of the cDNAs were partially sequenced. All these were identical and had a high degree of sequence similarity with a PME cDNA from peach (Table 5.2). One of the cDNAs was subcloned and fully sequenced using the strategy shown in Figure 5.5.

The resulting nucleotide sequence and deduced amino acid sequence of the putative PME are shown in Figure 5.6. This PME clone, named VvPME1, has a cDNA insert which is 1827 bp in length. Beginning with the first Met, the ORF extends from nucleotides 5 to 1597, encoding a polypeptide of 531 amino acids with a molecular mass of 58 kDa. A putative NH_2-terminal signal peptide of 32 amino acids is evident (von Heijne, 1983). The mature protein is predicted to have a molecular mass of 54 kDa and a pI of 7.2. Seven potential glycosylation sites exist in the sequence of the mature peptide (Fig. 5.6).

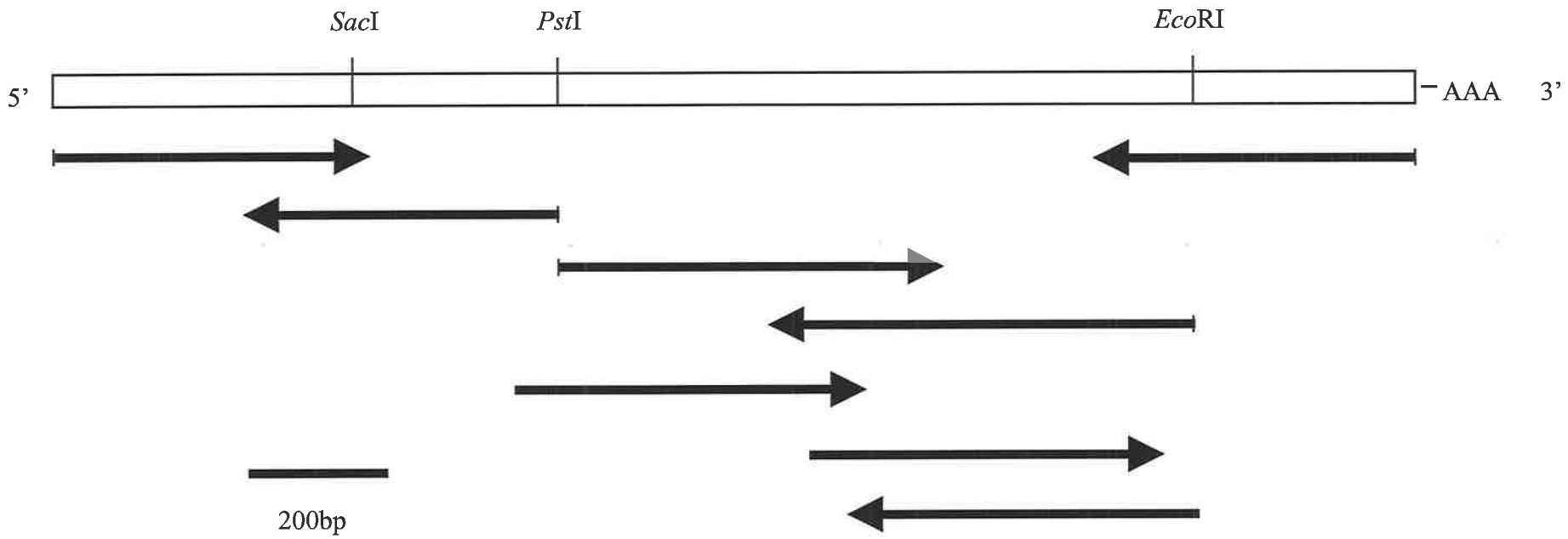

Figure 5.5 Sequencing and subcloning strategy for the VvPME1 clone (1827 bp). The arrows indicate the direction and length of the individual sequences. Arrows with a line are sequences obtained from a subcloned fragment. Arrows without a line are sequences obtained from primers designed to known sequences.

```
       CATAATGGCTTCTTTACTCAGGACAAGGTTGTCCCAATTCTCCCTTTTCTTTACTATCTTCTTCTTTTTCATTTCTTGCAGTTCTAGTAC    90
-32       M  A  S  L  L  R  T  R  L  S  Q  F  S  L  F  F  T  I  F  F  F  F  I  S  C  S  S  S  T
       ATTTGCTAGCAGTTCTGATGAAGAGGCTAATCTGAAGCTTCTTCAGTCTCAGTGTTTGGCGGTTCCTTCCTCGGCTTTTATCAGTTCACT   180
-3     F  A  S  S  S  D  E  E  A  N  L  K  L  L  Q  S  Q  C  L  A  V  P  S  S  A  F  I  S  S  L
       CAAGTCCACCATTGATGTACTTCGAGGGACTATGTCAGTTGTGTCCCAGTTCACTAAAGTGTTCAATGATTTTCGGCTTTCTAATGCCAT   270
28     K  S  T  I  D  V  L  R  G  T  M  S  V  V  S  Q  F  T  K  V  F  N  D  F  R  L  S  N  A  I
       TTCAGATTGCCTTGAGTTGCTGGATTTTGCAGCTGATGATCTGAGCTGGTCCCTTTCTGCCATTCAGAATCCTAAAGGTAAAGATAACGG   360
58     S  D  C  L  E  L  L  D  F  A  A  D  D  L  S  W  S  L  S  A  I  Q  N  P  K  G  K  D  N  G
       TACCGGCGATCTGGGGTCGGATTTGAAGACATGGTTGAGCTCTACTTTCACTAATCAGGACACATGCATAGAGGGATTTGTTGGTACCAA   450
88     T  G  D  L  G  S  D  L  K  T  W  L  S  S  T  F  T  N  Q  D  T  C  I  E  G  F  V  G  T  N
       TGGAATTGTCAAGACAGTGGTAGCTGAAAGCCTGAGCCAAGTAGCTTCATTAGTCCACAGCCTTCTTACTATGGTCCATGATCCAGCTCC   540
118    G  I  V  K  T  V  V  A  E  S  L  S  Q  V  A  S  L  V  H  S  L  L  T  M  V  H  D  P  A  P
       CAAAGGCAAATCCAATGGCGGTGGTGGCGGTGTGAAGCACGTGGGCAGCGGTGATTTTCCATCATGGGTTGGCAAACACAGCAGGAAGCT   630
148    K  G  K  S  N  G  G  G  G  G  V  K  H  V  G  S  G  D  F  P  S  W  V  G  K  H  S  R  K  L
       CCTTCAGGCCTCATCAGTGTCACCAGATGTTACAGTAGCTGCAGATGGAACTGGGAATTATACTACTGTGATGGATGCAGTCCAAGCAGC   720
178    L  Q  A  S  S  V  S  P  D  V  T  V  A  A  D  G  T  G  N  Y  T  T  V  M  D  A  V  Q  A  A
       CCCTGATTACAGCCAGAACCACTATGTAATCTACATCAAGCAAGGCATTTATAGGGAGAATGTGGAGATCAAGAAGAAGAAATGGAACCT   810
208    P  D  Y  S  Q  N  H  Y  V  I  Y  I  K  Q  G  I  Y  R  E  N  V  E  I  K  K  K  K  W  N  L
       GATGATGGTCGGAGATGGGATGGGCGCTACTGTCATTACTGGAAACCGGAGCTACATCGATGGCTGGACTACATATGCATCTGCAACCTT   900
238    M  M  V  G  D  G  M  G  A  T  V  I  T  G  N  R  S  Y  I  D  G  W  T  T  Y  A  S  A  T  F
       TGCTGTTAAGGGCAAAGGATTTATTGCAAGAGACATGACATTTGAGAACACGGCAGGACCAGAGAAGCACCAAGCGGTAGCATTGAGGTC   990
268    A  V  K  G  K  G  F  I  A  R  D  M  T  F  E  N  T  A  G  P  E  K  H  Q  A  V  A  L  R  S
       CGATTCAGACCTTTCGGTCTACTACAGATGCAGCATGAGGGGATACCAGGACACACTGTATCCCCACACGAATCGTCAATTCTACAGAGA  1080
298    D  S  D  L  S  V  Y  Y  R  C  S  M  R  G  Y  Q  D  T  L  Y  P  H  T  N  R  Q  F  Y  R  E
       GTGTAGAATCAGTGGCACCGTCGATTTCATATTCGGTGATGCCACGGTGGTCTTCCAAAACTGCCAAATCCTGGTGAAGAAGGGCTTACC  1170
328    C  R  I  S  G  T  V  D  F  I  F  G  D  A  T  V  V  F  Q  N  C  Q  I  L  V  K  K  G  L  P
       AAATCAAAAGAACACCATCACCGCCCAAGGCCGCAAAGACCCTGCTCAGCCCACGGGCTTCTCAATACAATTCTCCAACATATCAGCAGA  1260
358    N  Q  K  N  T  I  T  A  Q  G  R  K  D  P  A  Q  P  T  G  F  S  I  Q  F  S  N  I  S  A  D
       TTCTGATCTTCTGGCTTCAGTGAACTCCACACTCTCGTACCTTGGTAGGCCATGGAAGCAATATTCGCGTACCATCATCATGAAATCCTA  1350
388    S  D  L  L  A  S  V  N  S  T  L  S  Y  L  G  R  P  W  K  Q  Y  S  R  T  I  I  M  K  S  Y
       CATCAGTGATGCCATTAGGCCAGAAGGATGGCTAGAGTGGAATGGGGATTTTGCTCTGGATACACTCTACTATGGTGAATATATGAACTA  1440
418    I  S  D  A  I  R  P  E  G  W  L  E  W  N  G  D  F  A  L  D  T  L  Y  Y  G  E  Y  M  N  Y
       TGGACCCAGTGCAGGCCTAGGCAGCAGGGTCCAGTGGCCGGGTTTTCATCTGTTGAACAACTCCGCTCAGGCCGCCAACTTCACGGTGAC  1530
448    G  P  S  A  G  L  G  S  R  V  Q  W  P  G  F  H  L  L  N  N  S  A  Q  A  A  N  F  T  V  T
       CGAATTCATAGCCGGAAACCTGTGGCTGCCGTCGACCGGGGTGAAATATTCAGCAGGATTAGCAGTTTAAAGCAGTGAAAAGAGAGTATA  1620
478    E  F  I  A  G  N  L  W  L  P  S  T  G  V  K  Y  S  A  G  L  A  V  *
       TATATATATATAATGACACTATTGAGTTTGCAACTTCCAAGTCTCTAGCGTCTAGTAAATTGATTTCTCCAAAAGCAACGAGTCTTTCCA  1710
       TGTTACCATATATTTTTGGTAAATTTTAATGGTCCCAAGGGTCTTTGTCTTCTATAATTGGACCCCATCTCAACCACATTCAATGCTATC  1800
       ATGTGATTTGTTCTTTGAAAAAAAAAA                                                                1827
```

Figure 5.6 Complete nucleotide sequence and deduced amino acid sequence of the cDNA VvPME1. The first in-frame Met is in bold. The putative signal peptide is underlined. Potential glycosylation sites are boxed in grey and possible polyadenylation signals are boxed. The stop codon is marked by an asterisk. Amino acids are numbered on the left relative to the putative first amino acid of the mature protein, and nucleotides are numbered on the right.

The alignment of VvPME1 with other plant PME sequences reveals a high degree of sequence similarity in the COOH-terminal region of the sequences, but little similarity is detected in the NH_2-terminal part of the sequences (Fig. 5.7). The VvPME1 cDNA shows a 68.3% positional identity and 81% similarity with the amino acid sequence of the peach PME. The next most similar sequence to VvPME1 is the tomato PME, where there is 44.5% identity and 65.4% similarity, indicating that plant PME amino acid sequences are not as highly conserved as plant β-galactosidases.

5.3.3 Isolation of PGase cDNAs

The Shiraz berry cDNA library was screened with a probe that encoded a peach PGase. Twenty-eight strongly hybridizing plaques were isolated, although there was high background on the filters. Eight clones were taken through a secondary screen and five were partially sequenced. None of these clones had homology with any other plant PGase sequences (Table 5.2).

The cDNA library was rescreened using a cDNA encoding an apple PGase as a probe. Nineteen strongly hybridizing plaques were isolated; seven of which were taken through a secondary screen, and a third screen was required to obtain single plaques for some clones. Six clones were partially sequenced, of which five had sequences which were homologous to other plant PGase sequences. The longest cDNA, named VvPG1 was subcloned and sequenced as shown in Figure 5.8. The nucleotide sequence and the corresponding amino acid sequence of VvPG1, are shown in Figure 5.9. The ORF from nucleotide 5 to 1386 encodes a polypeptide with a molecular mass of 47 kDa. A potential signal peptide of 23 amino acids is evident (von Heijne, 1983). The mature protein has a molecular mass of 45 kDa and a pI of 8.6.

Other PGase cDNAs of similar length or shorter than VvPG1 were partially sequenced. The P3 cDNA was found to have the same sequence as the P4 cDNA. Figure 5.10 shows the P2, P3 and P5 cDNAs aligned to the VvPG1 cDNA where the sequences match or are the most similar. Overall, there were some differences in the

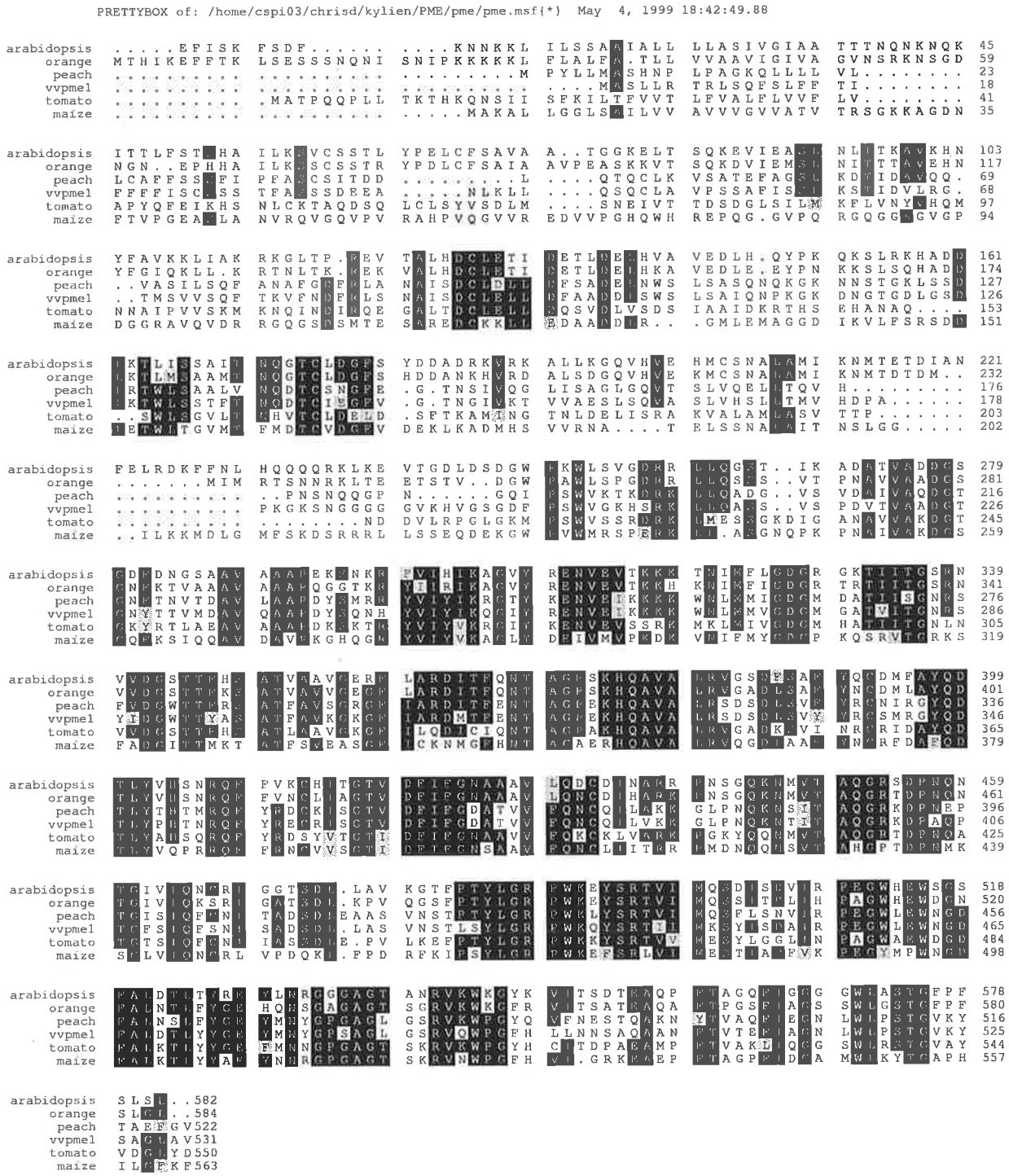

Figure 5.7 Alignment of the deduced amino acid sequence of the grape clone VvPME1 with the amino acid sequences of other plant PMEs using the PILEUP and PRETTYBOX programs (Genetics Computer Group, Madison, WI). Residues that are identical in at least four of the six sequences are highlighted in black and residues that are similar are highlighted in grey. The EMBL accession numbers of the amino acid sequences are U82976 (orange), U25649 (*Arabidopsis*), X95991 (peach), U70675 (tomato), and Y13285 (maize).

