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Genetic Manipulation of Fruit Ripening

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Introduction

The application of recombinant DNA technology promises to revolutionize agricultural practices in the near future. This is particularly true for the genetic engineering of crop plants, and indeed this new 'green revolution' is currently well in progress. The two obvious major advantages of this technology are: first, the breakdown of species, and even kingdom, barriers to the transfer of useful genetic material and, secondly, the provision of faster and more controlled plant breeding programmes. However, it should be emphasized that although genetic engineering will undoubtedly be of enormous benefit to plant breeders, it can not in itself replace conventional breeding programmes.

The genetic manipulation of plants can be achieved by several means (Cocking, 1990), and need not necessarily involve recombinant DNA technology. Useful traits can often be transferred by protoplast fusion, or selected from amongst mutants generated via either somoclonal or gametoclonal variation (Cocking, 1990).

However, in most instances the genetic manipulation of plants does involve recombinant DNA technology and subsequent transformation of the plant. Several techniques are available to mediate these transformations, including electroporation and transformation vectors based on viruses or the Ti plasmid of the bacterium *Agrobacterium tumefaciens*. For a recent comprehensive review on the general topic of foreign genes in plants, their structure, transfer, expression and application the reader is referred to Weising, Kahl and Schell (1988).

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; CaMV 35S, cauliflower mosaic virus 35S; CAT, chloramphenicol acetyltransferase; EFE, ethylene-forming enzyme; EPSP synthase, 5-enolpyruvylshikimate-3-phosphate synthase; M_r, weight average molecular weight; mRNA, messenger RNA; PE, pectinesterase; PG, polygalacturonase; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; tRNA, transfer RNA.

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Table 1. Attributes for manipulation by genetic engineering

Attributes
Factors affecting primary yield
Improved photosynthetic capacity
Reduced photorespiratory loss
Altered assimilate partitioning
Improved biological fixation
Improved disease/pest resistance
Herbicide resistance
Cold/drought/salt tolerance
Factors affecting post-harvest losses
Extension of shelf-life by controlled ripening/senescense
Improved tolerance of refrigeration and controlled-atmosphere storage
Increased disease resistance
Factors affecting food quality
Improved nutritional value
Improved texture/flavour/colour
Improved processing qualities
Enhancement of useful or novel products

The number of crop attributes that could be genetically manipulated is potentially enormous, some of the most obvious of these are listed in *Table 1*. Progress in many of these areas is already significant, as exemplified by the production of novel transgenic plants resistant to either insects or herbicides. Insect-resistant plants have been created by using recombinant DNA technology to transfer the gene for *Bacillus thuringiensis* δ-endotoxin into tobacco plants (*Nicotiana tabaccum*) (Barton, Whiteley and Yang, 1987). The use of bacterial proteins may meet with some consumer resistance. Some plant species have also developed effective protein-based defence mechanisms against herbivorous insects, for example digestive enzyme inhibitor proteins such as the cowpea (*Vigna unguiculata*) trypsin inhibitor. This, along with other plant-derived protective products, is a good candidate for manipulating crop plants for insect resistance (Hilder, Gatehouse and Boulter, 1990). Effective resistance to a variety of herbicides has also been developed. One example is resistance to the 'knockdown' herbicide glyphosate. In this case the molecular target for the herbicide is known. This is the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP synthase) (EC 2.5.1.19), which is involved in the biosynthesis of amino acids. Resistance has been conferred on a plant by transferring the gene for EPSP synthase from glyphosate-resistant *Salmonella typhimurium* (Comai *et al.*, 1985) or by engineering the plant's own EPSP synthase gene for overproduction by inserting a secondary copy under the control of a strong promoter (Shah *et al.*, 1986). An alternative approach to generating herbicide resistance is to introduce genes for herbicide detoxification pathways into plants. Such an approach is being investigated for the herbicide 2,4-D (Llewellyn *et al.*, 1990). In this instance the molecular target for herbicide action is unknown. In the case of fruit and vegetable crops, genetic manipulation could usefully be employed to enhance nutritional value (increase vitamin content), extend shelf-life, improve aesthetic qualities such as colour, flavour and texture, and also introduce attributes such as increased solids for more effective processing (Knight, 1989).

As with all genetic engineering projects, having identified an attribute for manipulation, the next stage is to isolate suitable genes for transformation. This often, but not always, requires an understanding of the biochemical processes underlying the trait to be manipulated, as demonstrated in the development of glyphosate-resistant plants. One example of such essential co-operation between biochemistry and molecular biology is in the current interest in manipulating tomato fruit ripening.

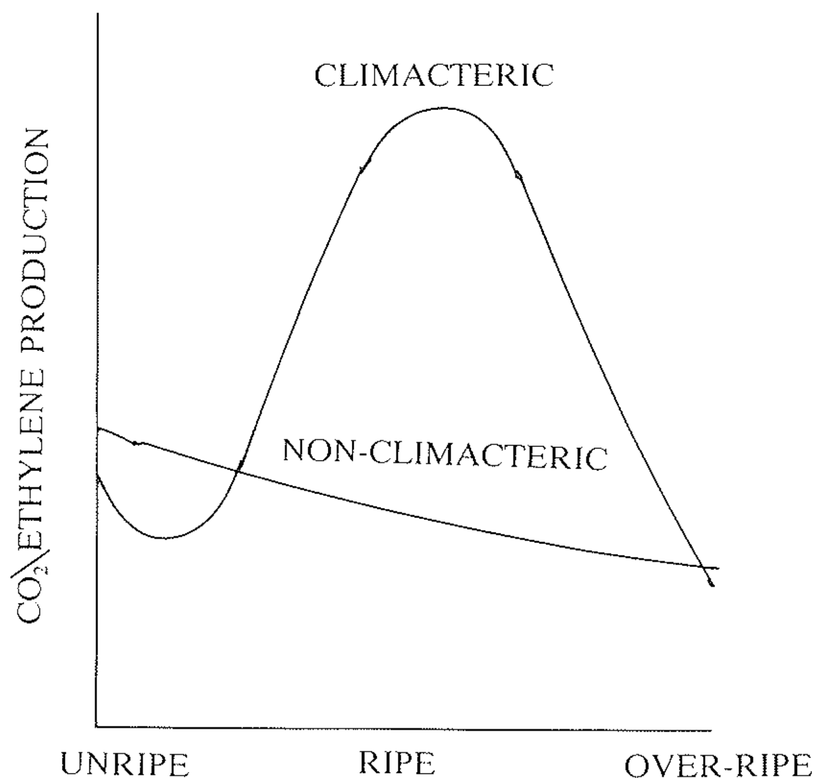
Biochemistry of fruit ripening

Fruit ripening is a complex process involving the co-ordinated functioning of several independent biochemical pathways, and has been comprehensively reviewed recently (Brady, 1987; Tucker and Grierson, 1987). It was once thought that fruit ripening was a senescence process brought about by a breakdown in the organizational resistance within the cells (Blackman and Parija, 1928). However, it is now apparent that ripening, rather than being the result of uncontrolled breakdown of cellular organization, is actually under specific genetic regulation (Grierson, 1985). This is important since it means that ripening can be manipulated at a genetic level using recombinant DNA techniques. The realization that ripening occurs via a group of independent biochemical pathways is also important since this implies that manipulation of one trait, for instance softening, is unlikely to adversely affect others, such as colour or flavour.

