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Transgenic Tomato Technology: Enzymic Modification of Pectin Pastes

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Introduction

Polysaccharides have been manipulated chemically, or by the use of added enzymes, for many years in order to improve their functionality for various industrial processes (Tucker and Woods, 1995). However, this chemical modification is often difficult to control. Genetic engineering presents the prospect to manipulate polysaccharide structure actually within the organism of interest. It also allows the prospect for these modifications to be more tightly controlled. This modification cannot take place directly but can via the manipulation of enzymes involved in either synthesis or degradation of the polysaccharides. Manipulation of synthesis is currently difficult given the relative scarcity of biochemical, and in particular genetic, information on the enzyme systems involved. However, advances in this area are being made and no doubt manipulation of polysaccharides by the modification of genes encoding biosynthetic enzymes will occur in the near future. In contrast, the genetic modification of polysaccharide degrading enzymes has been achieved and has already resulted in commercial products.

Cell wall polysaccharides are often major determinants in the rheological properties of plant foods and their processed products. This is particularly the case for pectin in determining the texture of intact fruit and the viscosity of pastes made from fruit. The structure of these plant cell wall polymers, in processed products, can be modified by the application of exogenous enzymes. Alternatively, structural alterations can be brought about by the modification of endogenous enzyme activities. In many instances the action of endogenous cell wall hydrolytic enzymes may be detrimental for food quality and in these cases heating is often used to inactivate these enzymes. A particular instance is the use of a '*hot-break*' process for the inactivation of pectolytic enzymes during the production of tomato pastes.

The plant cell wall is a complex structure (Carpita and Gibeaut, 1993) and it is beyond the scope of this paper to describe this structure. Basically, the plant cell wall

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is composed of a network of cellulose microfibrils. These fibrils consist of chains of linear β(1–4)-linked glucans held together by intra-polymer hydrogen bonds. The fibrils within any one plane of the wall are often orientated parallel to each other: a typical cell wall being composed of several such planes with adjacent planes of fibrils set at an angle to each other to give a laminated structure to the wall. The typical cell wall also contains hemicellulose polymers. In dicotyledonous plants, such as the tomato, the major hemicellulose is usually xyloglucan. This is composed of a linear β(1–4)-linked glucan backbone substituted with glucose, galactose and some fucose residues. The hemicellulose can hydrogen bond to the surface of the cellulose microfibrils but is also large enough to actually span the gap between two adjacent fibrils. In this way the cellulose and hemicellulose act to form a kind of interconnected framework. This is then embedded in a gel-like matrix composed of pectins and proteins. The major pectins are galacturonan polymers containing a backbone of β(1–4)-linked galacturonic acid residues. These can have either the free carboxylic acid group at the C6 carbon or alternatively this carbon can be methyl esterified. The galacturonic acid backbone can be interrupted by rhamnose residues. In the case of the pectic polymer-rhamnogalacturonan I – these rhamnose residues occur alternate with galacturonic acid residues in runs of up to several hundred residues. The rhamnose residues can also be linked to neutral sugar side chains of galactose and/or arabinose residues.

Fruit softening is often accompanied by degradation of the pectin components of the cell wall. These changes include a decline in the degree of esterification, a loss of neutral sugars such as galactose and arabinose and an increased solubility and depolymerisation (Tucker, 1993). These changes in pectin structure are brought about by the action of cell wall associated enzymes during ripening. In particular polygalacturonase (E.C 3.2.1.15), which has the capacity to depolymerise the polyuronide chains in pectin, pectinesterase (E.C 1.1.1.11), which can de-esterify the galacturonic acid residues and β-galactosidase thought to be important during the loss of neutral sugars. Pectinesterase and β-galactosidase are associated with most fruit. Polygalacturonase is found in a large number of, but not all, fruit. All three enzymes often undergo significant changes in expression during ripening. The expression and molecular biology of these enzymes have been most extensively studied in tomato fruit. Tomato fruit during ripening show all the typical changes in pectin structure outlined above.

This review describes work largely carried out at Nottingham University to reduce the expression of these cell wall degrading enzymes in tomato fruit. This has been achieved in each case by the application of gene silencing techniques. It is again beyond the scope of this review to consider the mechanism of action of gene silencing. Instead, the reader is directed to a recent conference proceedings for further details (Lycett *et al.*, 1996). Silencing can in effect be brought about by two mechanisms: *antisense* or *co-suppression*. Antisense involves the creation of an artificial gene in which the coding region for all, or part, of an endogenous gene is placed in a reverse (antisense) orientation under the control of a suitable promoter. This antisense gene is then transformed into the plant. In the cell the antisense gene directs the transcription of an antisense RNA molecule. This, by definition, is complimentary to the mRNA product of the normal endogenous gene. The theory is that these two complimentary RNA molecules base pair within the nucleus thus preventing the translation of the

mRNA and formation of the target enzyme. A similar gene silencing also occurs if the transgene, instead of containing coding sequences in an antisense orientation, has the sequence in the normal orientation. In this instance, both the transgene and the target gene are silenced, resulting in a phenomenon known as co-suppression. The mechanism(s) for co-suppression have not been fully elucidated.