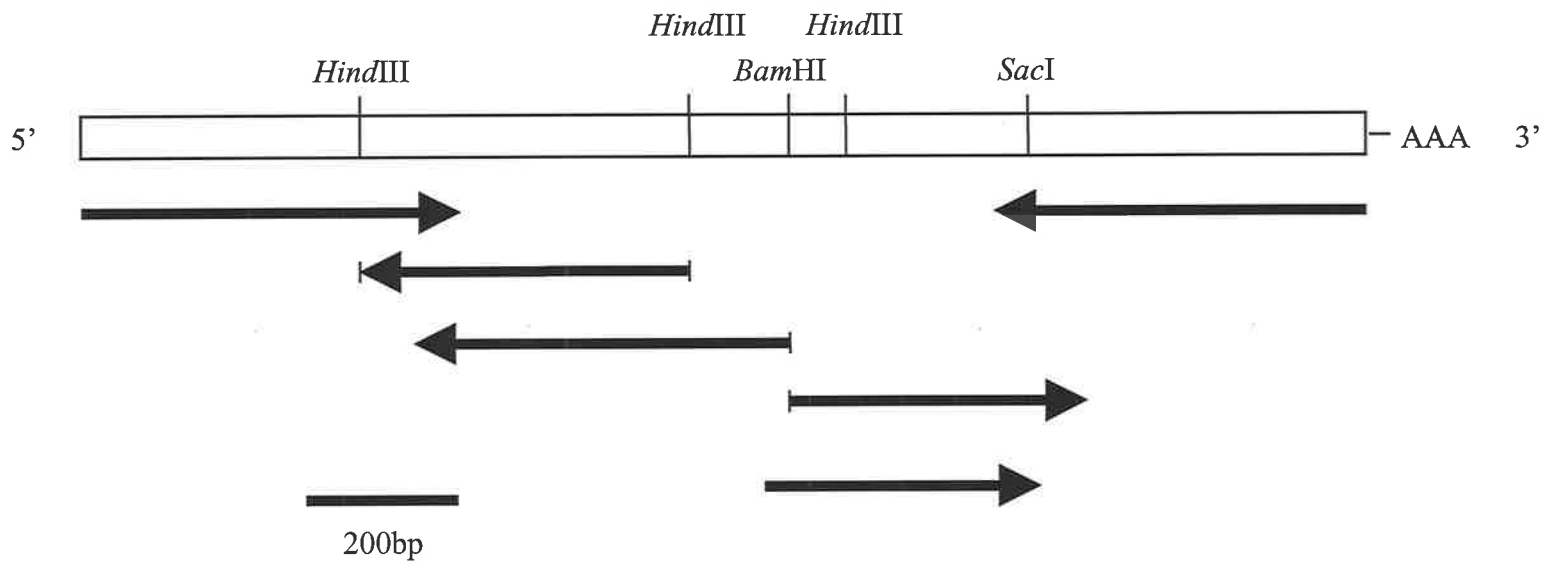

Figure 5.8 Sequencing and subcloning strategy for the VvPG1 clone (1609 bp). The arrows indicate the direction and length of the individual sequences. Arrows with a line are sequences obtained from a subcloned fragment. Arrows without a line are sequences obtained from primers designed to known sequences.

```
        CTTCCATATATACTTTTCCGAATATTATCTCTCTCCTTTAGTAAAGAAGAAGCAATGTCTCTACAAAGGCGCTTCGTTCCATGTCTCATT    90
-23                                                            M  S  L  Q  R  R  F  V  P  C  L  I
        GTCTTTCTCCTTTCTTTCCCATGTTACTTCAGTTTGCAAGCGAGCCCACCTTATGATTATCTTGGTGAGGCATCTGGCTATGATTCTCAG   180
-11     V  F  L  L  S  F  P  C  Y  F  S  L  Q  A  S  P  P  Y  D  Y  L  G  E  A  S  G  Y  D  S  Q
        GCGAATCATTTGCATTTCAGCACCATTCAAAGCCGTGAGACTGAGAACTGGCCATGGCGCAAATTTGGAAGTAAAACGGGGAAATCTTTG   270
20      A  N  H  L  H  F  S  T  I  Q  S  R  E  T  E  N  W  P  W  R  K  F  G  S  K  T  G  K  S  L
        GCTTCAGTTAAAATGGTTAACGTGAATTATTATGGAGCTAAAGGTGATGGAAGTGATGCCACAGAGGCATTCAAGAAAGCTTGGAAGGCA   360
50      A  S  V  K  M  V  N  V  N  Y  Y  G  A  K  G  D  G  S  D  A  T  E  A  F  K  K  A  W  K  A
        GCTTGTTCATCTCCAGGATCTGTCCTAGTGGTGCCCAAAAACAAGAACTATCTTCTTAAGCCAATTACATTTCAAGGTCCTTGTAAATCC   450
80      A  C  S  S  P  G  S  V  L  V  V  P  K  N  K  N  Y  L  L  K  P  I  T  F  Q  G  P  C  K  S
        AGTATTACAGTGCAGATATATGGAACTGTCCAGGCATCTACTGATCGATCCGCCTACAGTAACGATATGACCCACTGGCTTATCTTTGAA   540
110     S  I  T  V  Q  I  Y  G  T  V  Q  A  S  T  D  R  S  A  Y  S  N  D  M  T  H  W  L  I  F  E
        AATGTCCAAAATTTAGCGGTTCAAGGTGGTGGCACCATCAATGGAAATGGGAAGACGTGGTGGGAAAACTCTTGCAAAGTGAACTATGAT   630
140     N  V  Q  N  L  A  V  Q  G  G  G  T  I  N  G  N  G  K  T  W  W  E  N  S  C  K  V  N  Y  D
        CTTCCTTGCAAGGGTGCACCAACGGCCTTAACCTTCTATAACTGCAAGAACTTGGCAGTGAAGAACCTCAAGATCCAAAATGCCCAGCAA   720
170     L  P  C  K  G  A  P  T  A  L  T  F  Y  N  C  K  N  L  A  V  K  N  L  K  I  Q  N  A  Q  Q
        ATGCATGTTTCTTTTGAAAAATGCGTGGGTGTTCAAGCTTCGGGTCTCACCATAACTGCACCGGGGAACAGTCCCAACACTGATGGAATC   810
200     M  H  V  S  F  E  K  C  V  G  V  Q  A  S  G  L  T  I  T  A  P  G  N  S  P  N  T  D  G  I
        CATGTGAGTTACACCAAAATTATCCAGATTTCAAGCTCGGTTATTGGAACAGGTGATGACTGTATTTCAATTGTGAGTGGATCCCAGAAG   900
230     H  V  S  Y  T  K  I  I  Q  I  S  S  S  V  I  G  T  G  D  D  C  I  S  I  V  S  G  S  Q  K
        GTGCAAGTCAATGGTATCACCTGTGGACCAGGCCATGGAATCAGCATTGGAAGCTTAGGATCTGGAGATTCAGAGGCTCATGTTTCGGAT   990
260     V  Q  V  N  G  I  T  C  G  P  G  H  G  I  S  I  G  S  L  G  S  G  D  S  E  A  H  V  S  D
        GTTACAGTGAACGGCGCAACGCTTTCGGGAACCACAAATGGAGTTAGAATCAAGACATGGCAGGGAGGGTCTGGAAGCGCAAGCAACATC  1080
290     V  T  V  N  G  A  T  L  S  G  T  T  N  G  V  R  I  K  T  W  Q  G  G  S  G  S  A  S  N  I
        AAATTTCAGAACATTGTAATGCATAACGTGGAGAACCCTATAATAATAGACCAAAAGTACTGTGACCAAAGCAAACCATGCAAGTCACAG  1170
320     K  F  Q  N  I  V  M  H  N  V  E  N  P  I  I  I  D  Q  K  Y  C  D  Q  S  K  P  C  K  S  Q
        AGCTCGGCTGTTCAAGTGCAAAATGTGTTGTACCAGAACATAAAAGGAACAAGTTCTTCGAAGGAGGCTATATCGCTTGATTGCAGTGCG  1260
350     S  S  A  V  Q  V  Q  N  V  L  Y  Q  N  I  K  G  T  S  S  S  K  E  A  I  S  L  D  C  S  A
        AAGTTTCCATGTCAAGGGATTCTGCTGCGAGACATTGATATAAAAGTTGGAGGAGGAAAGGCAGCCAAAGCTGTATGCAGCAATGCCAGA  1350
380     K  F  P  C  Q  G  I  L  L  R  D  I  D  I  K  V  G  G  G  K  A  A  K  A  V  C  S  N  A  R
        GTGACTGTAATGGGGGATGTGTCTCCAAATTGCGCCTGAGAAATGCATGGAAGCTAGGATTATTGGTTCTGAATCCTCTGATCACACACT  1440
410     V  T  V  M  G  D  V  S  P  N  C  A  *
        TACATATATATATTTTTGTATATATGTATACAGTACTGAAAAATAATATATGAGTTTGTCATATTTGTTTATCAAAGAATAAGGTCCAATA  1530
        ATACTAGAATGTAGCCAACACAATAATACTAATCTGATCATCTCTTATTGTTTATCATCAAAAAAAAAAAAAAAAAAAA              1609
```

Figure 5.9 Complete nucleotide sequence and deduced amino acid sequence of the cDNA VvPG1. The first in-frame Met is in bold. The putative signal peptide is underlined. Potential polyadenylation signals are boxed. The stop codon is marked by an asterisk. Amino acids are numbered on the left relative to the putative first amino acid of the mature protein, and nucleotides are numbered on the right.

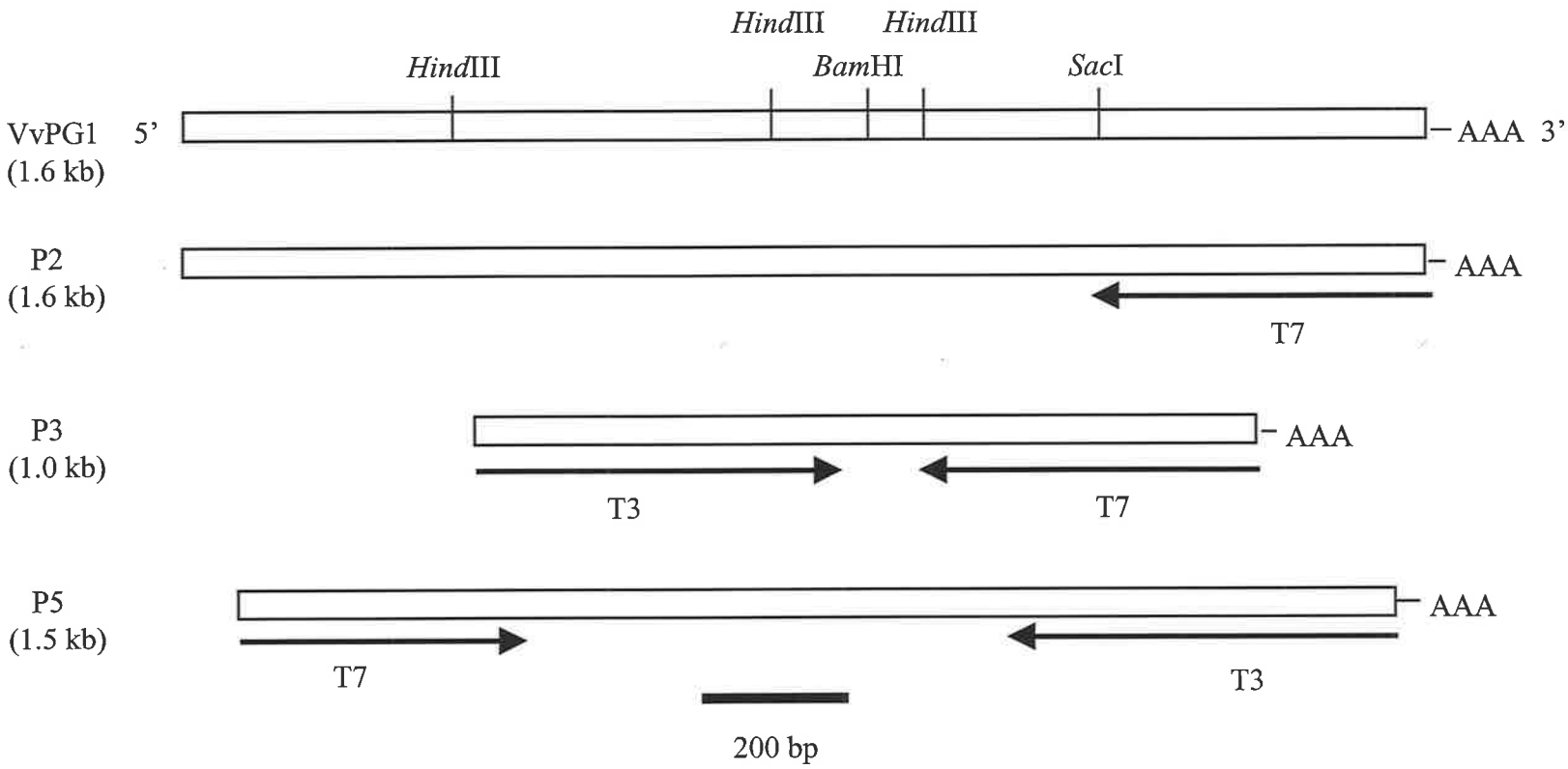

Figure 5.10 Comparison of the four PGase cDNAs isolated from grape. VvPG1 has been fully sequenced and the arrows indicate the direction and length of the individual sequences for the other three PGase clones. Approximate sizes of the clones are also shown.

sequences when compared to VvPG1. The sequence for the P2 cDNA matched exactly with VvPG1 with the exception of a deleted amino acid. The P3 cDNA was approximately 1.0 kb in length, much shorter than VvPG1, but the sequence from both ends of the P3 cDNA exactly matched internal sequences of VvPG1, suggesting that P3 is a partial-length cDNA of the VvPG1 clone. However, the poly(A) tail of P3 started 178 bases before that of the VvPG1 poly(A) tail, indicating that multiple polyadenylation signals (Fig. 5.9) are used during mRNA processing. The P5 cDNA was 65 bp shorter than VvPG1 at the 5' end and the poly(A) tail started 15 bp earlier than in the VvPG1 sequence. In addition to these differences, the same amino acid that was absent from the P2 cDNA was also missing from the P5 cDNA, and four base changes were found in the 5' untranslated sequence of P5 when compared to VvPG1. These results suggest that there are at least two PGase genes expressed in ripening grapes and that different polyadenylation sites are used.

Alignment of the VvPG1 sequence with other plant PGases shows a relatively high level of similarity, especially within the central region of the proteins (Fig. 5.11). VvPG1 is most similar to the kiwifruit PGase, showing 64.6% positional identity and 77.7% similarity at the amino acid level. When the deduced amino acid sequence of VvPG1 was aligned with other plant amino acid sequences, it could be seen that the VvPG1 cDNA was probably full-length (Fig. 5.11).

5.3.4 Isolation of XET cDNAs

The berry cDNA library was screened with a probe that encoded a partial-length XET cDNA from kiwifruit. Nine strongly hybridizing plaques were isolated and six were taken through secondary screening. Five clones were partially sequenced but none were related to other plant XET sequences. One clone was similar to a heat shock protein (plastid-specific HSP90 homologue) from rye (Schmitz *et al.*, 1996).

Following the failure to isolate an XET cDNA from the cDNA library, degenerate primers were designed against highly conserved regions of XET amino acid sequences

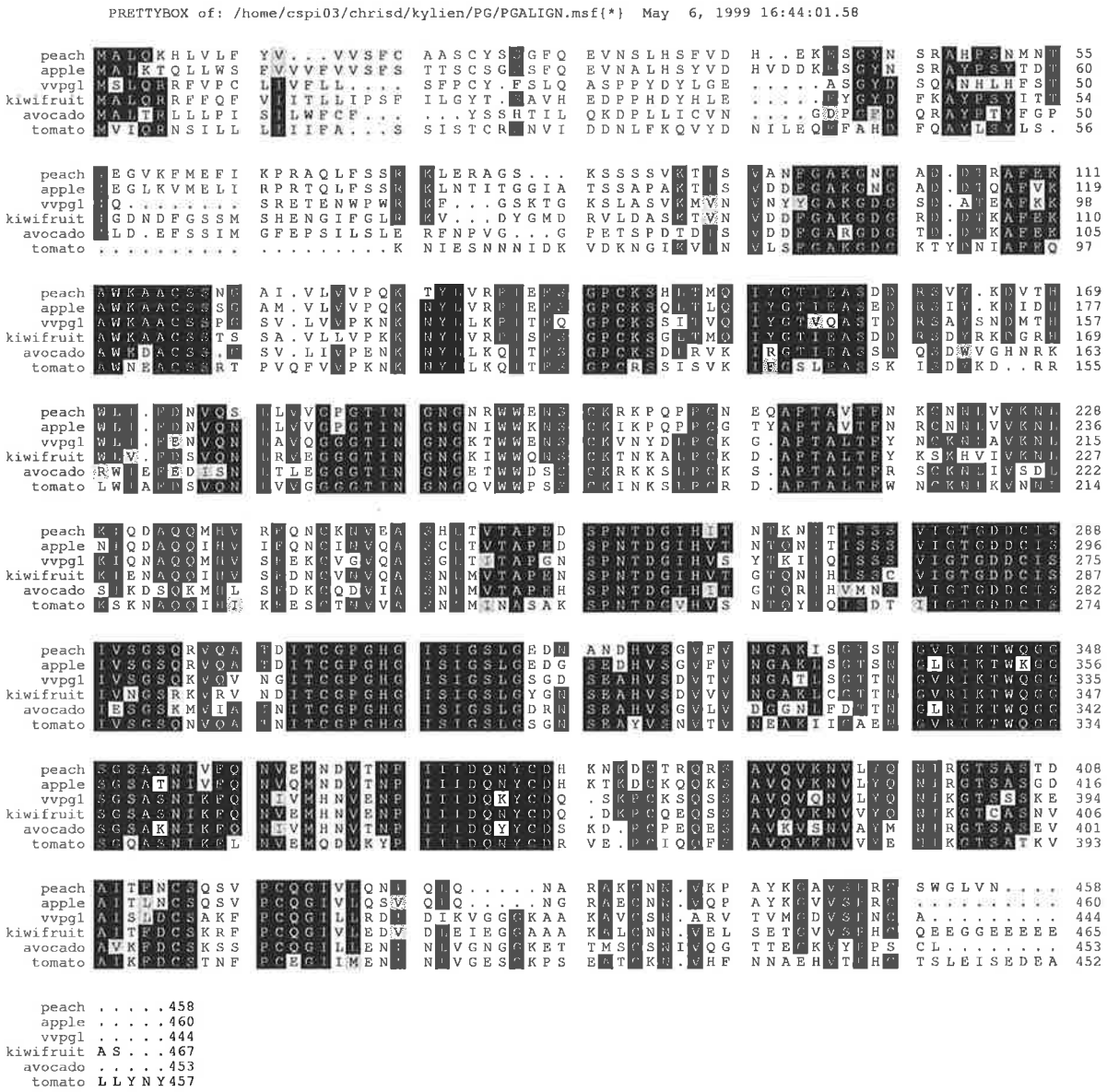

Figure 5.11 Alignment of the deduced amino acid sequence of the grape clone VvPG1 with the amino acid sequences of other plant PGases using the PILEUP and PRETTYBOX programs (Genetics Computer Group, Madison, WI). Residues that are identical in at least four of the six sequences are highlighted in black and residues that are similar are highlighted in grey. The EMBL accession numbers of the amino acid sequences are X77231 (peach), L27743 (apple), L12019 (kiwifruit), L06094 (avocado), and X04583 (tomato).

from other plants. These primers were used in PCR with single-strand cDNA made from poly(A^+)-RNA isolated from 42 and 70 dpa berries. A DNA band of approximately 500 bp was detected using the 70 dpa berry cDNA as a template, and this band was isolated and subsequently cloned. After transformation, colonies were selected and the plasmid DNA isolated. When the various clones were digested with restriction enzymes, three different banding patterns were observed. Subsequently, cDNAs from three clones, representing each of the different banding patterns, were partially sequenced and all were found to have sequence similar to other plant XETs (Table 5.3). These sequences comprised two groups. One cDNA (XET1) was closely related to a soybean XET, while the best match for the other two cDNAs were with a kiwifruit XET. This indicated that at least two genes encode XET isoforms in grapes. Two partial-length cDNAs representing the two divergent sequences were fully sequenced and were named VvXET1 and VvXET2 (Table 5.3).

Alignments of the nucleotide sequences and the deduced amino acid sequences of the two XET cDNAs are shown in Figures 5.12A and 5.12B, respectively. An overall sequence comparison reveals that VvXET1 and VvXET2 have 59.4% identity at the nucleotide level, and 59.3% identity and 71.3% similarity at the amino acid level. VvXET2 has an insertion of nine amino acids which is not present in VvXET1. Both XETs have a potential N-glycosylation site (Smith *et al.*, 1985) at a similar position (Fig. 5.12B).

The deduced amino sequences of VvXET1 and VvXET2 were aligned with a selection of other plant XETs, as shown in Figure 5.13. VvXET1 is most similar to the soybean XET with 82.7% identity and 90.7% similarity at the amino acid level, whereas VvXET2 shares the highest similarity with kiwifruit XET, with 88.7% identity and 93.7% similarity at the amino acid level. A dendrogram was assembled using the VvXET1 and VvXET2 sequences and the majority of known plant XET sequences, which were truncated to match the corresponding grape XET sequences (Fig. 5.14).