CHANGES IN RESPIRATION AND ETHYLENE

The ripening of fruit can be broadly classed as either climacteric or non-climacteric in nature. This division of fruit into two distinct classes is based on their respiratory behaviour during ripening (*Figure 1*). Non-climacteric fruit, such as strawberry (*Fragaria* x *Ananassa*) or grape (*Vitis vinifera*), show a steady decline in respiration during ripening. Climacteric fruit, such as tomato (*Lycopersicon esculentum*), apple (*Malus* × *domestica* Borkh.) and banana (*Musa* cv.), have a relatively low rate of respiration just prior to the onset of ripening and this increases to a peak during ripening and then declines. In many fruits the peak of respiration coincides with optimum eating quality, the post-climacteric decline representing in such cases overripeness of the fruit. This respiratory pattern is common to all climacteric fruit, although, as indicated in *Figure 1*, the size of the peak is highly variable between fruits. From the point of view of shelf-life it is interesting to note that, in general, the greater the respiratory rate of the fruit, the faster those fruit ripen and hence the shorter the shelf-life. This generalization also holds for the non-climacteric fruits, which while demonstrating a common respiratory pattern also exhibit markedly different respiratory rates.

Although the classification of fruit as climacteric or non-climacteric is based on their respective respiration patterns, these two classes of fruit also differ in respect of the so-called 'ripening hormone' ethylene. This simple hydro-



Maximum CO_2 and ethylene production of selected fruit

Fruit	CO_2 (ml kg^{-1} h^{-1})	Ethylene (p.p.m.)
Climacteric		
avocado (*Persea americana*)	155	500
banana (*Musa* cv.)	60	40
cherimoya (*Anona cherimolia*)	170	219
pear(*Pyrus communis*)	33	40
tomato (*Lycopersicon esculentum*)	20	27
Non-climateric		
cherry (*Prunus avium*)	25	–
grape (*Vitis vinifera*)	20	–
lemon (*Citrus limon*)	5	0·15
pineapple (*Ananas comosus*)	17	0·30
strawberry (*Fragaria x Ananassa*)	21	–

Figure 1. Climacteric and non-climacteric patterns of respiration and ethylene synthesis in fruit.

carbon has long been known to influence fruit ripening (Denny, 1924) as well as having diverse effects on other aspects of plant physiology (Abeles, 1985). Most plant tissues produce ethylene but this is usually in very small amounts unless the tissue is wounded. Climacteric fruit are characterized by a burst of ethylene synthesis associated with the respiratory climacteric, whereas in

non-climacteric fruit no such increase in ethylene production is detectable (*Figure 1*). The two classes of fruit also differ in their response to applied ethylene or its analogue, propylene. Given sufficient exposure to exogenously applied propylene, unripe climacteric fruit respond by increasing their own endogenous ethylene output and initiating ripening (McMurchie, McGlasson and Eaks, 1972). Non-climacteric fruit do not respond to propylene by increasing their ethylene production (McMurchie, McGlasson and Eaks, 1972) but, like climacteric fruit, show increases in the rates of both respiration and ripening. This is interpreted to show that ethylene synthesis is 'autocatalytic' in climacteric fruit and not in non-climacteric fruit (*Figure 2*).

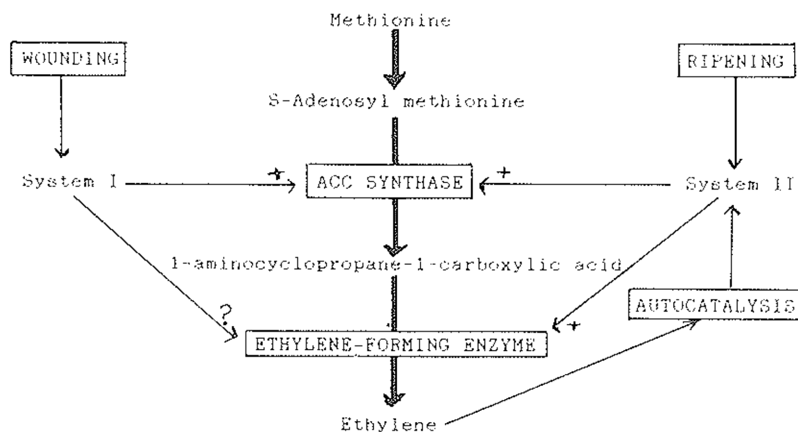


Figure 2. Outline of the key steps and control of ethylene biosynthesis.

The biosynthetic pathway for ethylene is known and, indeed, this was first established in apple fruit (Adams and Yang, 1979). The pathway is summarized in *Figure 2*, there being apparently two key enzymic steps, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (EC 4.4.1.14) and the so-called ethylene-forming enzyme (EFE). Hoffman and Yang (1980) demonstrated that ACC synthase activity was limiting in preclimacteric fruit and that both ACC synthase and EFE activity increased during ripening. This biosynthetic pathway seems to be common to both climacteric and non-climacteric fruit (Yu and Yang, 1980), the differences being in the relative control of the pathway in the two types of fruit. During ripening of climacteric fruit ethylene synthesis is autocatalytic whereas that of non-climacteric fruit is not. The biochemical basis for this differential control is unknown. Both

unripe climacteric and non-climacteric fruit respond to wounding with an increase in ethylene synthesis. The same biosynthetic pathway is responsible for both wound- and ripening-induced ethylene increases (Yu and Yang, 1980). The wound-induced ethylene synthesis in green tomato is not inhibited by silver ions (Tucker and Grierson, 1987) whereas ripening of the fruit is (Hobson *et al.*, 1984). Silver ions are thought to block the ethylene receptor and hence perception of this hormone (Bayer, 1976). These results imply that although ripening in climacteric fruit requires the ability of the fruit to perceive ethylene, the increase in ethylene production associated with wounding is independent of such perception. This presumably implies that wound-induced ethylene synthesis is not autocatalytic in climacteric fruit and is therefore subject to a control mechanism different from that for ripening. These observations support the suggestion of McMurchie, McGlasson and Eaks (1972) for the presence of at least two ethylene-synthesizing systems, I and II. In each case the biosynthetic pathway is the same but is subject to different controls. System I is common to all tissues and is involved in basal and wound-induced ethylene synthesis, and its control is not autocatalytic in nature. System II is that involved in the autocatalytic ethylene production peculiar to climacteric fruit (*Figure 2*).