Table 5.3 PCR results for the amplification of partial-length cDNAs encoding XETs and cellulases

PCR primers[1]	Clone name[2]	Best match
XET1F/XET1R	XET1 (VvXET1)	Soybean XET
XET1F/XET1R	XET2 (VvXET2)	Kiwifruit XET
XET1F/XET1R	XET3	Kiwifruit XET
(1F/5R) CEL3F/CEL4R	CEL1 (VvCEL1)	Tomato cellulase
(1F/4R) CEL2F/CEL4R	CEL2 (VvCEL2)	Poplar cellulase
(1F/4R) CEL2F/CEL4R	CEL3	Tomato cellulase
(1F/5R) CEL2F/CEL4R	CEL4	Tomato cellulase

[1] Primers in parenthesis are the initial primers used in the original PCR
[2] Names in parenthesis are the final names of the fully sequenced clones

A

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CTTGGGAAACCTAAGTGGAGATCCTTATATCCTCCACACCAATGTATTCATTCAAGGGAA 60
CTTGGGGAACAGGTCCGGCCAACCCTACATTTTACAGACAAATGTCTTCACGGGAGGAAA 60

GGGCAACAGGGAGCAACAATTCTACCTCTGGTTCGACCCCACTCGTAACTTCCACACCTA 120
AGGAGACAGAGAGCAGAGAATCTATCTTTGGTTTGACCCAACCAAAGCTTCTCACTCATA 120

CTCCATCGCCTGGAGCGCCCAACACATCATTTTGTTGGTGGACAACGTTCCCATAAGGCT 180
CTCAGTTCTATGGAATACGTTTCTGATAATATTCTTGGTGGACGATGTCCCAATAAGAGT 180

ATTCAAGAATGCAGAATCAATGGGGGTTCCCTTCCCAAAGAACCAGCCAATGAGGATATA 240
GTTCAAGAACAGCAAAGATTTGGGAGTTAAGTTTCCATTCGACCAACCCATGAAGATATA 240

CTCAAGCCTCTGGAATGCGGATGACTGGGCCACAAGAGGAGGGTTGGTGAAAACTGACTG 300
CTCAAGCCTGTGGAATGCGGATGACTGGGCCACAAGGGGTGGGCTGGAGAAGACCGACTG 300

GTCAAAGGCACCCTTCACAGCATACTACAGGAACTTCAGAGCCAGCACTTCCACCTCCAC 360
GTCCAAGGCTCCATTTGTGGCTTCGTACAAGAGTTTCCACGTGGATGGGTGTGAGGCTTC 360

AAGCACATTCTCTGACAGTGCCTTCCAGACC.........................CA 393
AGTGGAAGCCAAATTTTGTGCGACCCAGGGCAAGAGATGGTGGGACCAAAGGGAATTTCA 420

GGAACTTGATGCCTACGGCAGAAGAAGGCTGAGATGGGTTCAGAAGAACTTCATGATT   451
AGATCTCGACTCCCTTCAGTACAGGAGACTCGCCTGGGTCCGCCAGAAATACACCATC   478
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B

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LGNLSGDPYILHTNVFIQGKGNREQQFYLWFDPTRNFHTYSIAWSAQHIILLVDNVPIRL  60
LGNRSGQPYILQTNVFTGGKGDREQRIYLWFDPTKASHSYSVLWNTFLIIFLVDDVPIRV  60

FKNAESMGVPFPKNQPMRIYSSLWNADDWATRGGLVKTDWSKAPFTAYYRNFRASTSTST  120
FKNSKDLGVKFPFDQPMKIYSSLWNADDWATRGGLEKTDWSKAPFVASYKSFHVDGCEAS  120

STFSDSAFQT.........QELDAYGRRRLRWVQKNFMI 150
VEAKFCATQGKRWWDQREFQDLDSLQYRRLAWVRQKYTI 159
```

Figure 5.12 Comparison of the VvXET1 and VvXET2 sequences. Alignment of the nucleotide sequences **(A)** and deduced amino sequences **(B)** of VvXET1 and VvXET2. The shaded box indicates a potential glycosylation site.

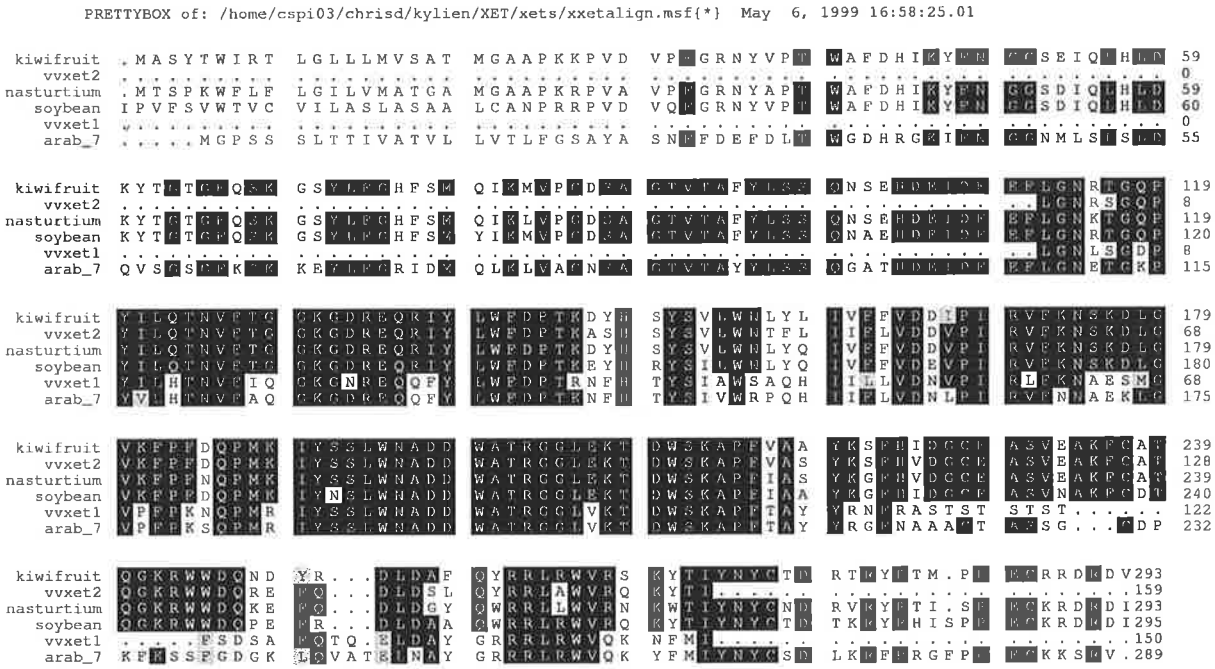

Figure 5.13 Alignment of the deduced amino acid sequences of the grape clones VvXET1 and VvXET2 with the amino acid sequences of other plant XETs using the PILEUP and PRETTYBOX programs (Genetics Computer Group, Madison, WI). Residues that are identical in at least four of the six sequences are highlighted in black and residues that are similar are highlighted in grey. The EMBL accession numbers of the amino acid sequences are L46792 (kiwifruit), L43094 (nasturtium), L22162 (soybean), and U43489 (arab_7).

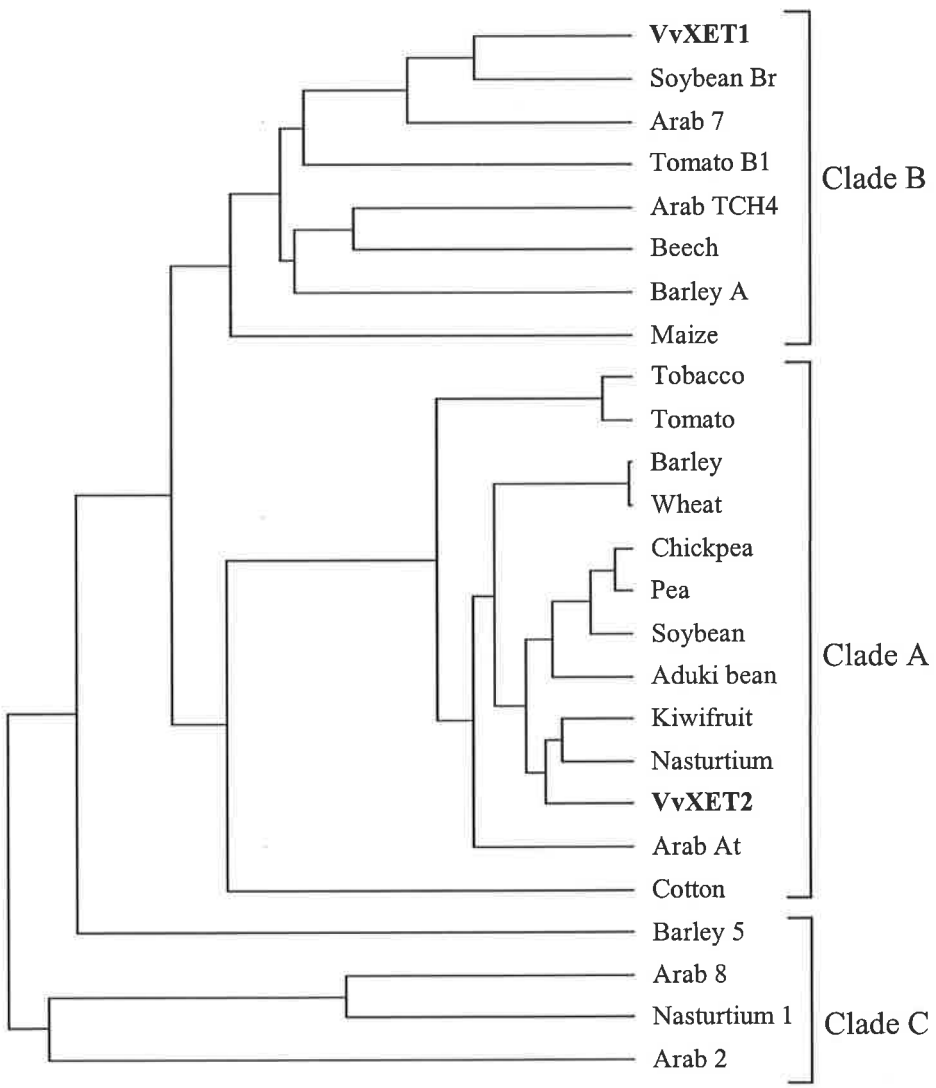

Figure 5.14 Phylogenetic tree of plant XETs. The unrooted tree was prepared by PILEUP and Growtree programs and shows the evolutionary distances. Each sequence was truncated to match the equivalent grape XET sequences before analysis. Accession numbers are L22162 (Soybean br), U43489 (Arab 7), X82685 (Tomato b1), U27609 (Arab tch4), X93174 (Barley a), D86730 (Tobacco), L46792 (Kiwifruit), L43094 (Nasturtium), D16456 (Tomato), D16455 (Soybean), D16454 (Arab at), X19659 (Barley), D16457 (Wheat), U15781 (Maize), D16458 (Adzuki bean), D88413 (Cotton), X93173 (Barley 5), X92975 (Arab 8), X68254 (Nasturtium 1), U43487 (Arab 2), E1354157 (Beech), O65734 (Chickpea) and D1035930 (Pea).

The grape XET isoforms are grouped differently; VvXET1 is in Clade B and VvXET2 is in Clade A.

5.3.5 Isolation of cellulase cDNAs

A heterologous probe for cellulase was not available to screen the berry cDNA library, so degenerate primers were designed against highly conserved regions of other plant cellulase sequences (Fig. 5.1). These primers were used in PCR, and cDNA made from poly(A^+)-RNA isolated from 70 dpa berries was used as the template. DNA bands of expected sizes ranging from approximately 450 bp to 800 bp were isolated for all combinations of primers. Nested PCR was performed on the original PCR products. DNA bands of expected sizes resulted and some of these were ligated into plasmid vectors and transformed into *E. coli*. Plasmids were isolated from selected colonies and analysed by restriction enzyme digestion. Four cDNAs were partially sequenced and were most closely related to either tomato or poplar cellulase cDNAs (Table 5.3). This suggests that at least two cellulase genes are expressed in grapes. The two cellulase cDNAs were fully sequenced and subsequently named VvCEL1 and VvCEL2 (Table 5.3).

The nucleotide sequences and deduced amino acid sequences of the grape cellulase cDNAs are aligned in Figures 5.15A and 5.15B, respectively. Comparison of the grape cellulase sequences revealed 61% identity at the nucleotide level, and 54% identity and 70.8% similarity at the amino acid level. Both contain a potential N-glycosylation site (Smith *et al.*, 1985) at a similar position (Fig. 5.15B). The grape cellulase cDNA fragments were aligned with other plant cellulases (Fig. 5.16). VvCEL1 was most similar to a tomato cellulase with 81% identity and 90.5% similarity at the amino acid level. The next most similar sequence was avocado, with 57.7% identity at the amino acid level. VvCEL2 was most similar to orange cellulase, showing 86.1% identity and 91.2% similarity at the amino acid level. A dendrogram of plant cellulases reveals that both VvCEL1 and VvCEL2 cellulase isoforms fall into the group of cellulases which

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                                        .         .         .         .         .         .
.................................GTGCTGCATGGTTGCACAAAGCCAC 25
                                 | ||  ||||| | ||||  ||  |
CTCCTACTCTGGATATCATGATGAGCTTCTCTGGGGGGCATCATGGATTCACAGGGCGTC 60

                                        .         .         .         .         .         .
CAGGAACCCCACCTTCTTGAGCTACATTCAAGTAAATGGCCAGACCCTTGTGGCTGATGA 85
 |  ||| || | |   ||   ||||| ||   ||||||||| ||  | |  || || ||
AAATAACGCCTCATATCTGGCTTACATCCAGTCAAATGGCCAAACAATGGGCGCGGACGA 120

                                        .         .         .         .         .         .
TTCTGACAATACATTTGGATGGGATAACAAGCATGTTGGAGCAAGGATTCTTCTCTCTAA 145
|  |||| |  | ||  | |||||  ||||||  | |||| ||| | | ||||| || ||
TGATGACTACTCCTTCAGTTGGGACGACAAGCGAGCTGGAACAAAGGTCCTTCTTTCCAA 180

                                        .         .         .         .         .         .
GGCATTCCTTCTCCAAAGGGTGCAGTCCCTCCATGATTACAAAGGTCATGCAGATAACTT 205
||  ||| |     |||      ||    | ||    || ||| | ||| |||| ||||
GGACTTCTTGGAGAAAAACACCGAGGAATTTCAGCTGTATAAACGACATTCAGACAACTA 240

                                        .         .         .         .         .         .
CATATGTTCTCTGGTCCCAGGAACTCCTTTCTCCCAAGCCCAGTACACCCCAGGTGGGTT 265
|||||| |||||| | ||||||||  |   ||  || ||||| ||||| ||||||||| |
CATATGCTCTCTGATTCCAGGAACATCCAGCTTTCAGGCCCAATACACTCCAGGTGGGCT 300

                                        .         .         .         .         .         .
GCTGTTCAAGATGAGTGACAGCAACATGCAGTATGTGACCTCCACTTCATTCCTGCTTGT 325
  | | |||   ||| || |||||  |||| |||||||| |||||  | ||||| ||  |
TTTCTACAAAGGGAGCGAGAGCAATCTGCAATATGTGACTTCCACAGCTTTCCTCCTCTT 360

                                        .         .         .         .         .         .
AACCTATGCCAAGTACTTAACTTCAGCCCACAAAGTTGTGAACTGCGGTGGAACTATTAT 385
||| || ||||||||  |||  |||         |   |     || ||     | | | |
AACATACGCCAAGTATCTAAGCTCAAATGGAGGATCCGCCTCATGTGGGACTTCAACTGT 420

                                        .         .
CACCCCAAAGAGGCTTCGAGTCATTGCT 413
| |  || |  ||||        | |||
CGCAGCAGAACGGCTCATCTCACTGGCT 448
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B

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                                        .         .         .         .         .         .
............AAWLHKATRNPTFLSYIQVNGQTLVADDSDNTFGWDNKHVGARILLSK 48
            |.|:|:|..|:.:|.||| ||||:.|||.| .|:||:|:.|.::||||
SYSGYHDELLWGASWIHRASNNASYLAYIQSNGQTMGADDDDYSFSWDDKRAGTKVLLSK 60

                                        .         .         .         .         .         .
AFLLQRVQSLHDYKGHADNFICSLVPGTPFSQAQYTPGGLLFKMSDSNMQYVTSTSFLLV 108
.|| ...:.:: || |.||:||||:|||.  |||||||||::| |:||:||||||.|||:
DFLEKNTEEFQLYKRHSDNYICSLIPGTSSFQAQYTPGGLFYKGSESNLQYVTSTAFLLL 120

                                        .         .
TYAKYLTSAHKVVNCGGTIITPKRLRVIA 137
||||||.|.   ..||...:.:.||  :|
TYAKYLSSNGGSASCGTSTVAAERLISLA 149
```

Figure 5.15 Comparison of the VvCEL1 and VvCEL2 sequences. Alignment of the nucleotide sequences **(A)** and deduced amino acid sequences **(B)** of VvCEL1 and VvCEL2. The shaded box indicates a potential glycosylation site.

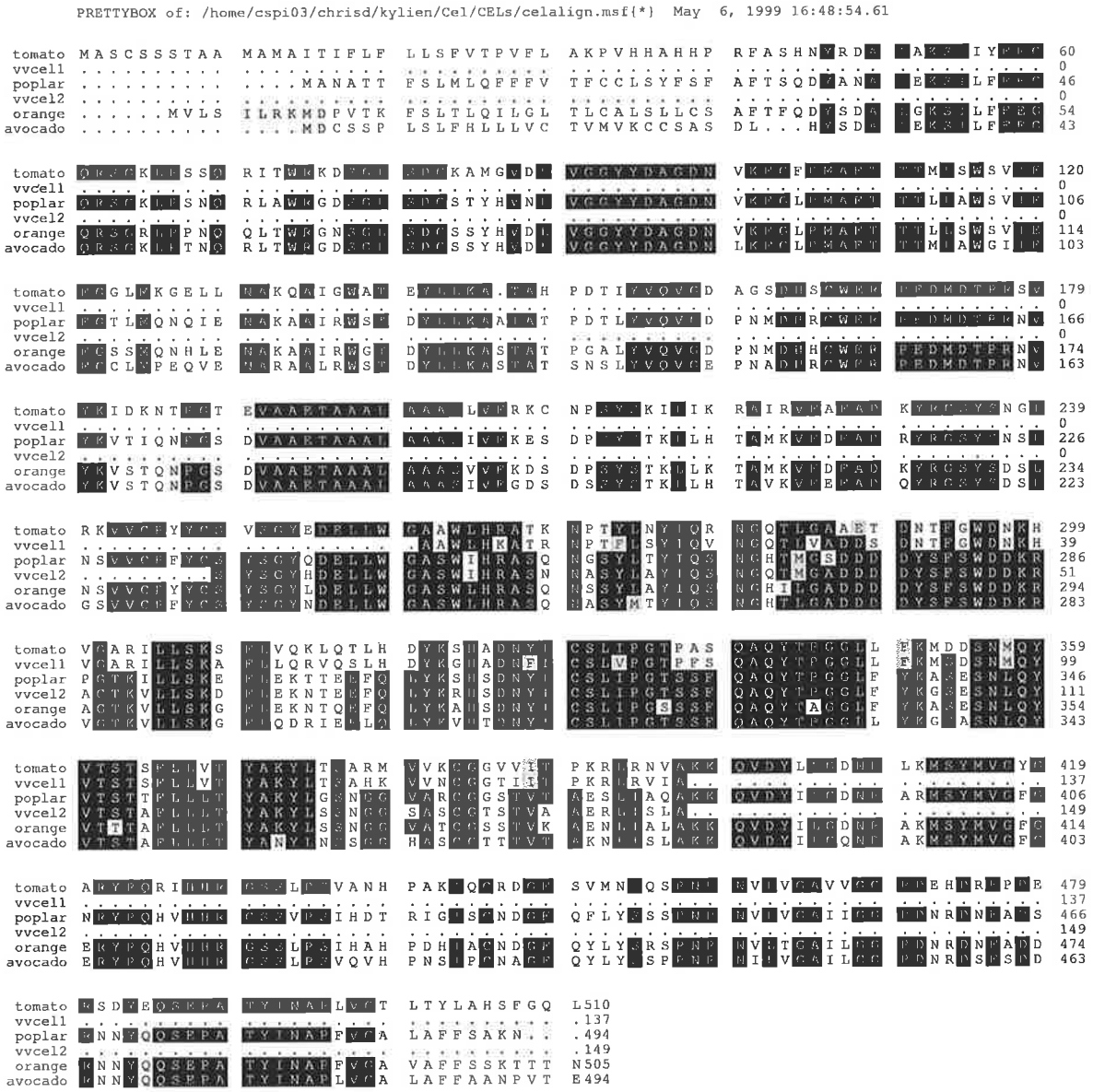

Figure 5.16 Alignment of the deduced amino acid sequence of the grape clones VvCEL1 and VvCEL2 with the amino acid sequences of other plant cellulases using the PILEUP and PRETTYBOX programs (Genetics Computer Group, Madison, WI). Residues that are identical in at least four of the six sequences are highlighted in black and residues that are similar are highlighted in grey. The EMBL accession numbers of the amino acid sequences are U20590 (tomato), D32166 (poplar), AF000135 (orange), and M17634 (avocado).

are involved in fruit ripening and expansion of young tissue groups, as reported by Catalá *et al.* (1997), although the grape cellulases are in different subgroupings within that group (Fig. 5.17).

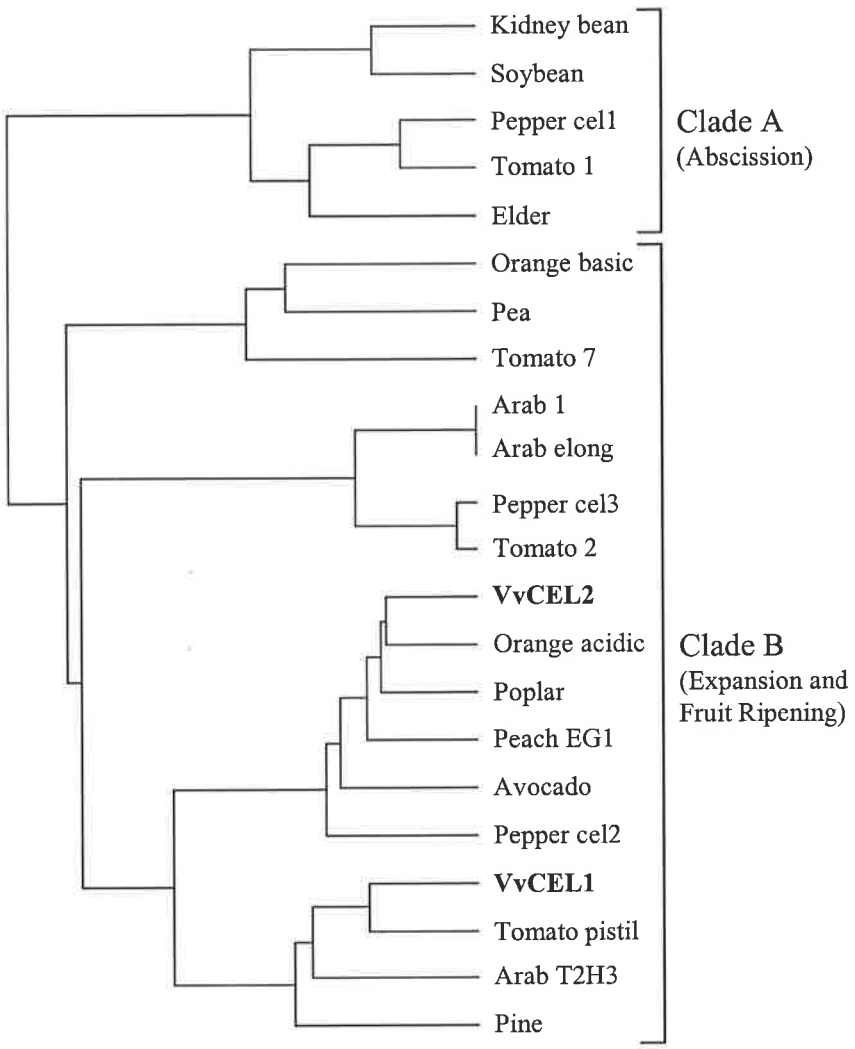

Figure 5.17 Phylogenetic tree of plant cellulases. The unrooted tree was prepared by PILEUP and Growtree programs and shows the evolutionary distances. Each sequence was truncated to match the equivalent grape cellulase sequences before analysis. Accession numbers are AF000135 (Orange acidic), D32166 (Poplar), M17634 (Avocado), X96853 (Peach eg1), X97190 (Pepper ccel2), U20590 (Tomato pistil), L41046 (Pea), U13055 (Tomato 2), Af000136 (Orange basic), X98544 (Arab elong), Y11268 (Tomato 7), X83711 (Pepper ccx3), X97189 (Pepper ccel3, X98543 (Arab 1), X74290 (Elder), X87323 (Pepper cel1), U00730 (Soybean), U13054 (Tomato 1), U34754 (Kidney bean), O64401 (Pine), O81416 (Arab T2H3) and U17888 (Arab).