Although much is known about the biosynthesis of ethylene, little biochemical data is available on how ethylene acts to influence ripening. Both climacteric and non-climacteric fruit respond to ethylene with an increase in respiration rate (Biale, 1964). In non-climacteric fruit this increase is dependent on the continuous presence of exogenous ethylene. In climacteric fruit, providing sufficient exposure to ethylene is given to trigger ripening, endogenous ethylene production ensues and respiration becomes independent of exogenous ethylene. Exogenous ethylene not only initiates ripening in climacteric fruit but also speeds up several of the ripening processes in both climacteric and non-climacteric fruit. For instance, ethylene can be used to enhance degreening of citrus fruit and this is often done commercially (Proctor and Caygill, 1985). Obviously ethylene is perceived by both climacteric and non-climacteric fruit and thus both types of fruit must have 'ethylene receptors'. The nature of the receptor is unknown. It is thought that silver ions can act as inhibitors of ethylene action by blocking the receptor (Bayer, 1976). The application of silver ions to mature green tomato fruit completely prevents the initiation of ripening (Hobson *et al.*, 1984; Hobson, Nichols and Ford, 1985). Application of silver ions after the onset of ripening stops ripening-related events such as colour change and cell wall solubilization (Tucker and Brady, 1987; Smith, Seymour and Tucker, 1989). Several of the ripening-related changes in gene expression can be both switched on by exogenous ethylene (Maunders *et al.*, 1987) and either prevented or attenuated by silver ions (Davies, Hobson and Grierson, 1987). These results show that ethylene perception is required for both the initiation and maintenance of ripening, and that this perception is probably responsible for initiating at least some of the gene expression responsible for ripening.

COLOUR, FLAVOUR AND TEXTURE CHANGES DURING RIPENING

The three major aesthetic qualities associated with fruit are colour, flavour and texture. Types of fruit differ in the biochemical mechanisms by which changes in these attributes are brought about. For instance, colour change in lemon and banana arises primarily from a degradation of chlorophyll and subsequent unmasking of pre-existing pigments. Colour changes in other fruit are due to both chlorophyll degradation and the *de novo* synthesis of new pigments, such as the carotenoid lycopene in tomato or the anthocyanin cyanidin in apple. Similarly, flavour changes result from the synthesis of complex mixtures of volatile compounds and alterations in sugars and organic acids within the fruit. More details of these pathways and the enzymes involved can be found in the recent review of fruit ripening by Tucker and Grierson (1987). Textural changes during ripening may also result from different pathways in different fruit but, in general, the major causative factor appears to be the degradation of the cell walls.

FACTORS FOR MANIPULATION DURING RIPENING

Having surveyed the basic biochemistry behind ripening, what are the key attributes that genetic engineering could be used to manipulate? The major problem preventing the full economic potential of many fruit from being realized is their relatively short shelf-life. Thus reduction in post-harvest losses due to either overripening or microbial attack is of paramount importance. Where possible, fruit are harvested at the immature or mature green stages of development for transportation and storage. This both increases the shelf-life and reduces losses due to microbial attack. However, for some fruit, such as strawberry, this approach cannot be used since these fruit will only ripen normally on the vine and must be harvested once ripening is well advanced. Also, for several other fruit, such as mango (*Mangifera indica*), even harvesting at the mature green stage of development only allows a relatively short 'green life' which is insufficient for effective marketing. Thus one priority for manipulation would be the extension of 'green life' or means to arrest ripening once commenced. This might best be achieved by the manipulation of either ethylene biosynthesis or perception. There are several candidates for this type of work, namely ACC synthase, EFE or the receptor itself, presuming that this is actually proteinaceous in nature. To date our knowledge of any of these is insufficient for the purpose of genetically engineering fruit, but advances in this area can be expected very soon.

Current storage technologies employ low temperature, low O_2 or high CO_2 either separately or in combination (Wills *et al.*, 1989). A major problem for many fruit is a susceptibility to damage under these storage conditions. Increased tolerance of these conditions would be a useful trait to manipulate, but since little is known about the biochemical basis of this damage, progress in this area is not likely to be rapid.

The spoilage of fruit by overripening or microbial decay is often regulated by textural changes within the fruit. Thus although fruit are unlikely to attain

excessive colour or flavour development due to becoming overripe, the fact that they oversoften is often detrimental. Excessive softening diminishes the acceptance of the fruit by the consumer and also increases the susceptibility of the fruit to microbial attack. The biochemical basis of cell wall breakdown, and hence softening, in fruit has been studied extensively, especially in tomatoes, and some possible key enzymes identified. Thus manipulation of softening has become a major aim for fruit biotechnologists.

Tomato biotechnology

Tomato is a major commercial crop with a value exceeding one billion dollars a year in the United States alone (Martin and Olmstead, 1985). Tomato also has many advantages as a model system for both biochemical and molecular biological investigations (Nevins, 1987). Seed is readily available and the plants are easy to grow either under controlled glasshouse conditions or in the field. The growth and development of the plant and fruit is predictable and the short generation time enables up to three generations a year. The tomato has a relatively small genome which has been extensively mapped (Stevens and Rick, 1986) and several well-characterized mutants are available (Rick, 1987). The apparent lack of any protease activity and low phenolic content enables relatively easy protein purification. Also, the lack of starch allows the production of relatively clean cell wall preparations. Finally, tomato cells are readily transformed by *Agrobacterium tumefaciens* and transgenic plants can be regenerated quite successfully (Koorneef *et al.*, 1987).

It is therefore not surprising that tomato has become perhaps the most intensively studied fruit from the point of view of biotechnology. This is exemplified by the fact that an entire symposium volume has recently been devoted to this topic (Nevins and Jones, 1987). Several aspects of the plants' growth and development are targets for genetic manipulation, including softening, and indeed the application of biotechnology to the improvement of this crop is well advanced. One example of this is the generation of tomato plants resistant to the herbicide glyphosate by the transfer of a mutant EPSP synthase gene (Fillatti *et al.*, 1987). A large amount of work has been carried out on the mechanism of softening in tomato fruit, and this has led to attempts to manipulate this attribute by genetic engineering.

Textural changes in tomato fruit

The softening process in tomato fruit is accompanied by visual changes in the ultrastructure of the pericarp plant cell walls (Crookes and Grierson, 1983). Electron micrographs from green and ripe fruit can be interpreted to show that during ripening the middle lamella region of this wall becomes diffuse, possibly due to the action of cell wall hydrolases on specific wall polymers. At a biochemical level two major changes in the composition and properties of the cell wall are apparent. First, during ripening there is a large loss of galactose and a smaller loss of arabinose from the cell wall (Gross and Wallner, 1979). Secondly, there is an increase in the solubility of the

galacturonic acid-containing component of the cell wall (Gross and Wallner, 1979). Galacturonic acid is a characteristic component of the acidic pectin or rhamnogalacturonan polymers of the cell wall and is solubilized in association with rhamnose and other neutral sugars (Gross, 1984; Seymour *et al.*, 1987). This increase in soluble pectin is consistent with the ultrastructural changes observed under the electron microscope, since the middle lamella is known to be a pectin-rich region of the cell wall. As well as becoming more soluble, this pectin component is also depolymerized during ripening. The soluble pectin is heterogeneous with respect to size, that from green fruit having a weight average molecular weight (M_r) of about 200 000 whereas that from ripe fruit has an M_r of about 98 000 and is more polydisperse (Seymour and Harding, 1987; Seymour *et al.*, 1987).

Although there is a wealth of compositional data on fruit cell walls, actual detailed biochemical structures of the various polymers is lacking. Thus the

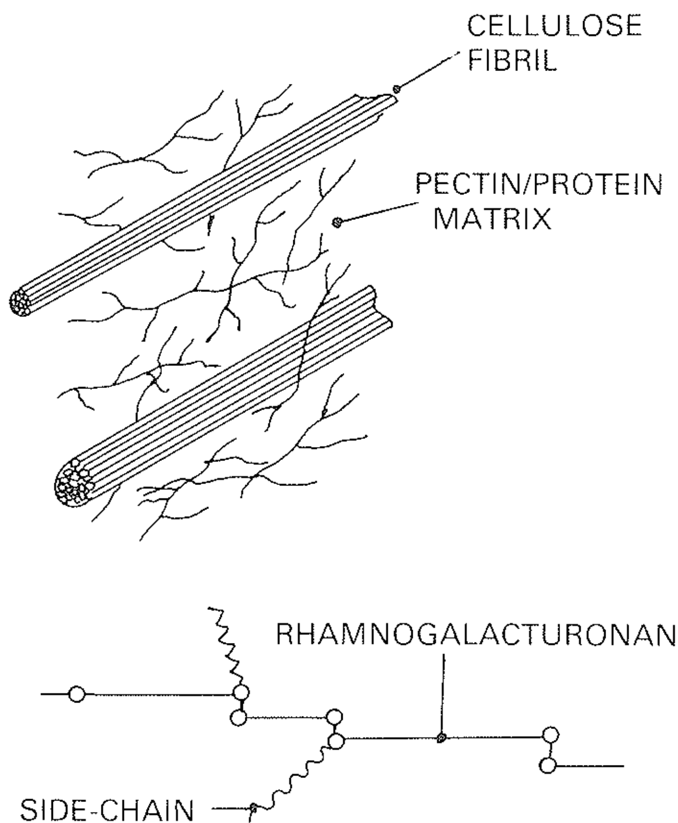


Figure 3. Simplified models for the plant cell wall and middle lamella pectins. Cell wall model (above) and enlarged fragment of a pectin molecule showing 1, 4-linked galacturonic acid chains (——) interspersed with rhamnose (O) and containing irregular side-chains of neutral sugars (∼).

pectin structure shown in *Figure 3* is only a model based on available compositional and linkage data (Seymour *et al.*, 1990) and evidence from other plants (Darvill *et al.*, 1980). Plant cell walls are extremely complex structures consisting not only of pectin but also of other carbohydrate polymers such as hemicellulose and cellulose, as well as protein. The interconnections between these various components and the actual three-dimensional structure of the wall is far from understood. It is beyond the scope of this review to cover this cell wall structure. The simplified model depicted in *Figure 3* is sufficient for the purposes of this paper. For more detailed information several excellent reviews are available (Aspinall, 1980; Darvill *et al.*, 1980; McNeil *et al.*, 1984; Fry, 1986).

The basic model of the cell wall depicted in *Figure 3* shows the wall to consist of fibrils of cellulose polymers coated with hemicellulose and embedded in a matrix of pectins and proteins. The fruit middle lamella is thought to consist of a structure something like the basic model but with a relatively high level of pectic polymers compared to the other wall components. The most obvious changes during ripening occur in these pectic polymers, and in particular in the rhamnogalacturonan. A model structure for the fruit middle lamella pectin is shown in *Figure 3*. The α-1,4-linked galacturonic acid backbone is interspersed with rhamnose residues, the observed ratio of galacturonic acid to rhamnose being 43 : 1 (Seymour *et al.*, 1990). It is unclear whether these rhamnose residues occur randomly throughout the polymer or are organized in clusters within so-called 'hairy regions' (Darvill *et al.*, 1980). The galacturonic acid residues can carry either a free carboxyl group (de-esterified) or an *O*-methyl group (esterified) at the C-6 position. The extent of esterification is thought to be in the region of 50–60% (Seymour *et al.*, unpublished). Again, whether the distribution of these esterified residues is either ordered or random in nature is unknown. A proportion of the rhamnose residues have side-chains of neutral sugars, primarily galactose and arabinose. The actual structure and distribution of these side-chains is again subject to some debate. Using this model we can suggest putative enzyme activities capable of causing the observed changes in solubilization and depolymerization of the rhamnogalacturonan during ripening.

Tomato fruit have been shown to contain a wide range of cell wall hydrolases. Several comprehensive surveys of these and their possible involvement in wall degradation are available (Huber, 1983; Tucker and Grierson, 1987). In this review we shall concentrate on those capable of degrading the acidic pectins. There are two key enzymes found in tomato fruit which can degrade rhamnogalacturonan, namely pectinesterase (EC 3.1.1.11) and polygalacturonase (EC 3.2.1.15). The action of these two enzymes on pectin is depicted in *Figure 4*.

Pectinesterase (PE) is present in green fruit and its activity increases only slightly during ripening (Hobson, 1963; Tucker, Robertson and Grierson, 1982). However, this activity is associated with several isoenzymes (Pressey and Avants, 1972) and the ratio of the two isoenzyme forms found in the cultivar Ailsa Craig has been shown to alter dramatically during ripening (Tucker, Robertson and Grierson, 1982). In contrast, polygalacturonase

PECTINESTERASE

POLYGALACTURONASE

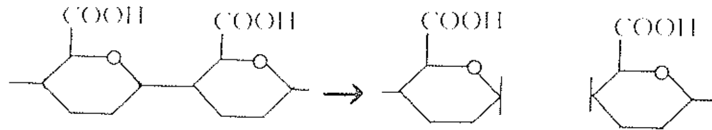


Figure 4. Mode of action of pectolytic enzymes.

(PG) activity is absent in green fruit and appears during ripening (Hobson, 1964; Tucker, Robertson and Grierson, 1980) due to *de novo* synthesis (Grierson and Tucker, 1983). This PG activity is also associated with two isoenzyme forms, PG1 and PG2, which appear sequentially during ripening (Tucker, Robertson and Grierson, 1980). Early in ripening, PG1 is the only isoform present, but PG2 rapidly accumulates and is the major isoform in ripe fruit. This PG2 can also be separated into two distinct forms, PG2A and PG2B, which are thought to differ only in their relative extents of glycosylation (Mohd Ali and Brady, 1982).

One model for the action of these two enzymes is that they act in a sequential and synergistic manner. The action of PE in the de-esterification of galacturonic acid residues, might generate sites for PG action, the latter being inactive against methylated polyuronides. However, if this does occur during ripening, then it is only to a very limited extent, as is shown by *in vitro* cell wall degradation studies (Seymour, Lasslett and Tucker, 1987). If cell walls are isolated from green fruit and subsequently digested with purified PG2, then the resultant solubilized pectin is almost identical in amount and size to that solubilized *in vivo* during ripening. In comparison, treatment with both PG2 and purified PE results in the extensive solubilization of polyuronide and its almost complete depolymerization, which is totally different from events during ripening. Thus although PE and PG may interact, this is severely limited *in situ*. One possible explanation for this limitation may be a spatial separation of these two enzymes within the wall. Immunolocalization of PE2,

the isoform of the enzyme which increases during ripening, places this enzyme in the primary wall adjacent to the cell membrane (Seymour *et al.*, unpublished), while it is presumed that PG resides in the middle lamella, where it acts to degrade the rhamnogalacturonan.