5.4 Discussion

In this study, full- or partial-length cDNAs encoding grape β-galactosidase, PME, PGases, XETs and cellulases have been cloned. These cDNAs were identified by sequence homology to other plant cDNA clones. In other plant systems for all five enzyme classes, homologous cDNAs or genes have been isolated, the corresponding protein has been purified and the enzymatic activity confirmed. For example, a protein showing PGase activity was isolated from tomato, 80% of the protein was subsequently sequenced and the corresponding cDNA cloned and fully sequenced (Sheehy *et al.*, 1987). Moreover, heterologous expression of cDNAs in *E. coli* has allowed the characterization of some enzymes, such as tomato XET B2 (Arrowsmith and de Silva, 1995), confirming the links between enzyme activity and predicted proteins encoded by cDNAs. A number of relatively divergent protein sequences (i.e. less than 50% similarity), in some cases from the same plant species, have been shown to belong to the same enzyme class (Lashbrook *et al.*, 1994; Xu *et al.*, 1996). For example, two XETs (XTR2 and XTR6) from *Arabidopsis* were found to only have 37% identity at the amino acid level (Xu *et al.*, 1996).

β-Galactosidase cDNA

Only a relatively small number of genes and/or cDNAs encoding plant β-galactosidases have been isolated. In fruit, cDNAs encoding β-galactosidases have been isolated from apple (Ross *et al.*, 1994), papaya (Othman *et al.*, 1998), mango (unpublished, EMBL O04976) and tomato (Carey *et al.*, 1995). When the deduced amino acid sequence of VvBG1 is aligned against amino acid sequences of other plant β-galactosidases, it is evident that the plant β-galactosidases are very highly conserved, including the predicted cleavage site for the NH_2-terminal signal peptides (Fig. 5.4). The NH_2-terminal signal peptide of plant proteins typically contains 15-25 amino acids and consists of three structural regions: a basic NH_2-terminal region, a central hydrophobic region and a more polar region at the COOH-terminal end (von Heijne,

1985). Such a signal peptide is necessary to target the protein to the endoplasmic reticulum and hence to the secretory system of the cell (Chrispeels, 1991). Using the "(-3,-1)-rule", the most likely cleavage site of the signal sequence can be predicted from the cDNA sequence information (von Heijne, 1986).

PME cDNA

The alignment of PMEs (Fig. 5.7) reveals that PMEs tend to be highly conserved in the COOH-terminal region of the protein and not so highly conserved in the NH_2-terminal region. It is formally possible that the VvPME1 cDNA may not be full-length and therefore the prediction that the first in-frame Met is the translation start point may not be correct. However, the amino acid sequence corresponding to the putative signal peptide has characteristics normally attributed to signal peptides that target proteins to the ER (Fig. 5.6 compared with von Heijne, 1985).

In addition to the removal of the signal peptide, VvPME1 may also undergo further post-translational processing. For example, protein sequences deduced from two PME cDNAs isolated from tomato have NH_2-terminal extensions of 229 and 233 amino acids respectively (Hall *et al.*, 1994). The exact function of this NH_2-terminal extension is not known but it may be involved in the stabilization of the tomato PMEs, or in the inhibition of enzyme activity during transport or targeting of the protein to the cell surface or cell wall (Hall *et al.*, 1994). The predicted molecular mass of 54 kDa for the mature protein of VvPME1 is therefore more likely to correspond to the molecular weight of an unprocessed pre-protein form of the enzyme and a long NH_2-terminal extension is expected to be removed during post-translational modification.

One noteworthy feature of the grape VvPME1 amino acid sequence is that it has seven possible N-glycosylation sites, recognized by the consensus motif Asn-X-Ser/Thr (Smith *et al.*, 1985). The carbohydrate moiety of glycoproteins has been implicated in stability, molecular targeting and cell-cell recognition (Faye, 1988; Stryer, 1988). Similarly, nine potential N-glycosylation sites have been found for peach PME, seven of which are within the putative mature peptide (Glover *et al.*, 1996). However, three

peach PMEs purified from ripe fruit show no indication that they are glycosylated (Glover and Brady, 1994), suggesting that potential glycosylation sites are not necessarily utilised.

PGase cDNAs

Four different PGase cDNAs were isolated from grape, one (VvPG1) was fully sequenced and the others, with the exception of P2, were sequenced from both ends (Fig. 5.10). One notable feature of the PGase cDNAs was the different lengths of the 3' untranslated sequence. The P3 poly(A) tail started 178 bp earlier and the P5 poly(A) tail started 15 bp earlier than the VvPG1 poly(A) tail. The AATAAA polyadenylation signal sequence tends to be very highly conserved for animals (Wahle and Keller, 1992), but plant poly(A) signal sequences frequently deviate from the consensus (Heidecker and Messing, 1986). VvPG1 did not contain the conserved sequence. However, there were a number of possible sites in the VvPG1 cDNA sequence that may act as polyadenylation signals (Fig. 5.9). The presence of multiple polyadenylation sites is not uncommon. For example, the ribulose bisphosphate carboxylase and chlorophyll a/b binding protein genes from petunia and the bronze gene from maize show multiple polyadenylation sites (Dean *et al.*, 1986).

Long NH_2-leader sequences are found for some PGases. For example, the tomato PGase isoform, PG2A, has a 71 amino acid pre-sequence cleaved to give the mature protein (Grierson *et al.*, 1986), and this is believed to occur for a number of other fruit PGases (Kutsunai *et al.*, 1993; Atkinson, 1994; Hadfield *et al.*, 1998). However, not all PGases have this NH_2-terminal leader sequence. For example, the tomato abscission PGases (TAPG1, TAPG2 and TAPG4) have no NH_2-terminal extensions (Kalaitzis *et al.*, 1995; Kalaitzis *et al.*, 1997), nor does a peach PGase (PRF5) (Lester *et al.*, 1994) and two melon PGases (Hadfield *et al.*, 1998). From the alignment of VvPG1 with other plant PGases (Fig. 5.11), VvPG1 has similar features to the PGases that have this NH_2-terminal extension, suggesting that VvPG1 may undergo similar proteolytic processing.

XET cDNAs

The detection of two cDNA sequences for XETs in grape suggests the presence of an XET gene family. When the deduced amino acid sequences of the partial-length cDNAs were aligned with other fruit XETs, it was evident that the two grape XET isoforms can be classified into two different clades; VvXET1 falls within Clade B and VvXET2 within Clade A (Fig. 5.14). These clades are identified by particular conserved regions and not by defined expressions patterns or tissue locations (Xu *et al.*, 1996). Clade A XETs have a highly conserved region towards the COOH-terminus, and Clade C XETs are as for Clade A except they have an additional three amino acid residues upstream of the conserved region and two or five additional amino acids following the conserved region. Clade B contains the remaining XETs (Xu *et al.*, 1996). The divergence between the two grape XET sequences is not unusual. In the *Arabidopsis* XET family, members are found in each of the three clades (Fig. 5.14).

Cellulase cDNAs

Cellulases have been classified into two general groups; one comprises cellulases found in fruit ripening and young expanding tissues, while the other includes cellulases that are associated with abscission (Catalá *et al.*, 1997). The phylogenetic tree of plant cellulases shows that the grape cellulases are in the same large group that are associated with fruit ripening and expanding young tissues (Fig. 5.17). A closer examination of the tree suggests that there may be two subgroups within Clade B and, if so, VvCEL1 and VvCEL2 are in different subgroups. However, there is no easily distinguishing feature that separates these two subgroups, other than evolutionary distances projected by the phylogenetic tree.

Conclusions

In summary, cDNAs have been isolated and characterized from grape berries that encode the enzymes β-galactosidase, PME, PGase, XET and cellulase. Some were isolated by screening a cDNA library and are near full-length. Other cDNAs were isolated using PCR, and are 400-500 bp in length. All isolated cDNAs have high

sequence similarity, both at the nucleotide and amino acid level, to other cDNAs encoding the respective enzymes. A description of the cDNAs is summarized in Table 5.4.

Table 5.4 Description of cDNAs

cDNA name	Homology to	Method of isolation	Length (bp)
VvBG1	β-Galactosidase	cDNA library	2855
VvPME1	Pectin methylesterase	cDNA library	1827
VvPG1	Polygalacturonase	cDNA library	1609
VvXET1	Xyloglucan endotransglycosylase	PCR	451
VvXET2	Xyloglucan endotransglycosylase	PCR	478
VvCEL1	Cellulase	PCR	413
VvCEL2	Cellulase	PCR	448

The primary reason for isolating the grape cDNAs in this present study was to use the cDNAs as molecular probes to monitor transcriptional activity of genes encoding the five respective enzymes in developing grape berries. However, the isolation here of cDNAs encoding PGase and cellulase, neither of which were detected in the enzyme assays described in Chapter 4, already indicate that some transcription of PGase and cellulase genes were occurring in ripening grape berries. Transcriptional activity of the genes was investigated further in the experiments described in Chapter 6. The compiled information is subsequently related back to the detectable enzyme activities and to the modification of cell wall polysaccharides in ripening grape berries.

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Chapter 6

Gene Copy Number and Transcriptional Activity of Genes encoding Cell Wall-Modifying Enzymes

6.1 Introduction

In Chapter 5, the isolation of seven grape berry cDNAs encoding the enzymes β-galactosidase, PME, PGase, XET and cellulase was described. To study the spatial and temporal transcriptional patterns of the genes encoding each of these enzymes in grape berries and other grapevine tissues, the seven cDNAs could be used as probes in Northern hybridization analyses.

A major objective was to determine if any of the genes encoding cell wall-modifying enzymes showed upregulation of transcription when the berries begin to soften around veraison. In other fruit, increased transcription of certain genes encoding these enzymes occurs during ripening. For example, an mRNA transcript encoding the gene β-galactosidase is only detected in ripening apples and tomatoes (Ross *et al.*, 1994; Carey *et al.*, 1995) and increased levels of cellulase mRNA are found in ripe compared to unripe avocados and tomatoes (Christoffersen *et al.*, 1984; Lashbrook *et al.*, 1994). Some of these enzymes may also be associated with other physiological processes, such as rapid cell expansion (Smith *et al.*, 1996; Brummell *et al.*, 1997) and abscission (Kalaitzis *et al.*, 1995; Trainotti *et al.*, 1997), and may therefore be found in plant tissues other than fruit.

When studying the expression patterns of genes at the transcriptional level in grape berries, comparisons between the two cultivars Gordo and Shiraz are of interest because they soften to different extents during ripening. As described in Chapter 3, Gordo berries deform to an average of 1.8 mm at maturity, whereas the softer Shiraz berries have a deformability of greater than 3 mm when ripe (Boss *et al.*, 1996). Any differences observed in the transcriptional activity of the genes, assuming active enzymes are produced, might be correlated with the difference in softening.

However, interpretation of Northern analyses with respect to the genes that are being expressed relies on the availability of gene-specific probes for hybridization. Essential preliminary information is therefore required to define how many genes are recognised

by the probe to be used. Southern hybridization analyses were therefore performed to estimate the number of β-galactosidase, PME, PGase, XET and cellulase genes present in the grapevine genome.

Northern hybridization analyses were subsequently performed to compare the expression patterns of these genes at the transcriptional level in developing berries of Gordo and Shiraz, as well as in other Shiraz grapevine tissues. The determination of the transcriptional expression patterns of the genes allowed a comparison between known enzyme activities, reported in either Chapter 4 or in other studies, the respective mRNA levels, and the changes in cell wall polysaccharides described in Chapter 3. In making these comparisons, it must be emphasised that while steady-state mRNA levels might be related to transcriptional activity of corresponding genes, they do not necessarily reflect the final levels of enzyme expressed in the particular tissue from which the RNA was extracted. Turnover rates, translational efficiency and other factors will affect final enzyme activity.

6.2 Materials and Methods

Unless otherwise stated, all common molecular biology techniques were performed, and buffers prepared, as described by Sambrook *et al.* (1989).

6.2.1 Materials

6.2.1.1 Plant material

Total RNA was extracted from the five Gordo berry samples described in Section 4.2.1, i.e. 41, 63, 86, 105 and 126 dpa. Genomic DNA was extracted from Gordo leaves collected from the Waite Vineyard, Waite Campus, University of Adelaide.

6.2.1.2 Shiraz RNA

Flowers, berries (8 samples, 14 to 112 dpa), leaves at three stages of maturity (unexpanded, partly expanded and fully expanded), and seeds from *Vitis vinifera* L. cv Shiraz were collected from John Harvey's Slate Creek vineyard (Willunga, South Australia) in the 1993/1994 and 1994/1995 seasons. Shiraz root tips were obtained from canes that had been planted in pots. Total RNA was isolated by Dr. Chris Davies (CSIRO, Plant Industry, Horticulture Unit, Adelaide, South Australia) using the procedure of Rezaian and Krake (1987) as modified by Rathjen and Robinson (1992).

The *Vitis* ribosomal DNA repeat unit (pVvc-1) (Thomas *et al.*, 1993), which was used as a ribosomal RNA probe, was a kind gift from Dr. Mark Thomas (CSIRO, Plant Industry, Horticulture Unit, Adelaide, South Australia).

6.2.2 Extraction of genomic DNA

Genomic DNA was extracted from young Gordo leaves using the procedure described by Thomas *et al.* (1993). Two grams of ground leaves, prepared by placing the leaves in liquid nitrogen and grinding in a coffee grinder, were placed in a 50 ml tube containing 25 ml ice-cold 200 mM Tris-HCl buffer, pH 7.6, containing 250 mM NaCl, 2.5% (w/v) polyvinylpyrrolidone (PVP) 40,000, 50 mM EDTA and 0.1 % (v/v)

β-mercaptoethanol (BME). The tube was centrifuged for 10 min at 3000g at 4°C in swing-out buckets and the pellet resuspended in 5 ml cold 200 mM Tris-HCl buffer, pH 8.0, containing 500 mM NaCl, 2.5% (w/v) PVP 40,000, 50 mM EDTA, 3% (v/v) Sarkosyl, 20% (v/v) ethanol and 1% (v/v) BME. The tube was incubated for 30 min at 65°C with occasional shaking and 5 ml chloroform:isoamyl alcohol (24:1, v/v) was added. The suspension was mixed by vortexing and centrifuged for 10 min at 2500g. The supernatant was transferred to a Corex tube, 0.54 vol isopropanol was added, and the solution was gently mixed and left at room temperature for 10-15 min. After centrifugation for 10 min at 6000g, the pellet was washed with 70% (v/v) ethanol, dried and resuspended in 600 μl TE. The DNA was transferred to an Eppendorf tube, 3 μl 20 mg/ml RNase A was added, and the mixture was incubated for 30 min at 37°C. The tube was centrifuged for 10 min at 10,000g and the supernatant transferred to a clean Eppendorf tube. To the tube, 0.5 vol 7.5 M ammonium acetate was added, the tube was centrifuged for 15 min at 12,000g and the supernatant transferred to a clean Eppendorf tube. Isopropanol (0.54 vol) was added, mixed gently and left at room temperature for 10 min. The tube was centrifuged for 10 min at 12,000g, and the pellet washed with 70% (v/v) ethanol. The pellet was dried and resuspended in 200 μl TE. The absorbance was measured at 260 nm and the DNA concentration calculated assuming one Ab_{260} unit equals 50 μg DNA/ml using a 1 cm path length..

6.2.3 Extraction of total RNA

Total RNA was extracted from Gordo grape berries at the five stages of development, using the method of Levi *et al.* (1992) as modified by Tattersall *et al.* (1997). The extraction buffer contained 500 mM Tris-HCl buffer, pH 8.5, 1.5% (w/v) lithium dodecyl sulfate, 1.5% (w/v) Nonidet P-40 (ICN Pharmaceuticals, Costa Mesa, CA), 1.5% (w/v) sodium deoxycholate, 300 mM lithium chloride, 10 mM EDTA, 2% (w/v) PVP 40,000, 2% (w/v) PVPP, 10 mM DTT and 75 mM BME. The PVP, PVPP, DTT and BME were added to the extraction buffer after autoclaving.

Grape tissue (Gordo) was ground using liquid nitrogen and a coffee grinder. Ground tissue (5 g) was added to 10 ml ice-cold extraction buffer. Two vol chloroform was added and the tube shaken vigorously for 5 min. After centrifugation for 15 min at 2500g, the upper aqueous phase was transferred to a new tube and 2 vol chloroform was added and the above step repeated. The upper aqueous phase was transferred to a Corex tube and 500 µl 3 M NaCl and 10 ml ethanol added, mixed and stored at -20°C for 1 h. The tube was centrifuged for 15 min at 4000g, and the pellet resuspended in 500 µl TE 1 (50 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA). The suspension was transferred to an Eppendorf tube and clarified by centrifugation for 10 min at 12,000g. The supernatant was transferred to a new Eppendorf tube, and 500 µl 3 M NaCl and 275 µl isopropanol was added, the solution was mixed and stored at -20°C for 1 h. After centrifugation for 10 min at 12,000g, the pellet was washed with 70% (v/v) ethanol and dissolved in 300 µl TE 1. Lithium chloride (100 µl, 8M) was added, the tube incubated for 12 h at 4°C and centrifuged for 10 min at 12,000g. The pellet was resuspended in 300 µl TE 1, and 450 µl 5 M potassium acetate was added and incubated on ice for approximately 5 h. The tube was centrifuged for 10 min at 12,000g, and the resulting pellet resuspended in 300 µl TE 1. The RNA was precipitated by the addition of 30 µl 3 M NaCl and 660 µl ethanol and held for 1 h at -20°C. After centrifugation for 10 min at 12,000g, the pellet was washed with 70% ethanol and dried. The RNA pellet was resuspended in 50 µl TE 2 (10 mM Tris-HCl buffer, pH 8.5, 1 mM EDTA) and the concentration of RNA calculated from the measurement of the absorbance at 260nm assuming one Ab_{260} equals 50 µg RNA/ml using a 1 cm path length. The purity and quantity of RNA was checked by separation on a 1.2% (w/v) agarose gel.

6.2.4 Preparation of DNA for probes

Fragments of the full-length cDNAs encoding β-galactosidase, PME and PGase were used as probes for the Southern and Northern analyses (Table 6.1). The entire PCR products of the XET and cellulase cDNAs were used as probes. The melting

Table 6.1 T_ms and mismatches at 65°C for each probe used for Southern and Northern analyses

Probe	Probe size	2x SSC		1x SSC		0.1x SSC	
	(bp)	Tm (°C)	% mismatch	Tm (°C)	% mismatch	Tm (°C)	% mismatch
βG1f	624 (3'end)	91	26	86	21	70	5
PME1f	669 (5' end)	93	28	88	23	71	6
PG1f	440 (3' end)	88	23	83	18	67	2
XET1f	492	94	29	89	24	72	7
XET2f	519	93	28	88	23	72	7
CEL1f	446	93	28	88	23	72	7
CEL2f	479	93	28	88	23	71	6

temperatures (T_m) for each of the probes were calculated using the following equation (Meinkoth and Wahl, 1984) and the percentage mismatch at 65°C calculated (i.e. T_m minus 65) for each probe (Table 6.1).

$$T_m = 81.5 + 16.6 (\log_{10}[Na^+]) + 0.41 (\%GC) - (500/N)$$

where N = chain length (bp), GC = guanine and cytosine nucleotides

Probes were prepared as described in Section 5.2.3 using the Gigaprime DNA labelling kit. Approximately 100 ng DNA for each probe was labelled with [α-P^{32}]-dATP. The labelled DNA was separated from the unincorporated label on a 1 ml Sepharose G-50 column.