There is much evidence in the literature to support a key role for PG in pectin degradation and hence possibly fruit softening. This evidence can be summarized as follows. The action of PG is consistent with the observed changes in pectin. There is a correlation between PG content and rate of softening within a wide range of tomato cultivars (Hobson, 1965). This correlation extends into several of the available ripening mutants. Thus both never-ripe (*Nr*) (Hobson, 1980; Tucker, Robertson and Grierson, 1980) and alcobaca (*alc*) (Mutschler *et al.*, 1988) have much reduced PG activity and also exhibit slower softening. The ripening inhibitor (*rin*) mutant has little, if any, PG activity and does not soften (Tigchelaar, McGlasson and Buescher, 1978; Hobson, 1980; Tucker, Robertson and Grierson, 1980). Finally, purified PG1 or PG2 can bring about *in vitro* changes in cell wall polyuronides similar to those occurring *in situ* (Seymour, Lasslett and Tucker, 1987) and when applied to green tomato discs can mimic the ultrastructural changes occurring in the middle lamella (Crookes and Grierson, 1983). A fuller survey of this and other circumstantial evidence for the importance of PG for wall degradation and softening can be found in Brady (1987).

This evidence would indicate a key role for PG during texture development in the tomato fruit. However, although PG does undoubtedly play a role in this process, the results from transgenic and mutant fruit (covered in the next two sections of this chapter) demonstrate that other, as yet unidentified, factors are also important. Thus there is a need for further work to elucidate both enzymic and non-enzymic mechanisms of softening. Since PG is absent from green fruit and is synthesized *de novo* during ripening, this presumably implies that the gene for this protein is switched on. This and its probable importance in softening made PG a natural choice as a target for genetic manipulation.

Gene expression during tomato fruit ripening

Ripening in many fruit has been shown to be not only a degradative but also an anabolic process. Thus, continued incorporation of radiolabelled amino acids into proteins during ripening has been demonstrated in a variety of climacteric fruit, such as pear (*Pyrus communis*), banana and avocado (*Persea americana*) (Richmond and Biale, 1966; Frenkel, Klein and Dilley, 1968; Brady *et al.*, 1970; Brady and O'Connell, 1976) and this list includes tomato (DeSwardt, Swanepoel and Dubenage, 1973). The analysis of these radiolabelled proteins by two-dimensional gel electrophoresis clearly demonstrates that during tomato ripening the production of many proteins continues while some others decline or cease synthesis altogether, and that new proteins are synthesized (Baker, Anderson and Hruscka, 1985). This has been confirmed by Biggs, Harriman and Handa (1986) who analysed, using one-dimensional SDS-PAGE, total proteins extractable from tomato fruit at

Table 2. Major changes in proteins or translatable mRNA products during ripening

Proteins*			Translation products*			Translation product increases†	
Increase	Decrease	Fluctuate	Increase	Decrease	Fluctuate	Major	Minor
94	106	85	116	41	156	190	80
44	98	60	89	35	53	55	57
34	88	26	70		39	48	44
20	76	21	42		30	35	20
12	64	16	38		14		
	52		33				
	48		31				
	45		29				
	36		26				
	28						
	25						
	15						

* From Biggs, Harriman and Handa (1986).
† From Grierson *et al.* (1985).
All values quoted are in kDa.

various stages of development. This group reported increases in five polypeptides, decreases in 12 polypeptides and fluctuations in a further five polypeptides during ripening. These changes and their molecular weights are summarized in *Table 2*. Some of the proteins that decline are probably chloroplast proteins not required during ripening (Bathgate *et al.*, 1985). Those that appear are presumably key ripening enzymes such as PG.

These changes in total protein must be accompanied by corresponding changes in translatable mRNA. Labelling studies in a range of climacteric fruit again indicate continued synthesis of rRNA, tRNA and polyA-containing RNA (Richmond and Biale, 1967; Marei and Romani, 1971) and again this list includes tomato fruit (DeSwardt, Swanepoel and Dubenage, 1973; Rattanapanone, Grierson and Stein, 1977). That this polyA-containing RNA contained new, ripening-specific translatable messages was first demonstrated in tomato fruit by Rattanapanone, Speirs and Grierson (1978). This group isolated mRNA from fruit at various stages of ripeness and, following *in vitro* translation, analysed the protein products by SDS-PAGE. This analysis clearly demonstrated that some mRNAs persist throughout ripening while others either decline or appear. Since this initial investigation several groups have documented the observable changes in mRNAs during ripening. Grierson *et al.*, (1985) reported that at least six abundant mRNAs present in immature green fruit decline in quantity during maturation and ripening, while at least eight new translatable mRNAs appear during ripening (*Table 2*). Biggs, Harriman and Handa (1986) carried out a similar experiment but extended this by analysis of the translation products using both one- and two-dimensional electrophoresis. This group identified increases in nine, decreases in two and fluctuations in five mRNAs using a one-dimensional SDS-PAGE (*Table 2*) and even more extensive changes were evident using the two-dimensional approach. There are significant differences between these two sets of results, as summarized in *Table 2*. This may be due to the use of different tomato cultivars (Ailsa Craig and Rutgers, respectively), different gel conditions or by sampling at different stages of development. This type of analysis is relatively crude and will only detect the major mRNA species present in the tissue. A more sensitive analysis of changes in gene expression can be achieved by employing recombinant DNA technology.

Table 3. Properties of some ripening-related cDNAs (from Slater *et al.*, 1985; Maunders *et al.*, 1987)

Clone	Insert (kbp)	Translation product (kDa)	mRNA size	Ripening specific
pTOM5	1·64	48	1·96	+
pTOM6	1·65	55	1·58	+
pTOM13	1·40	35	1·40	+
pTOM36	1·30	52	1·47	+
pTOM75	0·95	28	1·20	–
pTOM99	0·90	44	1·52	+
pTOM137	1·00	57	1·68	–

Several groups, both in the UK and the USA, have generated ripening-related cDNA libraries from tomato fruit. One approach described by Slater

et al. (1985) was to extract mRNA from 'ripe' Ailsa Craig tomato fruit and then anneal the corresponding cDNAs into the *Pst*I site of the plasmid pAT153. Transformants in *Escherichia coli* strain C600 were then screened by differential hybridization to either 'green' or 'ripe' sequences, to yield 146 ripening-specific clones. These were shown to consist of 11 groups and eight unique clones. Seven of the families were chosen for further study (*Table 3*). The size of insert in each group representative was determined (Slater *et al.*, 1985; Maunders *et al.*, 1987; *Table 3*) and shown to range from 0·9 to 1·8 kbp. Hybrid release translation experiments were then used to determine the polypeptide molecular weight of the mRNA translation product corresponding to each cDNA (*Table 3*). A comparison of these products with the data of Grierson *et al.* (1985), which is summarized in *Table 2*, for the changes in translatable mRNAs during ripening shows a strong correlation. The size of the mRNA corresponding to each cDNA clone has been estimated by Northern blotting (Maunders *et al.*, 1987; *Table 3*). It can be seen that the insert represents near full-length copies of the mRNA in several cases, and especially in the case of pTOM6 and pTOM13. Maunders *et al.*, (1987) also tested the organ specificity of these seven cDNA clones (*Table 3*). All except pTOM75 and pTOM137 were specific to ripe fruit, dot blot hybridization failing to detect any hybridization to RNA extracted from unripe fruit, root or leaf tissue. Hybridization to both pTOM75 and pTOM137 was also detected using RNA from root and leaf tissue. Further work on the pTOM13 clone (Smith, Slater and Grierson, 1986) has shown that although absent in green fruit and undamaged leaves, expression of the mRNA corresponding to this clone can be initiated in both tissues in response to wounding. The resultant 35 kDa protein, which seems to be associated with both ripening and wound-induced ethylene, may in some way be involved in, or responding to, the synthesis or presence of ethylene, respectively. As such this would represent a good candidate for genetic manipulation of ethylene synthesis or perception in fruit.