6.2.5 Southern hybridization analysis

Genomic DNA (10 μg per sample) was digested with restriction enzymes for approximately 4 h at 37°C. The digested genomic DNA was separated on a 0.8% (w/v) TAE buffered agarose gel. The DNA was transferred onto a Zetaprobe membrane (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions for alkaline blotting. The gel was soaked in 0.25 M HCl for 10-15 min to depurinate the DNA and rinsed thoroughly with distilled water. The DNA was transferred from the gel onto Zetaprobe membrane for 16 h, using 0.4 M NaOH as the transfer solution. The membrane was rinsed in 2x SSC buffer and allowed to air dry before the DNA was fixed onto the membrane using a UV crosslinker (Stratagene).

The membrane was prehybridized in hybridization buffer as described in Section 5.2.4. The [^{32}P]-radiolabelled DNA probe was heated at 95°C for 5 min, added to the hybridization bottle and hybridized at 65°C for 16 h. The membrane was washed twice at low stringency in a solution of 2x SSC and 0.1% (w/v) SDS for 15 min at 65°C. The wet membrane was wrapped in plastic and placed in a cassette containing a phosphor imaging screen (Fujifilm). The labelled DNA was analysed using a phosphor imager (Storm 860, Molecular Dynamics, Sunnyvale, CA) and ImageQuant software

(Molecular Dynamics). If multiple bands were seen in each lane for the digested DNA, then the wet membrane was washed to a higher stringency at 0.1% SSC and 0.1% (w/v) SDS at 65°C for 2x 15 min and again analysed using the phosphor imager.

6.2.6 Northern hybridization analysis

Total RNA (2.8 μg) in 6 μl was added to 18 μl sample buffer (11.4 μl deionized formamide, 4.3 μl 40% v/v formaldehyde, 2.9 μl 10x Northern buffer [50 mM sodium acetate buffer, pH 7.0, 200 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), 10 mM EDTA,] and 0.01% w/v bromophenyl blue) to give a total volume of 24 μl. The samples were heated at 65°C for 10 min and cooled on ice. The RNA samples were resolved on a 1.2% (w/v) agarose gel containing 8% (v/v) formaldehyde and 1x Northern buffer. RNA markers were denatured as for the total RNA and were loaded with the RNA samples. To check equal loadings of total RNA from the Shiraz and Gordo berries, 1.5 μg of RNA isolated from each stage of development was separated on a 1.2% (w/v) agarose gel and stained with ethidium bromide.

The total RNA was transferred from the gels to Zetaprobe membranes as described for the Southern analysis (Section 6.2.5). However, the gel was not treated with HCl and 50 mM NaOH was used in place of 0.4 M NaOH during the transfer process. Hybridization conditions and probes were the same as for Southern analysis. The membrane was washed at low stringency in 2x SSC and 0.1% (w/v) SDS at 65°C for 2x 15 min, and at medium stringency in 1x SSC and 0.1% (w/v) SDS at 65°C for 1x 15 min. The membrane was placed wet into plastic wrap and analysed using the phosphor imager. If the results of the Southern analysis showed multiple bands, membranes were washed at high stringency in 0.1x SSC and 0.1% (w/v) SDS at 65°C for 2x 15 min. The results were analysed using the phosphor imager. To ensure equal loadings, a Northern hybridization membrane of the Shiraz berry developmental series was probed with radiolabelled ribosomal DNA, washed at low stringency and analysed using the phosphor imager.

6.3 Results

6.3.1 Southern analyses

Gordo genomic DNA was studied by Southern hybridization analysis using fragments of the cDNAs as probes (Table 6.1). Following hybridization, washes were performed using either a low stringency wash of 2x SSC at 65°C, or a high stringency wash of 0.1x SSC at 65°C, which allowed for 23-29% and 2-7% nucleotide mismatches respectively, as shown in Table 6.1. Detection of multiple bands may be due not only to the presence of very similar genes, but also to the existence of restriction sites within introns, sequence polymorphisms between the Gordo and Shiraz DNA, or incomplete digestion by the restriction enzymes.

6.3.1.1 β-Galactosidase

Using the 3' end of the VvBG1 cDNA (BG1f) as a probe under low stringency conditions, Southern analysis revealed that VvBG1 is encoded by a single gene (Fig. 6.1). Higher stringency washes did not remove the faint band seen in the *Eco*RI cut lane.

6.3.1.2 PME

The probe, PME1f, was employed in Southern hybridization analysis and identical banding patterns were obtained using both high and low stringency washes. The restriction enzyme *Sac*I cuts the cDNA probe into two fragments of 400 bp and 270 bp, and this presumably accounts for one of the extra bands seen in the *Sac*I lane (Fig. 6.2). Additionally, incomplete digestion of the DNA by *Sac*I may account for one of the fainter bands. VvPME1 also appears to have internal *Eco*RV and *Hind*III sites. It therefore appears that VvPME1 is encoded by one or possibly two genes.

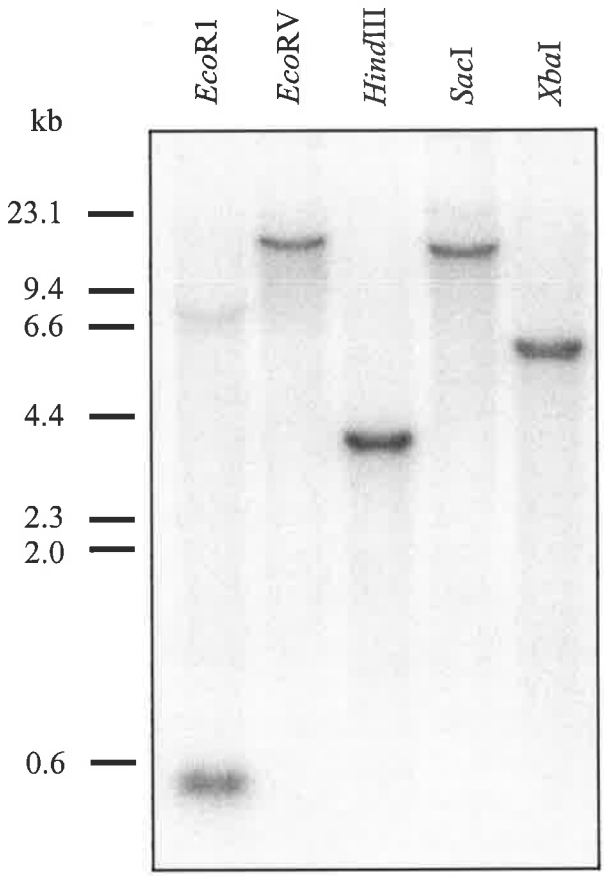

Figure 6.1 Southern hybridization analysis of VvBG1. Gordo genomic DNA (10 μg) digested with the indicated restriction enzymes was loaded in each lane. The blot was probed with a 624 bp fragment of the VvBG1 clone and washed at low stringency. DNA size markers are shown on the left.

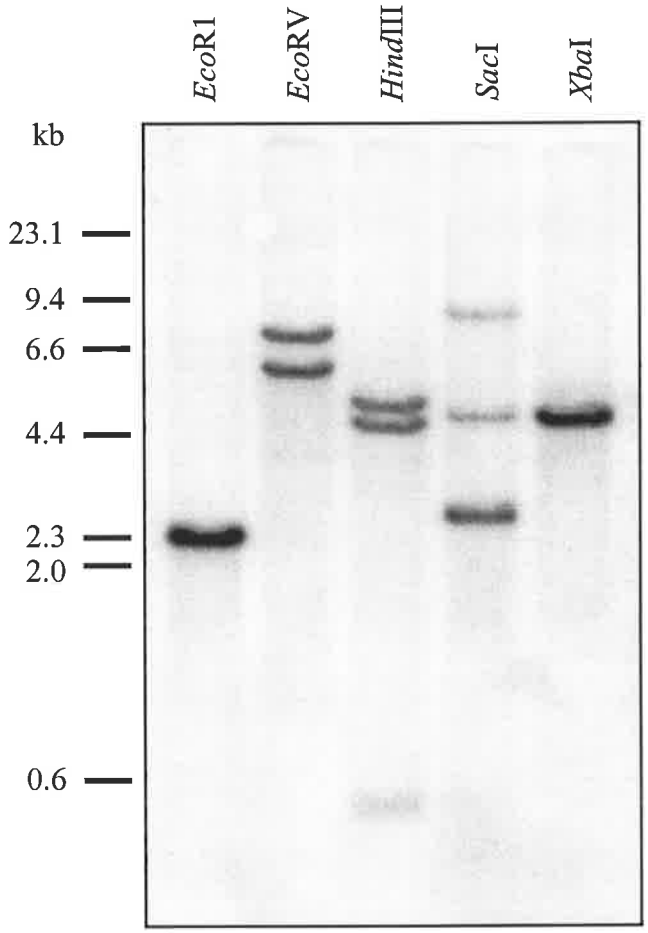

Figure 6.2 Southern hybridization analysis of VvPME1. Gordo genomic DNA (10 μg) digested with the indicated restriction enzymes was loaded in each lane. The blot was probed with a 669 bp fragment of the VvPME1 clone and washed at low stringency. A single site for *Sac*I was present in the VvPME1 cDNA. DNA size markers are shown on the left.

6.3.1.3 PGase

The 5' end of the VvPG1 cDNA (PG1f) was used as a probe for the Southern analysis. At high stringency, which allows for a calculated mismatch of 2%, a single banding pattern was seen for all the lanes (Fig. 6.3B) suggesting that VvPG1 is encoded by a single gene. However, under a relaxed stringency allowing up to 23% mismatch, multiple bands could be seen (Fig 6.3A), indicating that there are a number of closely related sequences in the Gordo genome.

6.3.1.4 XETs

The entire PCR-derived inserts corresponding to the grape XETs were used as probes for the Southern analyses for the XET genes. VvXET1 gave multiple banding patterns under both low (Fig. 6.4A) and high (Fig. 6.4B) stringency conditions, indicative of a small family of closely-related sequences. VvXET2 gave a single banding pattern under highly stringent conditions (Fig. 6.5A) revealing that it is encoded by a single gene. However, under less stringent conditions, which allowed for up to 28% mismatch, multiple bands were seen, indicating a number of closely related sequences (Fig. 6.5B). These results suggest that XETs are encoded by a multi-gene family.

6.3.1.5 Cellulases

Using the entire PCR fragments of VvCEL1 and VvCEL2 as probes under low stringency wash conditions, Southern analyses revealed that both VvCEL1 and VvCEL2 are encoded by single genes (Figs. 6.6A and 6.6B). No cross-hybridization was detected between the two probes under these conditions. These results indicate that there are at least two grape cellulase genes but no sequences that are closely related to either of the cellulase genes.

6.3.2 Expression patterns of genes at a transcriptional level

Total RNA was isolated from Gordo berry mesocarp and skin at different stages of development. Total RNA from the mesocarp of berries at 41 dpa could not be isolated

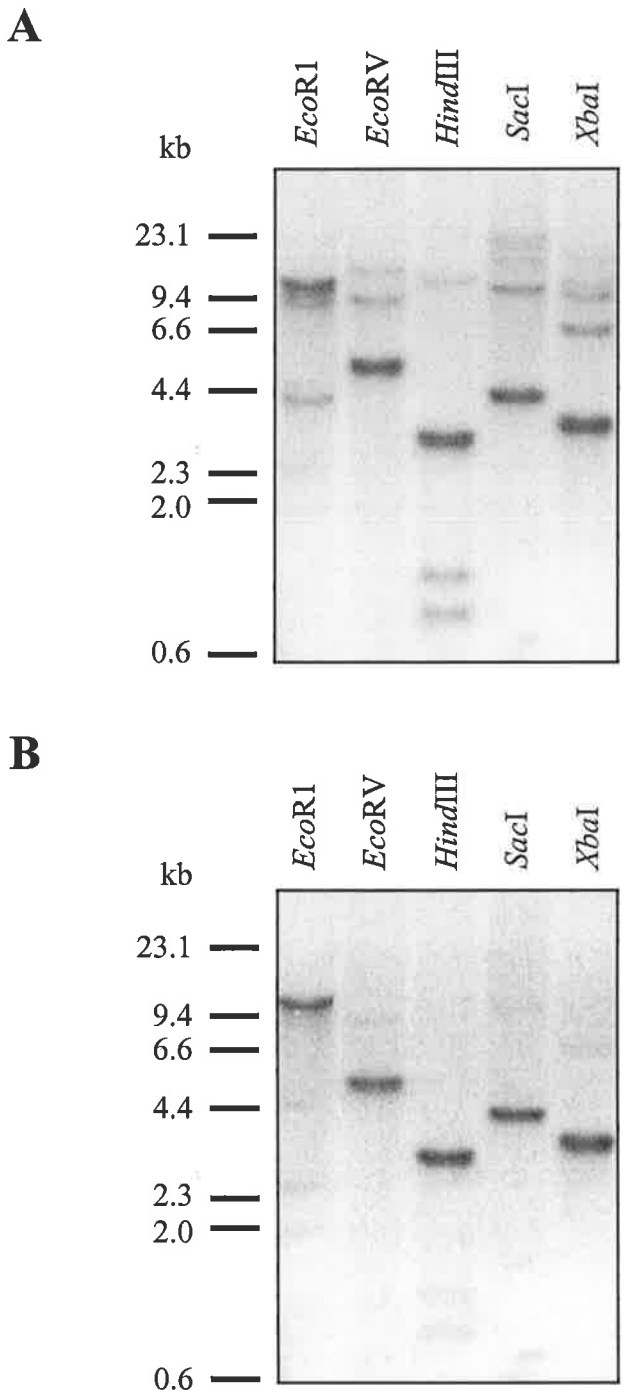

Figure 6.3 Southern hybridization analysis of VvPG1. Gordo genomic DNA (10 μg) digested with the indicated restriction enzymes was loaded in each lane. The blot was probed with a 440 bp fragment of the VvPG1 clone and washed at low **(A)** and high stringencies **(B)**. DNA size markers are shown on the left.

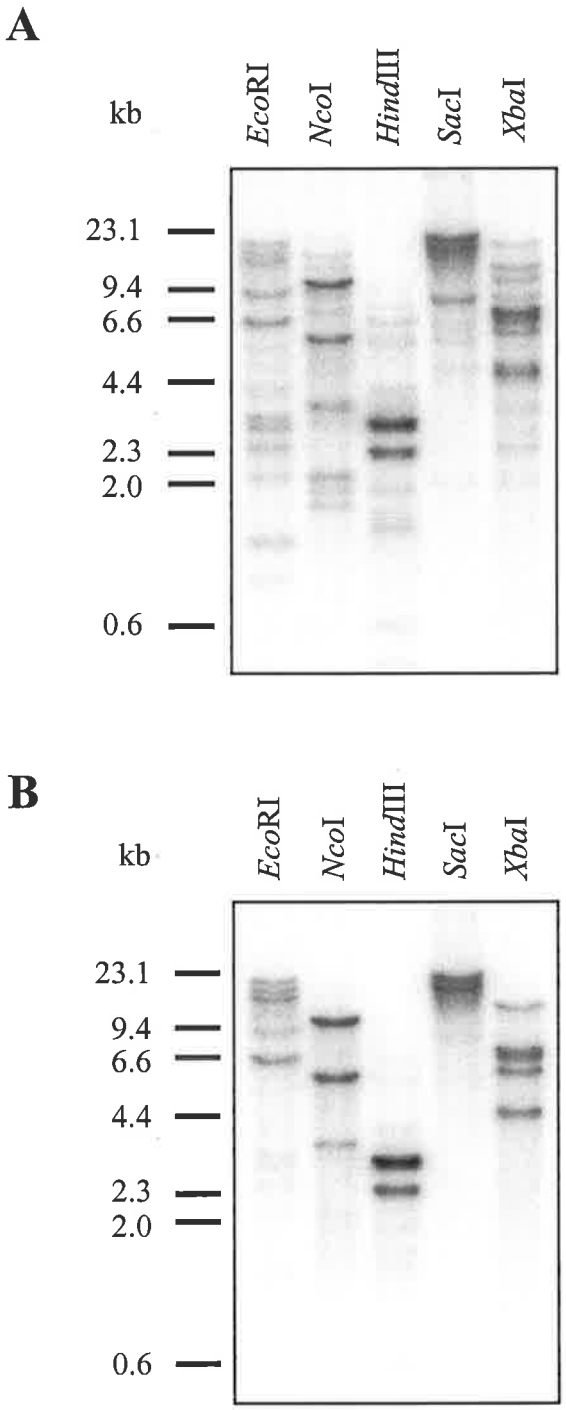

Figure 6.4 Southern hybridization analysis of VvXET1. Gordo genomic DNA (10 μg) digested with the indicated restriction enzymes was loaded in each lane. The blot was probed with the insert of the VvXET1 clone and washed at low **(A)** and high stringencies **(B)**. DNA size markers are shown on the left.

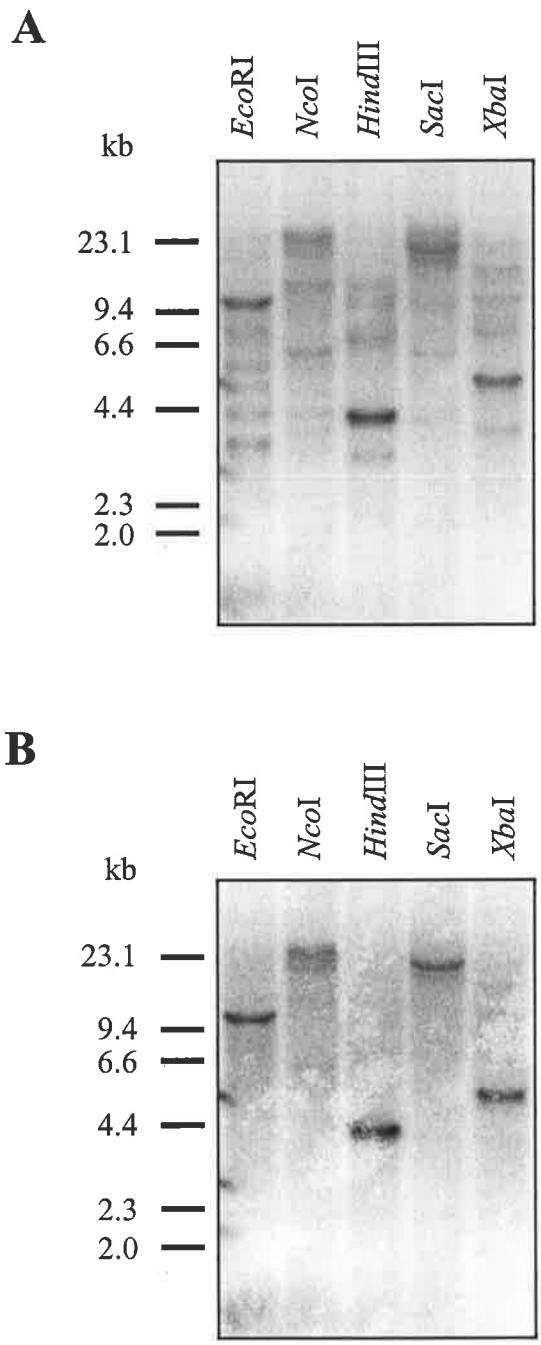

Figure 6.5 Southern hybridization analysis of VvXET2. Gordo genomic DNA (10 μg) digested with the indicated restriction enzymes was loaded in each lane. The blot was probed with the insert of the VvXET2 clone and washed at low **(A)** and high stringencies **(B)**. DNA size markers are shown on the left.