During ripening on the plant several different accumulation patterns were observed for the mRNAs corresponding to these various clones (Maunders *et al.*, 1987). For pTOM5, pTOM6 and pTOM137 expression peaked at the orange stage of ripening and thereafter declined slowly. The concentrations of pTOM13, pTOM6 and pTOM99 mRNAs rose more rapidly, then after a slight decline continued to rise to a peak, again corresponding to the orange stage of ripening, before declining. For pTOM75 expression was apparent at a low level in green fruit and rose very rapidly early on in ripening, a high level of expression was then maintained until the fully red stage of ripening. These results show that although these clones represent specific sequences which are all switched on during ripening, the differences in the kinetics of expression suggest that several control mechanisms may be active.

These seven clones have also been used to investigate the effect of exogenous ethylene, heat and silver ions on gene expression during ripening (Grierson *et al.*, 1987). If green fruit are exposed to exogenous ethylene, we again see variation in the kinetics of expression of mRNAs (Maunders *et al.*, 1987). For all clones except pTOM5 an increase in expression is detectable

within 24 h of treatment. Expression of pTOM5 is detectable after a lag of between 24 and 36 h. Differential effects on expression have also been noted in response to heat (Picton and Grierson, 1988) and silver ions (Davies, Hobson and Grierson, 1988).

In an alternative approach to the isolation of ripening-related cDNAs, Lincoln *et al.* (1987) concentrated on identifying genes in tomato fruit responsive to ethylene. This group defined four early stages of ripening, up to the first detectable colour change, called mature green 1–4 (MG1, MG2, MG3 and MG4). In MG1 fruit the locular tissue was firm, in MG2 a small amount of gel was present, in MG3 fruit formation of locular gel is complete and at the MG4 stage fruit pigmentation is just detectable. Only at MG3 and MG4 stages were detectable increases in ethylene production observed. This group prepared a cDNA library enriched for MG4-specific sequences, which was then screened with a probe enriched for sequences that appear when MG1 fruit are exposed to ethylene. This enabled the isolation of three ethylene-induced, ripening-specific clones, E4, E8 and E17. Although these are not as fully characterized as the 'pTOM' series of clones, Lincoln *et al.* (1987) have demonstrated a differential expression of this limited range of clones during ripening.

The effect of various ripening mutants on the expression of these ripening-specific clones has also been investigated (Dellapenna, Kates and Bennett, 1987; Lincoln and Fischer, 1988; Knapp *et al.*, 1989). Knapp *et al.* (1989) used the 'pTOM' range of clones to monitor expression in *rin* and *Nr* fruit compared to normal. In *rin* fruit expression of mRNA corresponding to pTOM5, pTOM6, pTOM13, pTOM36 and pTOM99 was reduced throughout ripening, and in the case of pTOM6 this reduction was particularly extreme. Those mRNAs homologous to pTOM75 and pTOM137 were not reduced in *rin* fruit; in fact pTOM137 expression was actually increased slightly in the mutant. In the case of the *Nr* mutant levels of all mRNAs except those corresponding to pTOM5 and pTOM6 were as normal. The two suppressed genes for pTOM5 and pTOM6 were expressed at 44% and 31% of maximum normal levels, respectively. Lincoln and Fischer (1988) also investigated the effect of the *rin* mutation on the expression of their ethylene-related genes. They found that E4 and E17 expression was barely detectable and that of E8 was significantly depressed in the *rin* fruit. However, a further clone J49, isolated by differential screening of a cDNA library generated from ethylene-treated MG1 fruit (Lincoln *et al.*, 1987) was not affected in the *rin* fruit.

Identification of clones corresponding to polygalacturonase

Presumably one at least of the ripening-specific clones corresponds to the putative softening enzyme, polygalacturonase (PG). This enzyme is synthesized *de novo* during ripening (Grierson and Tucker, 1983) and activity can be resolved into three isoenzyme forms PG1, PG2A and PG2B (Pressey and Avants, 1973; Tucker, Robertson and Grierson, 1980; Mohd Ali and Brady, 1982). The two isoforms PG2A and PG2B are thought to arise simply from the differential glycosylation of a common polypeptide (Mohd Ali and Brady,

1982) and so can, for the purpose of this discussion, be considered as a single isoform, PG2. Both PG1 and PG2 have been purified and analysed by SDS-PAGE (Tucker, Robertson and Grierson, 1980; Mohd Ali and Brady, 1982; Moshrefi and Luh, 1984). It has been demonstrated conclusively that PG2 is a single polypeptide with an apparent molecular weight of 46–47 kDa. PG1 consistently contains two polypeptides, one co-migrates with that found in PG2 and the other is slightly smaller, with an apparent molecular weight of 38–41 kDa. It is likely that the 46 kDa polypeptide in each case represents the catalytic subunit and in PG1 this is associated with an ancillary polypeptide of unknown function. One suggested function for this is that it acts as a targetting protein for PG within the cell wall (Knegt, Vermeer and Bruinsma, 1988). The premise that the 46 kDa polypeptide is closely similar in both PG1 and PG2 is supported by the fact that antibodies raised against purified PG2 cross-react with PG1 (Tucker, Robertson and Grierson, 1980; Mohd Ali and Brady, 1982) and that purified PG2 can be converted *in vitro* to a form similar to PG1 (Tucker, Robertson and Grierson, 1981). All the evidence to date therefore suggests that the catalytic subunits of all the PG isoforms are identical and are the product of a single gene.

Assuming a single gene product, several approaches have been employed to identify a cDNA clone corresponding to PG protein. These include the immunological approach of Dellapenna, Alexander and Bennett, (1986). This group prepared a cDNA library in pARC7 using mRNA isolated from ripe tomato fruit. Total library plasmid was then used to construct an expression library in λ Charon 16 by linearizing the plasmids with *Sst*I and ligation of the cDNA to the phage β-galactosidase gene. Screening of the resultant plaques was performed with PG-specific antiserum and immunologically positive clones selected. This approach generated several positive clones with inserts of between 550 and 900 base pairs. A representative clone, pPG16, was selected for further study. Hybrid release experiments using pPG16 selected an mRNA coding for a 54 kDa translation product, which was in turn immunoprecipitable by PG antiserum. This translation product co-migrates with a 54 kDa protein immunoprecipitable with PG antiserum from the *in vitro* translation products derived from the translation of total mRNA from ripe fruit and represents one of the major novel translation products previously identified in ripe fruit. Immunoblots of total *in vitro* translation products using this PG antiserum also specifically identified a 54 kDa polypeptide as the putative precursor of PG (Sato *et al.*, 1984). The pPG16 clone was later used by Fischers' group to identify a clone within one of their libraries, E41, as being a PG clone by cross-hybridization (Lincoln *et al.*, 1987).