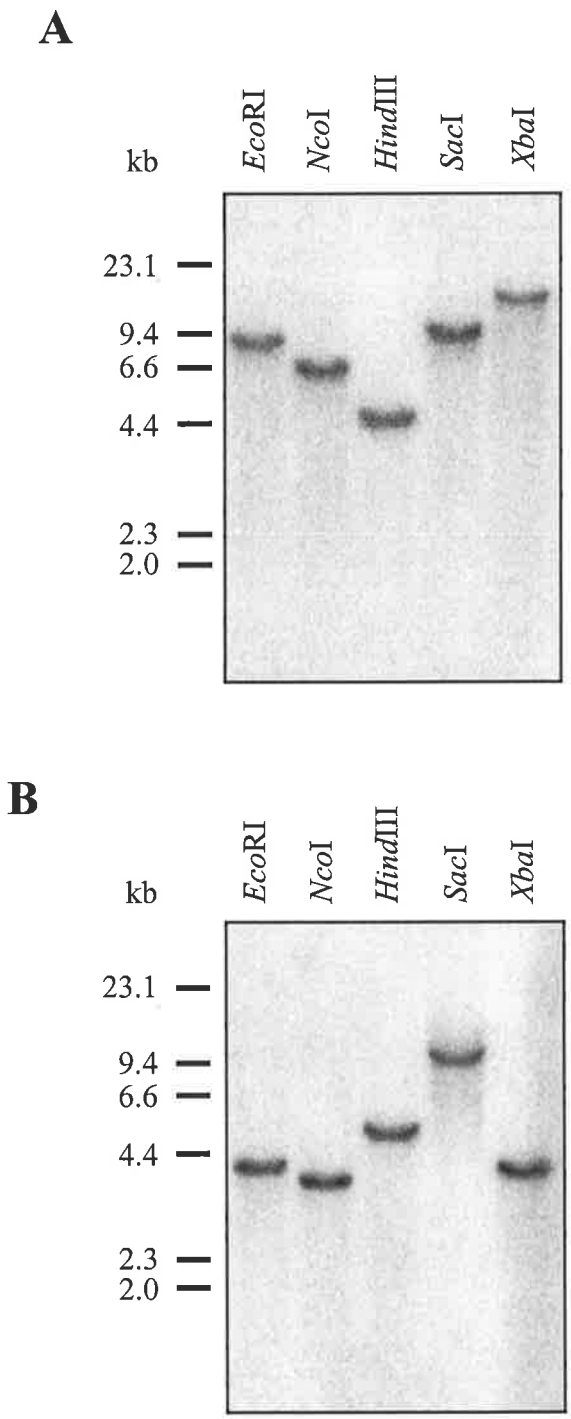

Figure 6.6 Southern hybridization analyses of VvCEL1 and VvCEL2. Gordo genomic DNA (10 μg) digested with the indicated restriction enzymes was loaded in each lane. The blot was probed with either the insert of the VvCEL1 clone **(A)** or the insert of VvCEL2 **(B)** and washed at low stringency. DNA size markers are shown on the left.

in an undegraded form using the procedure described in Section 6.2.3, possibly because the berries thawed at some stage during storage or whilst removing the skins. However, total RNA was successfully isolated from Gordo berry mesocarp at 63 (veraison), 86, 105 and 126 dpa and from Gordo skin at 126 dpa. To check equal loadings of RNA, 1.5 μg aliquots of RNA were separated on a TAE-buffered 1.2% (w/v) agarose gel and stained with ethidium bromide (Fig. 6.7A).

Total RNA was isolated from whole Shiraz berries every two weeks from flowering through to 112 dpa, and from the mesocarp and skin of berries at 84 dpa. The time of veraison was approximately 63 dpa for the Shiraz berries. To achieve equal loadings of RNA, samples were stained with ethidium bromide, as shown in Figure 6.7B. To confirm complete RNA transfer and equity in loading for the Shiraz berry Northern analyses, a ribosomal DNA probe was hybridized to the Northern hybridization membrane originally probed with βG1f (Fig. 6.7C). Total RNA was also isolated from Shiraz roots, leaves at three stages of maturity, and seeds, and equal loadings were confirmed as above (Fig. 6.7D).

For each DNA probe used, a separate Northern hybridization membrane was used. All membranes were hybridized using the same conditions, followed by washes of either medium (1x SSC, 65°C) or high (0.1x SSC, 65°C) stringency. Each Northern membrane was washed at a stringency for which the Southern hybridization analysis gave the fewest bands. Thus, the Northern membranes probed for VvBG1, VvCEL1 and VvCEL2 mRNA were washed at a medium stringency and those probed for VvPME1, VvPG1, VvXET1 and VvXET2 were washed at a high stringency.

Comparisons were also made between RNA isolated from the two cultivars Gordo and Shiraz. However, comparisons were made with caution because RNA was derived from the mesocarp tissue of Gordo berries and from both the skin and mesocarp tissue of Shiraz berries. To alleviate this problem, RNA samples isolated from separate skin and mesocarp samples of Shiraz berries at 86 dpa, and from the skin of Gordo berries at

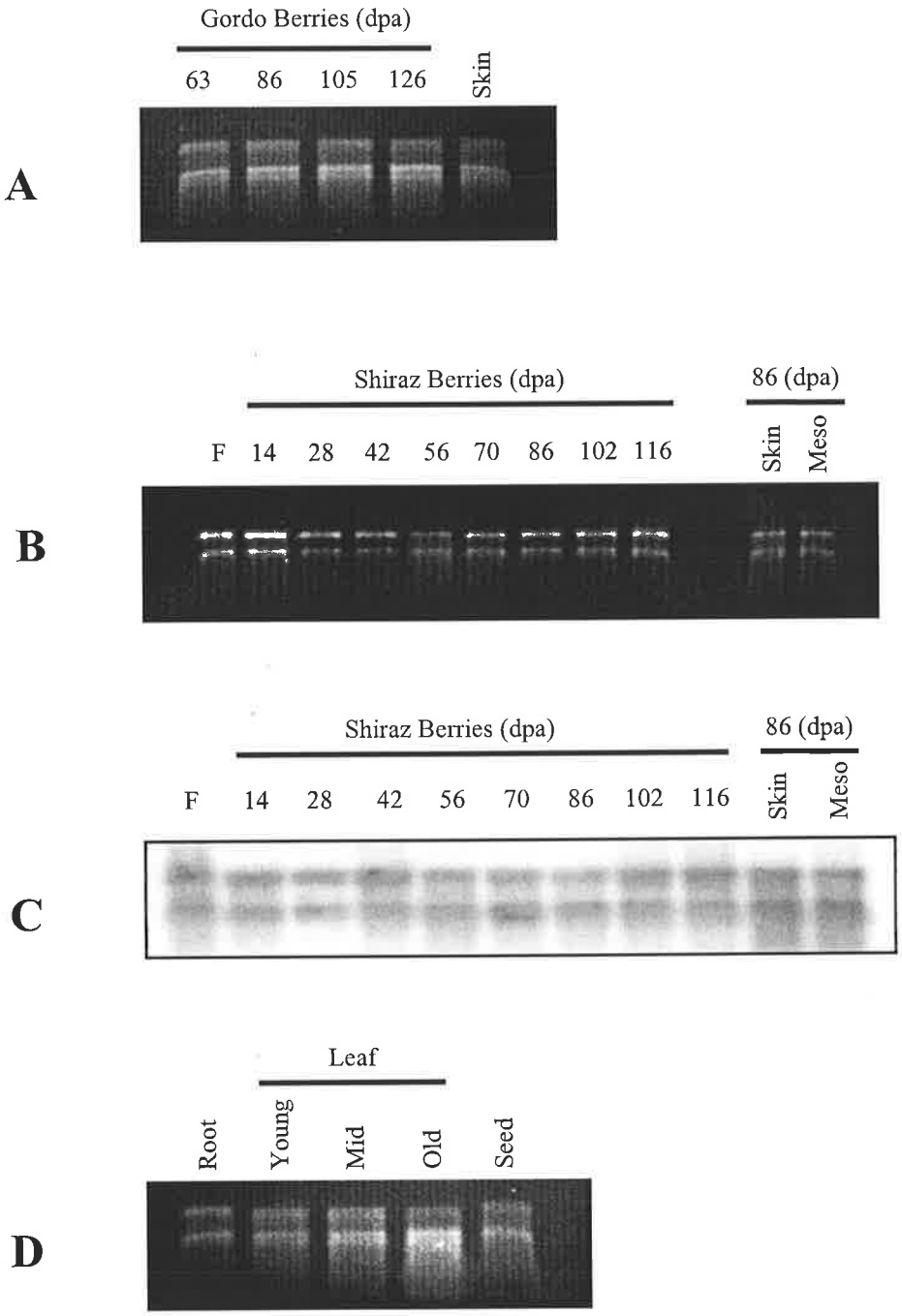

Figure 6.7 Evidence of approximate equal loading of total RNA. Total RNA isolated from Gordo berries, separated on an agarose gel and stained with ethidium bromide **(A)**. Total RNA isolated from Shiraz berries, separated on an agarose gel and stained with ethidium bromide **(B)**. Northern hybridization membrane probed with a ribosomal probe **(C)**. Total RNA isolated from Shiraz tissues, separated on an agarose gel and stained with ethidium bromide **(D)**.

126 dpa, were included in the Northern analyses. RNA samples isolated from other Shiraz grapevine tissues were also analysed.

6.3.2.1 β-Galactosidase

Low levels of VvBG1 mRNA were detected in Gordo berries at veraison (63 dpa) and 86 dpa but no transcripts were detected in 105 or 126 dpa mesocarp or in 126 dpa skin (Fig. 6.8A). Transcriptional expression of the gene encoding VvBG1 was found throughout Shiraz berry development (Fig. 6.8B). High levels of mRNA were seen in Shiraz berries at 14, 28, 42 and 70 dpa, and lower levels in 56 and 86 dpa berries. Little or no mRNA was detected for VvBG1 in berries at 98 and 112 dpa. There is a discrepancy between the Shiraz 86 dpa whole berry RNA which shows a reasonable level of transcript, and the mesocarp and skin RNA samples of 86 dpa in which very little transcript was detected. High levels of VvBG1 mRNA were detected in the seed and very low levels were detected in the root and leaf samples from Shiraz (Fig. 6.8C).

6.3.2.2 PME

The mRNA for VvPME1 were readily detected throughout softening of the Gordo berries, with a maximum level at 105 dpa (Fig. 6.9A). In contrast, lower levels of mRNA were detected for VvPME1 throughout development of the Shiraz berries (Fig. 6.9B). Similar levels of VvPME1 mRNA were found in the skin and mesocarp of 86 dpa Shiraz berries and of 126 dpa Gordo berries, indicating little difference in mRNA abundance of VvPME1 between the skin and mesocarp of grape berries. The highest level of VvPME1 transcript was found in 105 dpa Gordo berries as opposed to 70 dpa Shiraz berries. VvPME1 mRNA was detected in Shiraz seeds (Fig. 6.9C) while little or no VvPME1 transcript could be detected in roots and leaves.

6.3.2.3 PGase

Little or no mRNA hybridized to the VvPG1 cDNA probe at veraison for Gordo berries but low levels of VvPG1 transcript were detected in 86 and 105 dpa berries (Fig.

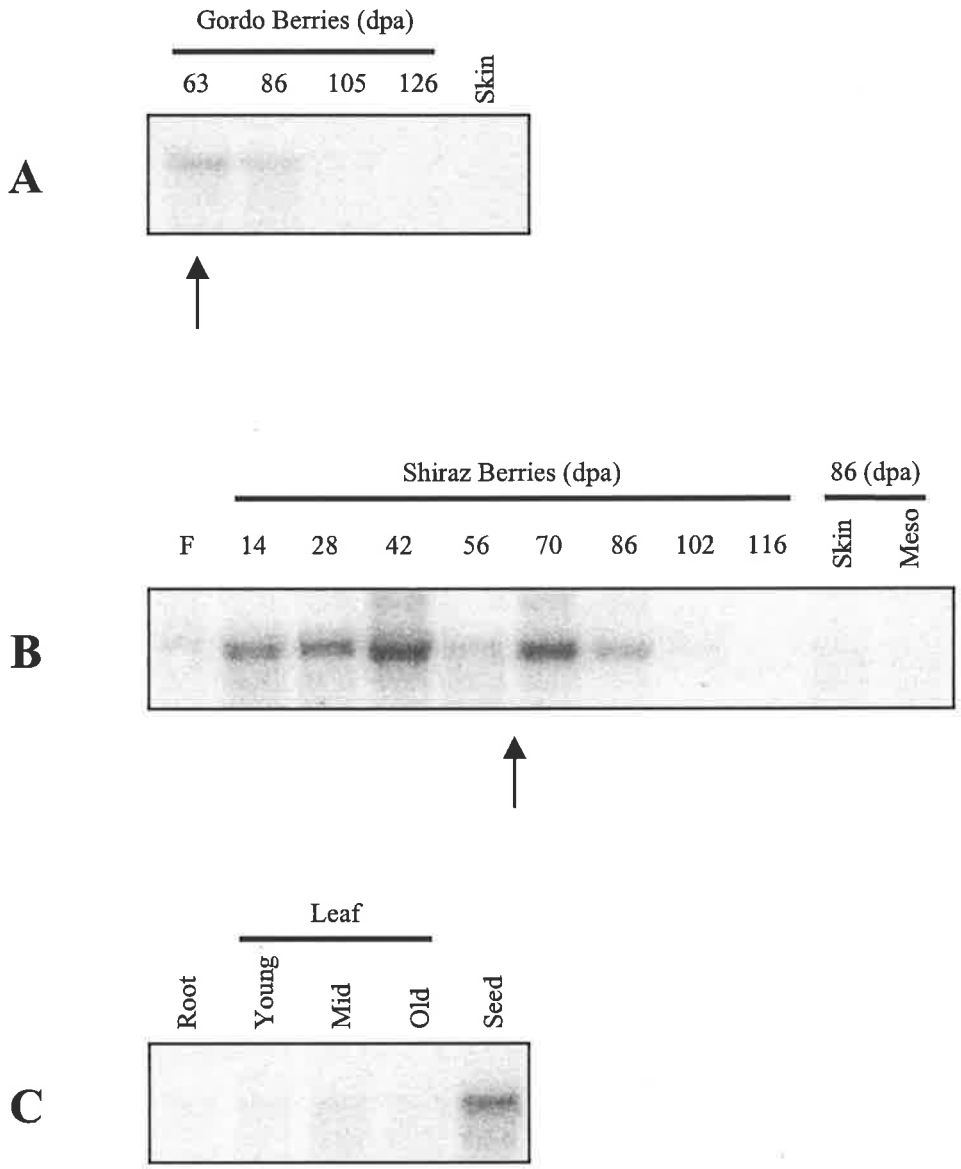

Figure 6.8 Northern hybridization analysis of VvBG1 mRNA. Each membrane contained total RNA extracted from Gordo berry mesocarp and skin **(A)**, Shiraz flowers, whole berries, berry skin and berry mesocarp **(B)** and Shiraz grapevine tissues **(C)** and was probed with BG1f. Each lane contained 2.8 μg total RNA. The arrows indicate time of veraison.

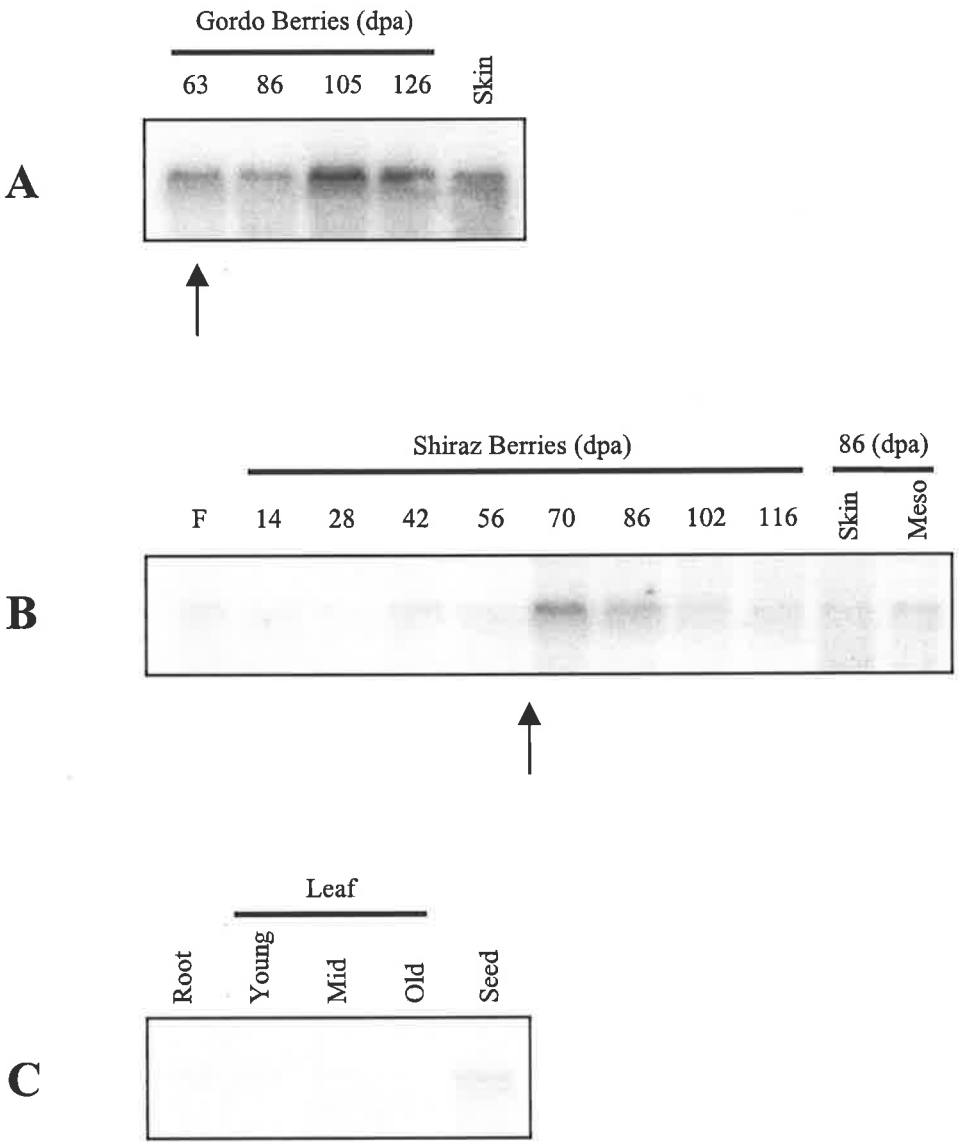

Figure 6.9 Northern hybridization analysis of VvPME1 mRNA. Each membrane contained total RNA extracted from Gordo berry mesocarp and skin **(A)**, Shiraz flowers, whole berries, berry skin and berry mesocarp **(B)** and Shiraz grapevine tissues **(C)** and was probed with PME1f. Each lane contained 2.8 μg total RNA. The arrows indicate time of veraison.

6.10A). Little mRNA could be detected in 126 dpa Gordo mesocarp or skin. No VvPG1 transcript could be detected in preveraison Shiraz berries, but after veraison there was an accumulation of the mRNA, with a maximum level reached at 86 dpa (Fig. 6.10B). This suggests that the transcription of the gene encoding VvPG1 may be associated with softening. The mRNA for VvPG1 was found mainly in the mesocarp of 86 dpa Shiraz berries. It should be noted that the skin RNA sample was possibly contaminated with a small amount of mesocarp RNA, accounting for the low level of VvPG1 mRNA detected in the skin. VvPG1 transcript could not be detected in any Shiraz tissues other than berry samples (Fig. 6.10C), indicating that VvPG1 transcription may be berry-specific, and possibly berry mesocarp-specific.

6.3.2.4 XETs

The levels of mRNA for VvXET1 increased in Gordo berry mesocarp from veraison (63 dpa) to maturity (126 dpa), with very little transcript detected in the skin (Fig. 6.11A). In contrast, mRNA encoding VvXET1 was detected in Shiraz berries mainly around veraison and not in later stages of ripening (Fig. 6.11B). In both cultivars, it appears that the VvXET1 transcript is mainly in the mesocarp tissue of berries. VvXET1 mRNA is also present in roots, young leaf and seed, and in lower levels in mid and old leaves (Fig. 6.11C). However, from the Southern analysis, it is known that more than one XET gene exists and may be detected using the hybridization conditions employed in the Northern analysis.