Unequivocal identification of a PG clone was provided by Grierson *et al.* (1986). This group purified PG2 protein and obtained the sequence of the first 30 N-terminal amino acids. Comparison of this data with sequence data obtained from the pTOM6 clone shows full sequence homology (*Figure 5*). As shown in *Table 3*, pTOM6 is complimentary to a mRNA coding for a 55 kDa translation product. Sheehy *et al.* (1987) have extended this sequence comparison even further. This group identified a PG cDNA clone using

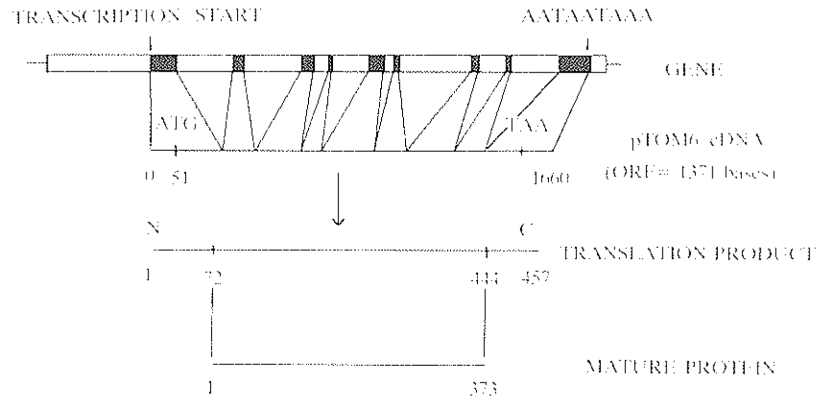


Figure 5. Expression of polygalacturonase gene during tomato fruit ripening.

immunological screening of an expression library in λgt11. Their 1·6 kb cDNA, like that of pTOM6, predicts an open reading frame encoding a polypeptide of 457 amino acids. This sequence has been compared with that for PG2 protein, which has had 80% of its amino-acid sequence determined, and the two match exactly. The difference in size between the 54 kDa translation product and the 46 kDa mature protein can be explained by extensive post-translational modification of the protein. The extent of this modification is shown by a comparison of the amino-acid sequence predicted from the cDNA with that actually observed in the mature protein (*Figure 5*). The 54 kDa contains a 71 amino acid N-terminal presequence (Grierson *et al.*, 1986; Sheehy *et al.*, 1987) which is cleaved during processing. The C-terminal region also contains a 13 amino acid extension which is also cleaved during processing (Sheehy *et al.*, 1987). The role of these sequences is unknown but presumably the N-terminal region contains signals for secretion and targetting of the PG to the cell wall. The first 22 amino acids of this N-terminal region are hydrophobic in nature and therefore could represent a classical secretion signal (Grierson *et al.*, 1989).

Having identified pTOM6 as the clone corresponding to PG, this was then used to screen a tomato genomic library and isolate genomic fragments containing the PG gene (Bird *et al.*, 1988). The gene has been sequenced and its general structure is summarized in *Figure 5*. The gene covers approximately 7 kb and contains eight intron sequences ranging in size from 99 to 953 bp. The genomic fragments also contain about 1·4 kbp of the 5′ untranscribed region of the gene and this has also been sequenced. Southern analysis of genomic fragments by Bird *et al.* (1988) would seem to indicate a single PG gene per haploid genome and this supports the theory of only one gene product representing the active subunit in all the isoforms of PG.

At present no firm identification for any of the other ripening-related

clones can be made. Lincoln *et al.* (1987) tentatively, on the basis of sequence homology, linked the gene product of E17 to tomato proteinase inhibitor I (Graham *et al.*, 1985), an enzyme thought to protect plants from insect predators. Other clones have been sequenced, for instance pTOM13 (Holdsworth *et al.*, 1987), and others are in the process of being sequenced.

Transformation of tomato plants

The availability of defined cDNA clones and genomic clones for PG has made this an obvious choice for transformation experiments to investigate tissue-specific gene expression and to attempt to regulate softening by genetic engineering (Kramer, Sheehy and Hiatt, 1989). Foreign DNA constructs can be integrated into the tomato genome by *Agrobacterium*-mediated transformation (Koorneef *et al.*, 1987). The DNA to be transferred is incorporated, along with a selection gene often coding for antibiotic resistance, into a disarmed Ti plasmid from *Agrobacterium tumefaciens*. Following infection, transformed cells are selected by growth in the presence of the antibiotic and plants regenerated. This approach has been employed by several groups to successfully transform tomato plants.

The identification of genomic clones for PG (Bird *et al.*, 1988) allowed the isolation of the 5′ flanking region of the gene. Since PG expression is known to be both fruit and ripening specific, this 5′ region may contain control sequences responsible for regulating this expression. This hypothesis was tested using a DNA construct in which a transcription fusion between a putative 1·4 kbp PG promoter fragment and the bacterial reporter gene, chloramphenicol acetyltransferase (CAT) (EC 2.3.1.28), was transformed into tomato cells (Bird *et al.*, 1988). Regenerated tomato plants were tested for CAT expression, which was found exclusively in ripening fruit. No CAT activity was detectable in unripe fruit or any other tissue tested. Non-transformed plants exhibited no CAT activity at all, whereas control plants transformed with the CAT gene but under the control of the constitutive cauliflower mosaic virus 35S (CaMV 35S) promoter showed CAT expression in all tissues tested (Grierson *et al.*, 1990). This ability to engineer plants for tissue- or developmentally regulated expression is useful for future applications, especially if the gene being transferred would be detrimental to some parts of the plant yet beneficial to others.

The manipulation of softening may be achieved by the downregulation of the PG gene. This has been accomplished independently by two groups, each using similar antisense RNA technology. This approach has been used to successfully downregulate other plant enzymes, for instance chalcone synthase (EC 2.3.1.74) (van der Krol *et al.*, 1988), and has also been used in animals. A recent review of the applications of this powerful technology is that by van der Krol, Mol and Štuitze (1988). The precise mechanism of gene downregulation by antisense RNA is unknown. The basic methodology is to insert into the genome an inverted copy of the gene, or part thereof. The resultant expression of antisense RNA then somehow prevents full expression of the corresponding endogenous sense gene.

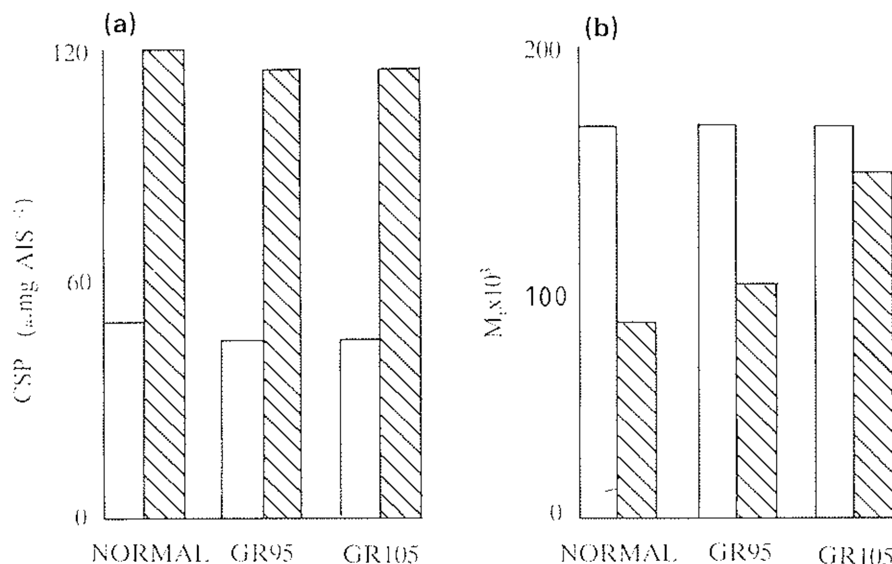


Figure 6. Effect of reduced polygalacturonase (PG) activity on cell wall pectin. (a) Levels of chelator-soluble polyuronide (CSP). (b) Weight average molecular weight (M_r) of soluble pectin. From normal or antisense fruit expressing 20% (GR95) or 1% (GR105) residual PG activity at either the green □ or ripe ▨ stages of development.

Smith *et al.* (1988) used a 730 bp *Hin*fI fragment from the 5′ end of the pTOM6 cDNA as the basis for their antisense experiments. This fragment contains 50 bp of the untranslated region, and was cloned in the reverse orientation between the CaMV 35S promoter (5′) and nopaline synthase terminator (3′) sequences. This construct was then inserted into the Bin 19 vector (Bevan, 1984), transformed cells selected by kanamycin resistance and plants regenerated. Sheehy, Kramer and Hiatt (1988) used a full-length PG cDNA in reverse orientation, again under the control of the CaMV 35S promoter. Both groups demonstrated the constitutive expression of antisense RNA throughout the transformed plants, and a consequent marked reduction in PG activity in transformed fruit. The level of PG reduction was variable between individual transformed plants, ranging from 50 to 95% and from 70 to 90% inhibition in the transformants generated by either Smith *et al.* (1988) or Sheehy, Kramer and Hiatt (1988), respectively. Subsequent work on the inheritance of these antisense genes has shown them to act in an apparently Mendelian fashion, and also generated a range of F_1 transformants in which PG activity has been reduced to less than 1% of the activity in corresponding normal fruit (Smith *et al.*, 1990).

The effect of these antisense transformations on pectin degradation and softening has been investigated (Smith *et al.*, 1988; Schuch *et al.*, 1989; Grierson *et al.*, 1990). There is little or no effect on the onset of the increase in chelator-soluble polyuronide or on the extent to which this solubilization proceeds (*Figure 6*). This soluble pectin can be analysed for both weight average molecular weight and polydispersity using gel filtration and analytical

ultracentrifugation techniques (Seymour and Harding, 1987). This analysis demonstrates a clear effect of reduced PG activity (*Figure 6*). The reduction in PG activity is accompanied by a reduction in pectin depolymerization. The effect on softening is difficult to assess since it is difficult to accurately quantify this parameter. Tests using a standard Stevens texture analyser to measure fruit compressibility failed to detect any appreciable differences between normal and transformed fruit (Smith *et al.*, 1988). It is difficult to assess the relative contributions of pericarp, locular, radial arms or skin firmness to these measurements, and it is likely that PG reduction will have an effect on only some of these. However, it is clear that PG reduction is having an effect on pericarp integrity as shown by the extended storage time and reduction in transport damage exhibited by transformed fruit (Robertson *et al.*, unpublished). The results from antisense fruit would implicate PG in the depolymerization of pectin but perhaps not in the solubilization of this pectin. However, it should be noted that PG levels have yet to be reduced to zero and it is possible that the low levels of PG present in antisense plants are sufficient to initiate pectin solubilization. It is clear, however, that the extent of this solubilization once initiated is not correlated with the amount of PG activity present in the fruit.

Similar conclusions can be arrived at by studying cell wall changes in the *Nr* and *rin* mutants during ripening (Seymour *et al.*, 1987). In this case, increases in chelator-soluble polyuronide commence concurrently with the appearance of PG activity but the extent of this solubilization cannot be correlated with the levels of PG activity. Thus normal (with 100% PG activity) and *Nr* (with 10% PG activity) have similar levels of chelator-soluble pectin after 10 days of ripening. However, depolymerization of this pectin in *Nr* fruit is reduced and these fruit can, like the antisense fruit, be stored for long periods. In *rin* fruit, levels of chelator-soluble polyuronide increase at about 77 days after the first change of colour and this coincides with the first detectable (0·5%) PG activity. No apparent depolymerization of the pectin occurs at this age and the *rin* fruit remain very firm.

It seems likely, therefore, that although PG may be required to initiate the increase in solubilization, it does not regulate the extent of this solubilization. It is important to note, however, that this work relates to chelator-soluble pectin. If similar experiments were carried out to determine the water-soluble pectin, then different results may be obtained. For instance, water-soluble pectin, unlike chelator-soluble pectin, may indeed be markedly reduced in the antisense PG fruit (Robertson, unpublished). The extent of pectin solubilization may not be related to softness of the pericarp; however, more work needs to be done to establish the significance of chelator- versus water-soluble pectin in this regard. In contrast to pectin solubilization there is clear evidence for the involvement of PG in pectin depolymerization, and this parameter could well be related to pericarp texture changes and, hence, shelf-life of the fruit.

These conclusions are partially supported by the work of Giovannoni *et al.* (1989). This group placed a PG gene under the control of their E8 promoter and then transformed *rin* plants with this chimeric construct. The E8

promoter is activated in *rin* fruit in response to either ethylene or its analogue, propylene. Exposure of transformed *rin* fruit to propylene resulted in the accumulation of PG activity up to about 60% of the level found in normal fruit. This increase in PG activity was accompanied by an increase in chelator-soluble polyuronide but no decrease in fruit compressibility. Unfortunately, this group did not analyse this pectin for depolymerization. This result could indicate that PG plays no role in softening since transformed *rin* fruit did not exhibit any softening despite increased PG synthesis. However, the *rin* is a pleiotropic mutant and could well be deficient in other key softening enzymes, for example it is known that *rin* fruit do not exhibit normal pectinesterase isoenzyme changes during ripening (Tucker, Robertson and Grierson, 1982).

Conclusion

This work provides a clear demonstration of how antisense technology can be used to alter a biochemical pathway (pectin depolymerization) and hence influence texture specifically and with no apparent effect on any other aspect of ripening. The accumulation of the pigment lycopene, ethylene synthesis and changes in enzymes such as invertase (EC 3.2.1.26) and pectinesterase are all unaffected by the antisense transformations (Sheehy, Kramer and Hiatt, 1988; Smith *et al.*, 1988; Smith *et al.*, 1990). The reduction in PG has had a detectable effect on the physiology of softening; however, the exact link between the pectin changes and softening remains to be elucidated, as does the involvement of other enzymes. These other enzymes, once identified, would be obvious candidates for future attempts to manipulate softening.

The work to date has been concentrated on tomato fruit. Similar approaches for other fruit would quite likely prove successful. It is already possible to transform apples and strawberries (James, Passey and Barbara, 1990) and undoubtedly techniques for other fruit are, or soon will be, available. Although the softening mechanisms in other fruit may differ slightly to that described for tomato, degradation of pectin does seem to be a fairly common phenomenon during ripening, and PG is found in a wide range of fruit (Huber, 1983; Tucker and Grierson, 1987). Hopefully, the experience gained from using tomato will eventually be used to extend this technology to other fruit.

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