VvXET2 transcript was detected at low levels in 63 dpa (veraison) Gordo berry mesocarp, and no mRNA was detected in the softening tissue of Gordo berries (Fig. 6.12A). In contrast, VvXET2 mRNA was readily detected in 70 dpa Shiraz berries and was present at lower levels in 42 and 86 dpa berries (Fig. 6.12B). This suggests that VvXET2 mRNA levels in Gordo berries might be higher in berries aged between 63 and 86 dpa, a period in which no Gordo berry mRNA was analysed. VvXET2 mRNA was present in Shiraz flowers and in berries around veraison, with little or no transcript

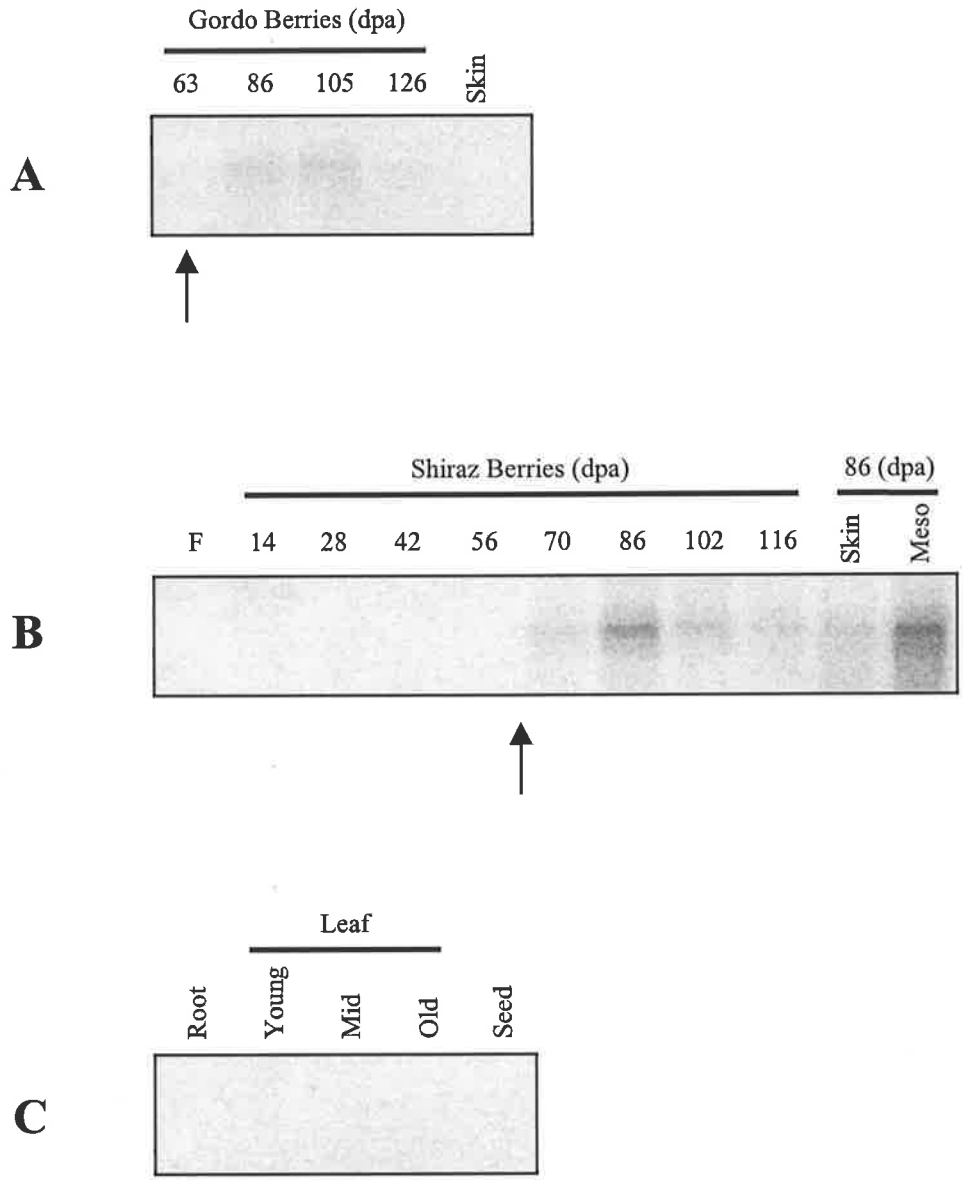

Figure 6.10 Northern hybridization analysis of VvPG1 mRNA. Each membrane contained total RNA extracted from Gordo berry mesocarp and skin **(A)**, Shiraz flowers, whole berries, berry skin and berry mesocarp **(B)** and Shiraz grapevine tissues **(C)** and was probed with PG1f. Each lane contained 2.8 μg total RNA. The arrows indicate time of veraison.

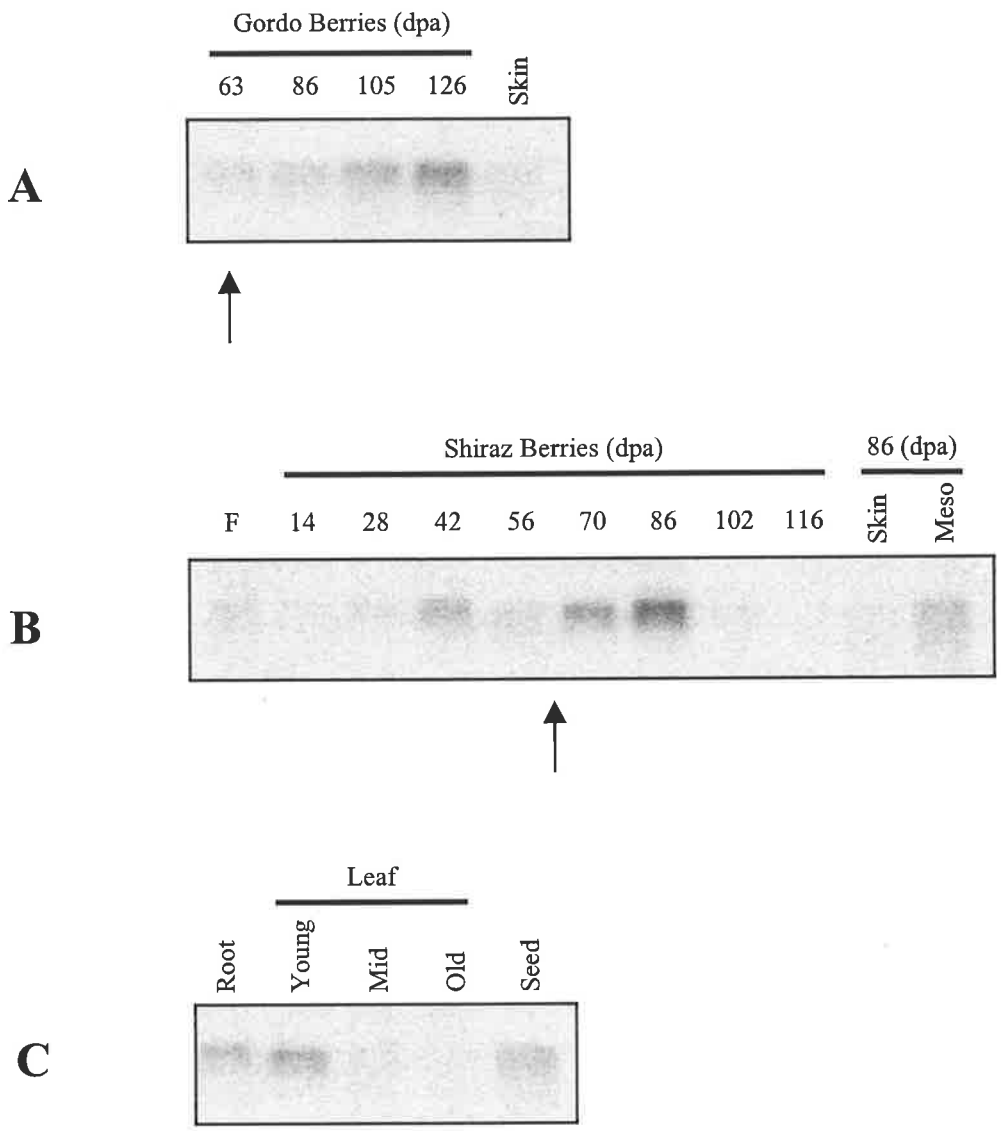

Figure 6.11 Northern hybridization analysis of VvXET1 mRNA. Each membrane contained total RNA extracted from Gordo berry mesocarp and skin **(A)**, Shiraz flowers, whole berries, berry skin and berry mesocarp **(B)** and Shiraz grapevine tissues **(C)** and was probed with XET1f. Each lane contained 2.8 μg total RNA. The arrows indicate time of veraison.

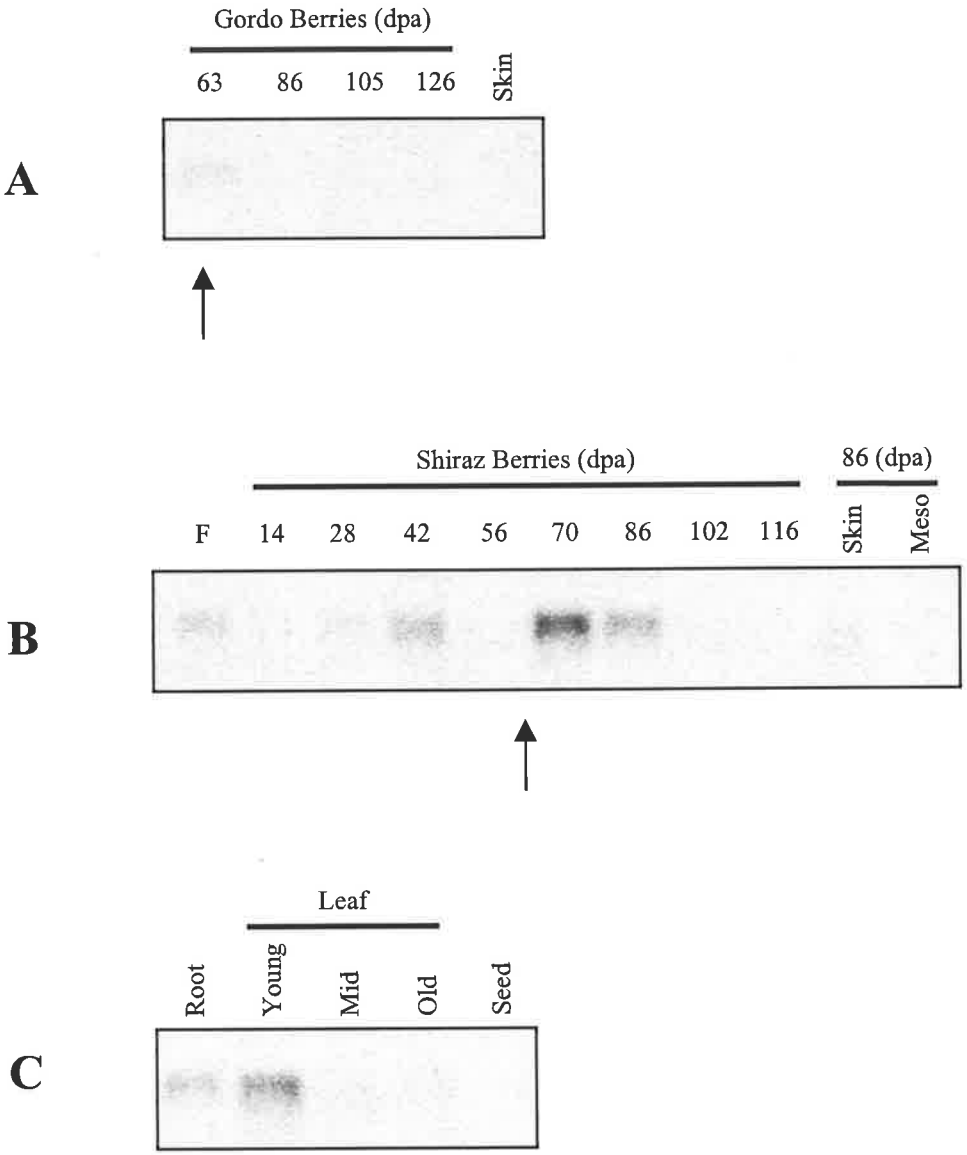

Figure 6.12 Northern hybridization analysis of VvXET2 mRNA. Each membrane contained total RNA extracted from Gordo berry mesocarp and skin **(A)**, Shiraz flowers, whole berries, berry skin and berry mesocarp **(B)** and Shiraz grapevine tissues **(C)** and was probed with XET2f. Each lane contained 2.8 μg total RNA. The arrows indicate time of veraison.

detected in young or mature berries. VvXET2 mRNA was detected in roots and young leaves but was not found in mid and old leaves, nor in seeds (Fig. 6.12C).

The patterns of transcriptional activity for VvXET1 and VvXET2 are very similar when the Northern hybridization analyses for the Shiraz berries developmental series are compared. The major difference relates to the time at which peak levels of mRNA are reached; 86 dpa for VvXET1 and 70 dpa for VvXET2. However, in Gordo berries the accumulation of VvXET1 and VvXET2 transcripts differs; VvXET1 mRNA accumulates in increasing amounts throughout softening, whereas VvXET2 mRNA is only detected at veraison. It must be remembered that the VvXET1 probe will hybridize to other sequences other than the VvXET1 mRNA, even at high stringency (Fig. 6.4B), whereas VvXET2 probably binds only to the VvXET2 transcript at high stringency, as shown by Southern analysis (Fig. 6.5B).

6.3.2.5 Cellulases

No mRNA was found to hybridize to the VvCEL1 probe in Gordo berries at veraison or postveraison (Fig. 6.13A). Low levels of VvCEL1 transcript were detected in Shiraz flowers and in 14 and 28 dpa (Fig. 6.13B), but at no other stage was mRNA detected. VvCEL1 mRNA was detected in young leaf, and lower levels of mRNA were also found in root and mid leaf (Fig. 6.13C). No VvCEL1 transcript was detected in old leaf or seed.

Transcriptional activity of the VvCEL2 gene was not detected at any stage of softening for Gordo berries (Fig. 6.14A) or Shiraz berry development (Fig. 6.14B). Low levels of VvCEL2 transcript were found in flowers. VvCEL2 mRNA was not detected in any of the Shiraz tissues (Fig. 6.14C). These results suggest that the VvCEL1 and VvCEL2 genes are not important for softening of the grape berry. However, the VvCEL1 gene may be involved with the expansion of young tissue.

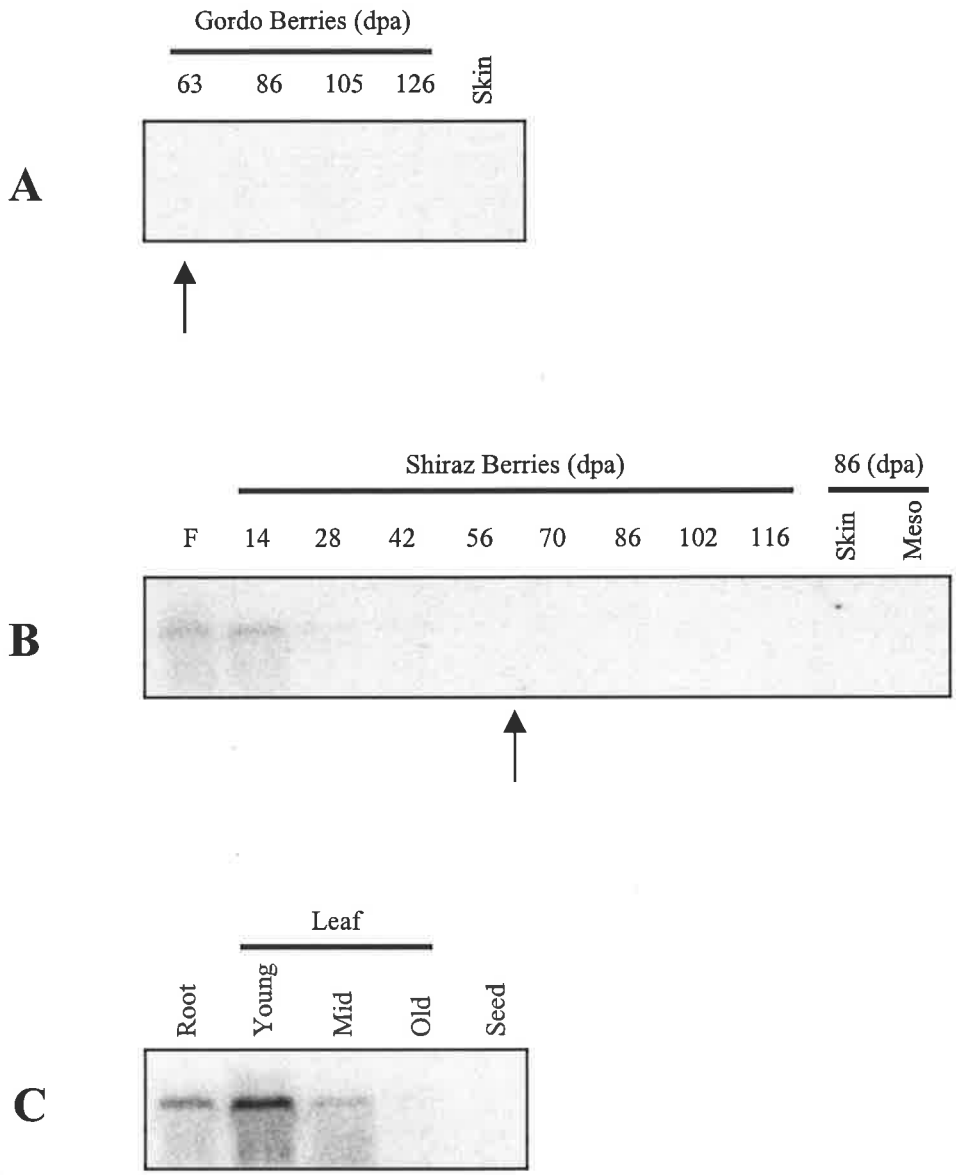

Figure 6.13 Northern hybridization analysis of VvCEL1 mRNA. Each membrane contained total RNA extracted from Gordo berry mesocarp and skin **(A)**, Shiraz flowers, whole berries, berry skin and berry mesocarp **(B)** and Shiraz grapevine tissues **(C)** and was probed with CEL1f. Each lane contained 2.8 μg total RNA. The arrows indicate time of veraison.

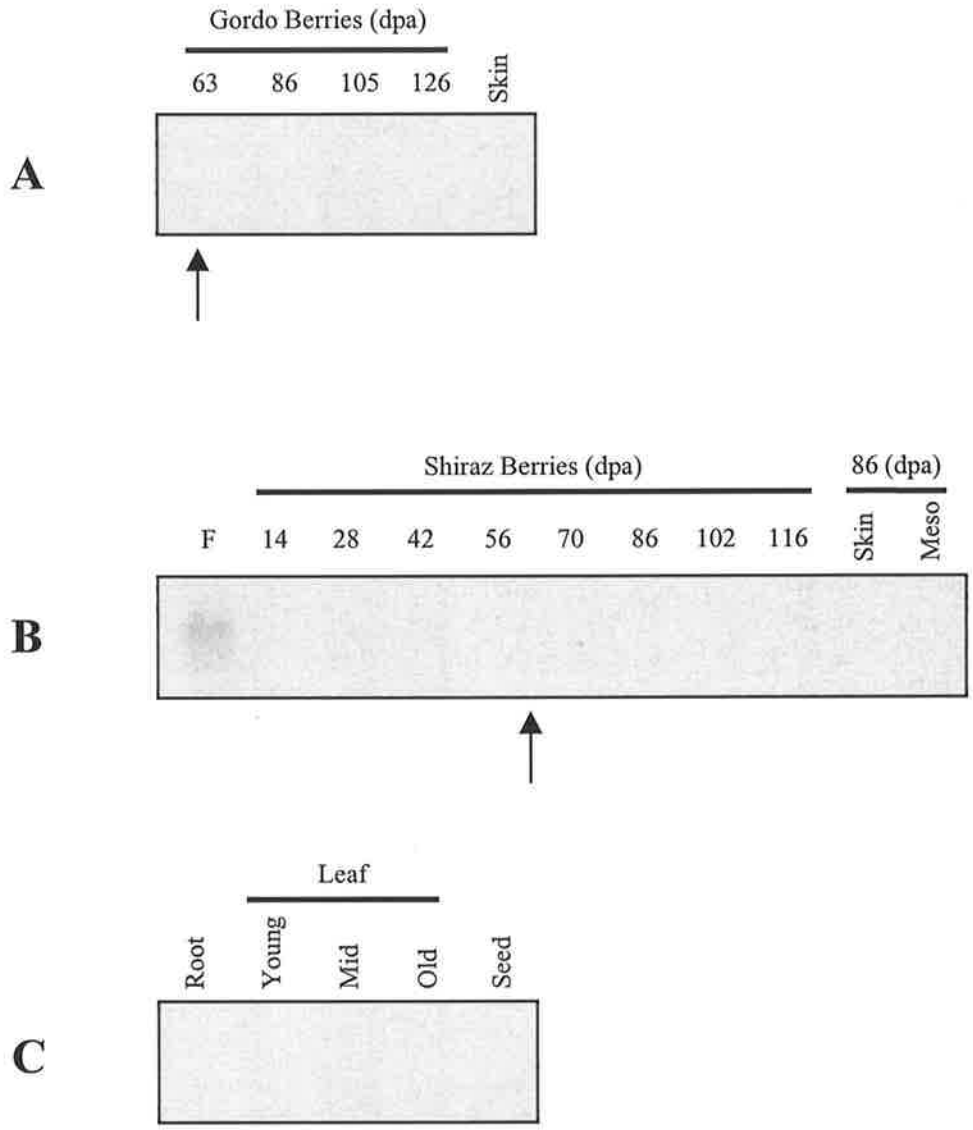

Figure 6.14 Northern hybridization analysis of VvCEL2 mRNA. Each membrane contained total RNA extracted from Gordo berry mesocarp and skin **(A)**, Shiraz flowers, whole berries, berry skin and berry mesocarp **(B)** and Shiraz grapevine tissues **(C)** and was probed with CEL2f. Each lane contained 2.8 μg total RNA. The arrows indicate time of veraison.

6.4 Discussion

The results of the Southern analyses are summarized in Table 6.2. The genes encoding VvPME1 and VvXET1 are probably members of multigene families. In high stringency washes, only one gene fragment was detected for VvBG1, VvPG1, VvXET2, VvCEL1 and VvCEL2. This data on gene copy number represented essential background information for the interpretation of Northern hybridization studies on the steady state levels of mRNA in grapes. By performing the Northern hybridization analyses at stringencies similar to those used for Southern analyses, it was possible to determine whether the mRNAs detected by the various probes were the products of single genes, or whether multiple mRNA species might be present.

Table 6.2 Estimated number of genes for each enzyme as determined by Southern analyses

Enzyme	cDNA	Estimated number of genes	
		High stringency wash	Low stringency wash
β-galactosidase	VvBG1	1	1
PME	VvPME1	1 or 2	1 or 2
PGase	VvPG1	1	> 3
XET	VvXET1	> 3	> 10
	VvXET2	1	> 5
Cellulase	VvCEL1	1	1
	VvCEL2	1	1

The transcriptional activity, as judged by mRNA abundance, of genes encoding each enzyme in various grapevine tissues and comparisons with the respective enzyme activities are summarized in Table 6.3.

The transcriptional activity of genes encoding the five cell wall-modifying enzymes in Shiraz berries revealed up-regulation of mRNAs for VvBG1, VvPME1, VvPG1,

Table 6.3 Enzyme activity *versus* transcriptional activity of genes encoding the different enzyme classes

Enzyme	Enzyme activity in berries	cDNA	Cultivar differences	mRNA levels in Gordo mesocarp from veraison to maturity	mRNA levels in various grapevine tissues					
					Berries[a]	Skin	Flower	Root	Leaves	Seed
β-Galactosidase	Yes - Increase	VvBG1	No	Decrease	+++	tr	tr	tr	tr	++
PME	Yes - Constant	VvPME1	Yes	Increase	+++	++	tr	-	-	+
PGase	Not detected	VvPG1	No	Low, constant	+	tr	-	-	-	-
XET	Not assayed	VvXET1	Yes	Increase	++	-	-	+	+	+
		VvXET2	Yes	Present at veraison	++	-	+	+	+	-
Cellulase	Not detected	VvCEL1	No	Not detected	-	-	+	+	++	-
		VvCEL2	No	Not detected	-	-	+	-	-	-

[a] General mRNA levels found in Gordo and Shiraz berries during development

- = no mRNA detected, tr = trace amounts of mRNA detected, + → ++++ = low to high mRNA levels detected

VvXET1 and VvXET2 after veraison. This implies that these genes could be involved in the softening and/or expansion of the berry during the postveraison period. In contrast, neither VvCEL1 nor VvCEL2 mRNAs were detected in softening berries.

One notable feature of the transcriptional activity of many of the genes studied here is an apparently temporary down-regulation of mRNA transcripts in 56 dpa berries, one week before veraison. This corresponds to the lag period in the growth of most grape cultivars, where no expansion of the berry occurs (see Chapter 1). The phenomenon of down-regulation before veraison and increased mRNA levels after veraison has also been reported for genes in the anthocyanin pathway (Boss *et al.*, 1996). In contrast, other genes such as the putative vacuolar invertase genes are expressed without interruption from berry set until shortly after veraison (Davies and Robinson, 1996).

β-Galactosidase

Levels of VvBG1 mRNA are relatively high in preveraison berries and tend to decrease in postveraison berries, whereas β-galactosidase activity increases only after veraison. Thus, although high levels of mRNA transcript are present before veraison, the majority of extractable active β-galactosidase enzyme accumulates after veraison. The decreasing abundance of VvBG1 mRNA in ripening grapes differs to patterns found for apple and tomato β-galactosidases, in which mRNA accumulates during fruit ripening (Ross *et al.*, 1994; Carey *et al.*, 1995).

VvBG1 mRNA was also detected in the seeds of berries and at very low levels in the roots and leaves of the grapevine (Table 6.3). In tomatoes, β-galactosidase mRNA was found in leaves but not in roots (Carey *et al.*, 1995). These results indicate that β-galactosidase may have a role in the softening of grape berries, but that the enzyme is likely to be involved in additional developmental processes in other tissues.

PME

The most notable feature of the Northern hybridization analyses of PME transcripts is the difference in VvPME1 mRNA levels between the two cultivars (Fig. 6.9). In Shiraz berries, levels of the VvPME1 transcript were relatively low throughout berry development, with a slight increase in berries 70 dpa (one week after veraison) (Fig. 6.9B). In contrast, mRNA levels in Gordo increased from veraison through to 105 dpa. A similar phenomenon is seen in beans, where mRNA levels for a gene encoding PME in developing bean pods differed between two cultivars (Ebbelaar *et al.*, 1996). Comparison of the transcriptional activity of the VvPME1 gene with PME enzyme activity detected in Gordo berries shows that VvPME1 mRNA accumulates during ripening, but PME activity remains relatively constant (Chapter 4). This finding emphasizes the fact that mRNA levels may not be good indicators of levels of active enzyme.

A large number of PME cDNAs and genes have been isolated from other plant species. An mRNA for the peach PME gene pPPE8B is detected through all stages of development (Glover *et al.*, 1996), as is the mRNA for the tomato PME gene B8 (Hall *et al.*, 1994). Additionally, mRNAs for genes encoding PMEs in *Arabidopsis* and tomato accumulate to high levels in young expanding tissues (Richard *et al.*, 1994; Gaffe *et al.*, 1997). Other than berries, VvPME1 mRNA was only detected in flowers and seeds of grapevines (Table 6.3).

PGase

VvPG1 mRNA was detected in postveraison berries, and comparison of skin and mesocarp of 86 dpa berries reveals that most of the VvPG1 mRNA is associated with the mesocarp of the grapes (Table 6.3). Contrary to the findings for transcriptional activity of the VvPG1 gene, no PGase activity was detected in ripening Gordo berries (Chapter 4), again highlighting the potential discrepancies between enzyme activities and mRNA abundance. Perhaps activity was lower than the threshold sensitivity of the enzyme assay. In avocado (Kutsunai *et al.*, 1993) and apple (Atkinson, 1994), mRNA

encoding PGase is only detected in ripe fruit, and in tomato, PGase mRNA levels increase 2000-fold in ripe fruit compared with unripe fruit (DellaPenna *et al.*, 1986). Thus, mRNAs encoding some PGases are correlated closely with fruit ripening.

PGases have been grouped into fruit-ripening PGases, pollen-specific PGases and abscission PGases (Kalaitzis *et al.*, 1995). From Figure 5.7, it is clear that the sequence of VvPG1 in ripening grapes is most closely related to the sequences of fruit-ripening PGases in other plants, in agreement with the conclusions made here that VvPG1 is involved in grape ripening and softening.

XET

The mRNAs for both VvXET1 and VvXET2 were detected in many of the grapevine tissues, although different patterns of mRNA accumulation occurred, both within the one cultivar and between cultivars (Figs. 6.11 and 6.12). It is important to note that both cDNAs were isolated by PCR using cDNA prepared from 70 dpa berry poly(A^+)-RNA, and the VvXET1 and VvXET2 mRNAs must therefore be present at this stage of berry development. XET enzyme activity was not monitored in grape berries and a comparison between enzyme and mRNA levels cannot be made.

In general, XETs are associated with expanding tissues of plants. For example, in barley, XET activity and the related mRNA levels were found to increase during leaf elongation (Smith *et al.*, 1996) and in tomato, high levels of mRNA encoding XET were found in elongating hypocotyls (Catalá *et al.*, 1997). XETs are also induced by environmental stresses, such as flooding, and by hormonal stimuli (Smith *et al.*, 1996; Xu *et al.*, 1996). There are only a few reports of XET cDNAs being isolated from fruit. Four related, but distinct, XET cDNAs have been cloned from tomato (Arrowsmith and de Silva, 1995). The mRNA of one of these tomato genes is abundant in ripening fruit pericarp, which suggests that it is involved in softening rather than expansion (Arrowsmith and de Silva, 1995). XET genes associated with ripening have also been identified in kiwifruit (Schröder *et al.*, 1998).

It is possible that the grape XETs examined here are actually involved in cell expansion. The berry has two phases of growth, and both cell expansion and softening is observed during the ripening phase (Chapter 1). However, in ripening tomato and kiwifruit, XET mRNA was detected after expansion had ceased and softening had begun, which indicates that some XETs may be involved with softening only (Arrowsmith and de Silva, 1995; Schröder *et al.*, 1998).

Cellulase

VvCEL1 mRNA was detected in flowers, young berries, roots and younger leaves (Fig. 6.13). In contrast, VvCEL2 mRNA was only detected in flowers (Fig. 6.14). No VvCEL2 mRNA was found in ripening berries. These findings are in agreement with the absence of detectable cellulase activity in ripening grape berries. However, it must be remembered that both VvCEL1 and VvCEL2 cDNAs were obtained by PCR from template cDNA prepared from 70 dpa berry poly(A^+)-RNA. Low levels of the cellulase mRNAs must therefore be present at this stage of berry development.

However, in avocado, cellulase mRNA levels increase at least 50-fold in ripe fruit compared with unripe fruit (Christoffersen *et al.*, 1984), and in tomato a transcript designated Cel2 also accumulates during ripening (Lashbrook *et al.*, 1994). Further work on Cel2 mRNA levels in ripening-impaired tomato mutants suggested that the Cel2 gene contributes to ripening-associated cell wall changes (Gonzalez-Bosch *et al.*, 1996). Cellulases are often associated with processes other than fruit ripening. For example, in tomato, high levels of cellulase mRNA have been found in developing pistils (Milligan and Gasser, 1995), expanding hypocotyls (Brummell *et al.*, 1997), and abscission zones of flowers, leaves and fruit (Kemmerer and Tucker, 1994; Del Campillo and Bennett, 1996; Trainotti *et al.*, 1997). From the results presented here, it seems that the VvCEL1 gene is more likely to be important in the expansion of young growing tissues of the grapevine than in berry softening. The role of VvCEL2 in the grapevine remains unclear.

Conclusions

In conclusion, the gene families studied here show complex transcriptional activities during grape berry development. It is possible to relate mRNA levels found in grapes back to the changes in composition of cell walls of ripening berries (Chapter 3). However, such connections must be viewed as speculative, because of the large number of events that occur between transcription and cell wall modification. Caution should also be exercised when relating mRNA abundance to detectable enzyme activity, because it is possible that the gene of interest may not be responsible for the activity and that one or more other isoforms are being measured instead.

Despite these interpretative constraints, the detection of VvBG1 mRNA in preveraison and early postveraison grapes is consistent with the decrease in cell wall galactan found in developing grapes (Chapter 3), and supports the notion that this mRNA is producing an active enzyme that acts on the cell walls early in development. VvBG1 mRNA accumulation also corresponds well to the accumulation of β-galactosidase activity. Thus, β-galactosidase is probably important in the hydrolysis of cell wall galactan during berry development and softening.

Similarly, the presence of VvPG1 mRNA in postveraison berries can be reconciled with the increase in water-soluble pectic polysaccharides found in Chapter 3. The VvPG1 gene could be transcribed and the resultant mRNA translated to produce an active enzyme that is modifying pectic polysaccharides. No PGase activity could be detected in grape berries, but only low activity of an endohydrolase would be necessary to initiate the increase in pectin solubility reported in Chapter 3. Thus, the method used to assay PGase activity may not have been sufficiently sensitive.

The transcriptional activities of the XET and cellulase genes suggest that they are involved with wall modification in other tissues rather than in berry softening. The potential role of XET in softening cannot be predicted from changes in XG content of cell walls during berry ripening (Chapter 3). XET activity would need to be measured

before reliable conclusions could be made about any possible role of XET in ripening berries.

Cellulase activity was not detectable in grapes, but again, only low activity would be required to have a significant impact on cell wall structure. Cellulose levels in cell walls of ripening grapes did decrease a little on a per berry basis (Chapter 3). However, changes in the degree of polymerisation of cellulosic polysaccharides might give a better indication of cellulase activity, especially if activity is relatively low. On the basis of the evidence that neither mRNA nor enzyme activity for cellulase was detected, it may be concluded that cellulase does not play an important role in the softening of grape berries. Similarly, the DE of pectic polysaccharides remains relatively constant during berry softening (Chapter 3), despite the detection of VvPME1 mRNA and PME activity (Chapter 4), which indicates that PME is of minor importance during berry softening.

Chapter 7

Summary and Future Directions

7.1 Summary

This study was undertaken to investigate the changes in cell wall composition that occur during grape berry development and to identify enzymes that might be important in cell wall modification. In particular, the study was focussed upon the processes that take place after veraison, the point at which berry ripening is initiated and softening begins, because it has been suggested in the past (Dey and Brinson, 1984; Seymour and Gross, 1996) that softening might involve partial degradation of cell walls.

Walls were therefore isolated from the mesocarp tissue of two grape cultivars, Ohanez and Gordo. Analyses of their monosaccharide, polysaccharide and protein composition showed that the main differences between the two cultivars were in the cellulose and PG content of the walls. The higher cellulose content of the Ohanez walls could be reconciled with the firmer texture of the berries of this cultivar.

Cell walls were subsequently isolated from Gordo berries at several stages of development and analyses revealed that a number of modifications occur during development (Chapter 3). These include an increase in water-soluble pectic polysaccharides, a decrease in galactan/AG-I and an increase in protein.

The changes in cell wall polysaccharide composition during berry ripening suggested that several classes of enzymes might be involved in the softening process; the activity of these enzymes was monitored throughout berry development (Chapter 4). Overall, β-galactosidase and α-galactosidase activities were the most readily detected and were found to increase markedly during berry softening. Indeed, it appears that β-galactosidase is probably responsible for the loss of galactan from cell walls observed as berries soften. The role of α-galactosidase in berry cell wall modification remains unclear and further definition of its function in plants is required. The only other enzyme detected during berry development was PME. However, the role of PME is also unclear, because the DE of pectic polysaccharides in cell walls remained constant during berry softening. Little or no PGase, galactanase, cellulase or XGase activity

could be detected in developing berries. However, their involvement in berry softening could not be ruled out because they are all endo-acting enzymes and low activities, which may only produce subtle changes in wall composition, have the potential to alter polysaccharide solubility and thus could greatly affect cell wall structure. In particular, it was concluded that very low levels of PGase might be responsible for the increased solubility of pectic polysaccharides during ripening.

The possible roles of cell wall-modifying enzymes in berry development were further investigated at the gene level. Several cDNAs encoding wall-modifying enzymes were isolated (Chapter 5) and these were subsequently used as probes to study the transcriptional activity of the respective genes in developing grape berries and in other tissues (Chapter 6). The gene families showed complex spatial and temporal transcriptional activities. The up-regulation of the gene encoding β-galactosidase was consistent with results obtained by measuring enzyme activity and with the decrease in galactan content in walls. Similarly, although no PGase activity could be detected, the presence of PGase mRNA was consistent with the increased solubility of pectic polysaccharides, which was probably mediated by levels of PGase enzymes too low to be measured in standard assays. The presence of XET mRNA also suggested that this enzyme plays some role in berry softening. PME mRNA was detectable during berry development, consistent with the low levels of PME activity measured. However, the DE of pectic polysaccharides remained constant during berry ripening. No mRNA transcripts encoding cellulases were detectable in ripening berries.

In conclusion, subtle changes in cell wall composition occurred during berry development, but the detectable activities of enzymes were generally low. This supports the theory that fruit softening is a complex process involving subtle changes to different components of the cell wall, and in many cases requiring only small amounts of enzyme activity.

7.2 Future directions

The work described in this study has provided a broad view of the changes that occur in the cell walls of mesocarp tissue of developing grape berries. Using this information, together with the cDNAs probes and other molecular tools produced, it should now be possible to focus on particular aspects of grape berry softening that the work described here has shown to be important. These include galactan breakdown, changes in molecular weights of cell wall polysaccharides and the characteristics of key enzymes that have been implicated in ripening. This, in turn, may result in a more detailed understanding of the softening process in ripening fruit, and in the longer term may be of benefit to the table grape and wine industries through the modification of cell walls *via* genetic engineering of grapevines.

7.2.1 Cell wall composition

Because this study was focussed on softening grape berries, most of the sampling was performed at, or after, veraison. It may also be useful to study cell wall modification earlier in berry development. For example, it is of interest to better define when and how much galactan is lost from preveraison berry cell walls, especially if this is presumed to be a major determinant in berry softening. Subsequently, a more complete comparison could be made between the cell walls isolated from berry samples taken from the first and second expansion phases of growth.

The decrease in molecular weights of polysaccharides is thought to be partially responsible for softening in fruit (Seymour and Gross, 1996). This process may also occur in grapes, particularly in view of the increase in water-soluble polysaccharides during berry ripening. However, in the present study the analysis only allowed the types and amounts of polysaccharides in the cell walls to be determined, and no determination of molecular weights was possible. Therefore, gel permeation chromatography of isolated wall polysaccharides could give an indication of changing molecular weight distributions of polysaccharides with respect to berry development,

and hence a more precise understanding of more subtle changes that occur to cell wall polysaccharides during ripening.

Additionally, individual polysaccharides could be isolated and linkage analyses performed to gain a greater understanding of the exact nature of polysaccharide changes during berry development. The structures of some pectic polysaccharides in grape berries have been well-documented in previous studies (Saulnier and Thibault, 1987b; Saulnier *et al.*, 1988), but the structures of many other polysaccharides remain to be elucidated.

It should be remembered that the detectable changes in cell walls of developing fruit are a net result of incorporation of newly synthesized components and the breakdown of existing components (Lackey *et al.*, 1980; Mitcham *et al.*, 1989). In grapes, the amounts of major polysaccharides decrease on a per berry basis during ripening, indicating that cell wall breakdown occurs more rapidly than cell wall synthesis. The incorporation of labelled substrates into cell wall polysaccharides, coupled with compositional analyses, would give an indication of turnover rates of important cell wall components during berry development.

Finally, the protein and amino acid content of walls in developing berries could also be studied in greater detail. The Hyp content of the walls increases as berries mature, which suggests an increase in the amount of extensin associated with the walls. Extensin is thought to be important in maintaining the integrity of plant cell walls and increased extensin deposition might strengthen the walls in expanding and softening berries. Other proteins considered important in the extension of cell walls are expansins, but their abundance and location in the walls have yet to be defined.

7.2.2 Cell wall-modifying enzymes

As discussed previously, β-galactosidase, α-galactosidase and PME activities were readily detectable in developing grape berries. Purification of these enzymes, and the isolation of individual polysaccharides to be used as substrates in assays, would allow

detailed characterization of the enzymes, and their various isoforms. This, in turn, might aid in determining the potential roles of individual enzymes in berry softening. Also, the purification of enzymes would enable the production of antibodies. Such antibodies could be used in western analyses or in sensitive enzyme-linked immunosorbent assays to examine the relative abundance of specific enzymes throughout development and could also be used in immunolocalisation studies to provide further information on their precise locations and potential roles in the ripening berry.

An alternative approach to the purification of key enzymes from ripening berries would be the heterologous expression of active recombinant proteins from cloned cDNAs. This would be particularly advantageous if purification of native enzymes from berries proved difficult, and would allow the production of large amounts of homogeneous enzyme for detailed characterization. Recombinant proteins could also be used to produce antibodies.

Finally, purification of other enzymes that may be involved in the softening of grape berries, such as XET, rhamnogalacturonase, pectate lyase, and other glycosidases, could be part of a future, more comprehensive study of cell wall metabolism in developing grape berries.

7.3 Future applications

In the longer term, the genetic modification of grapevines could be used either for the functional analysis of specific genes, or to modify cell wall structure for enhanced berry quality or improved productivity of grapevines. Genetic engineering of grapevines is possible (Nakano *et al.*, 1994; Scorza *et al.*, 1995; Franks *et al.*, 1998), although there are a number of broader issues that need to be addressed, including the acceptance of engineered cultivars by the wine and table grape industries.

The modification of cell walls to improve fruit quality is now a commercial reality. One of the most successful examples is the FLAVR SAVR™ tomato (Kramer and

Redenbaugh, 1994). A PGase gene isolated from tomatoes was reintroduced into the tomato in the "antisense" orientation (Sheehy *et al.*, 1988). This resulted in PGase activity being reduced to less than 1% of the activity in wildtype fruit which, in turn, slowed cell wall breakdown (Smith *et al.*, 1990). Tomatoes with reduced PGase activity have improved shelf life and are less susceptible to fungal infection because cell walls remain intact longer. Additionally, these tomatoes have enhanced flavour because they can be vine-ripened for a longer period of time (Schuch *et al.*, 1991). PME is also being targeted to improve tomato quality (Schuch, 1994). The viscosity and handling characteristics of tomatoes are determined by cell wall structure, and soluble solids are partly determined by pectins, and the modification of PME activity could affect both pectin and cell wall structure (Schuch, 1994).

All of these technological applications have relevance to the table grape and wine industries. For example, increasing the strength of cell walls could produce a crisper grape for the benefit of the table grape industry. Crisper grapes would satisfy consumer demand for improved texture. If the tougher cell walls were also associated with improved disease resistance, quality and productivity could be enhanced. Conversely, hastening wall degradation after harvest would improve juice extractability and filterability during winemaking.

The alteration of cell walls could also be achieved through the modification of enzymes involved with wall synthesis. Inhibition or up-regulation of polysaccharide synthases could generate cell walls with different polysaccharide compositions and this could change the rate and extent of grape berry softening. To accomplish this, a detailed knowledge of the polysaccharide synthases and their genes would be required. However, at this stage there are no reports of the purification or characterization of any polysaccharide synthase involved in plant cell wall synthesis, and genetic improvements of grape berry properties are more likely to be achieved through modification of hydrolases and other cell wall-modifying enzymes of the type described in this thesis.

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