These techniques have been used at Nottingham, and elsewhere, to reduce the expression of polygalacturonase, pectinesterase and β-galactosidase in tomato fruit. The effects on the cell wall metabolism, softening and processing of the fruit have been examined and attempts made to predict the structure/function relationships for pectin in this biological system.

Enzymes involved in fruit ripening and their down regulation

A wide range of enzymes with the potential to degrade cell walls have been found associated with ripening fruit (*Table 1*). In many cases these have been purified, characterised and corresponding DNA sequences identified. Work at Nottingham has concentrated on the pecteolytic enzymes polygalacturonase, pectinesterase and β-galactosidase.

POLYGALACTURONASE

Polygalacturonase (PG) activity is almost non-detectable in green tomato fruit (Hobson, 1964). However, the activity increases dramatically during ripening. This PG activity has been resolved into at least two isoforms (PG1 and PG2) using ion-exchange chromatography (Pressey and Avants, 1973; Tucker *et al.*, 1980). During ripening PG1 is the first detectable isoform, however, PG2 activity rapidly becomes the dominant isoform in ripe fruit (Tucker *et al.*, 1980). Both PG1 and PG2 have been purified and characterised (Moshrefi and Luh, 1984; Tucker *et al.*, 1980). The PG2 isoform has been shown to be composed of a single polypeptide chain with a molecular weight of around 43 kD, and this has been fully sequenced (Sheehy *et al.*, 1987). In contrast, purified PG1 appears to be composed of two heterologous polypeptide chains with molecular weights of around 43 kD and 38 kD (Moshrefi and Luh, 1984). The 43 kD polypeptide cross reacts with antibodies raised against purified PG2 and tryptic digestion of the 43 kD polypeptide from both PG1 and PG2 result in the same digest pattern of peptide fragments (Tucker *et al.*, 1980). These results suggest that the 43 kD polypeptide in each isoform is identical and presumably represents the catalytic subunit. Several groups have isolated clones corresponding to

Table 1. List of cell wall degrading enzymes commonly associated with ripening fruit

Arabinosidase
Cellulase
α-Galactosidase
β-Galactosidase
Mannosidase
Polygalacturonase
Pectinesterase
Pectate lyase
Xylosidase

Table 2. Enzyme activities in normal and various genetically modified lines of tomato fruit

Line	Polygalacturonase	Pectinesterase	b-Galactanase
Normal	550	82	13
PG antisense	4	67	ND
PE2 antisense	670	9	ND
PGPE chimeric antisense	7	5	ND
β-Galactanase co-suppressed	ND	ND	11

the 43 kD catalytic subunit of PG from fruit cDNA libraries (Grierson *et al.*, 1986; Sheehy *et al.*, 1987). Expression of the mRNA corresponding to these clones occurs in a fruit and ripening specific manner. There appears to be only a single gene encoding the fruit specific PG protein (Bird *et al.*, 1988). This finding suggests that the isoforms of PG in ripe tomato fruit are the product of a single gene and arise simply from differential post translational modification of a common polypeptide.

Several groups have down regulated fruit PG expression using either antisense RNA technology or co-suppression. (Sheehy *et al.*, 1988; Smith *et al.*, 1988). In both instances PG expression in transgenic fruit has been reduced to less than 0.5% of the activity occurring in normal fruit. Levels of activity in the transgenics produced at Nottingham are shown in *Table 2*. As expected both the isoforms of PG were effected by this transformation since only a single gene is present.

Recently, expression of the β-subunit has been down-regulated in transgenic tomatoes using antisense RNA technology (Watson *et al.*, 1994). This resulted in a reduction in the level of extractable PG1 isoform. In these transgenic fruit polyuronide solubilisation and depolymerisation were both significantly greater than in corresponding normal fruit. This suggests that whilst the β-subunit is not required for PG action it may have a possible regulatory role in limiting pectin degradation. The β-subunit has been immunolocalised to the cell wall in green fruit and as such a role in targeting the PG within the wall has been postulated (Pogson *et al.*, 1991). This targeting may represent the mechanism by which the β-subunit could limit pectin degradation.

PECTINESTERASE

Pectinesterase (PE) activity occurs in tomato fruit throughout both development and ripening (Hobson, 1963). Like PG, this activity can be resolved into several isoforms. Pressey and Avants (1972) showed that PE isoforms could be separated by ion-exchange chromatography and that the profile was different in different tomato cultivars. Tucker *et al.* (1982) demonstrated that the PE from *Ailsa craig* tomatoes could be separated into at least two isoforms, which they called PE1 and PE2. More recently, Warrilow *et al.* (1994) have separated three PE isoforms from tomato fruit which they termed PEA, PEB and PEC. Heparin affinity chromatography can be used to separate three PE isoforms from tomato fruit (*Figure 1*). This is a profile in which the fractions have been assayed using a kinetic programme to give a rate assay which shows the relative activities of the three isoforms. For the purpose of this paper these have been termed PE1, PE2 and PE3. The relative activities of these three isoforms in tomato fruit are shown in *Figure 2*. It can be seen that PE1 and PE2 both have peaks of activity at around the mature green-breaker stage of development. In contrast, PE3

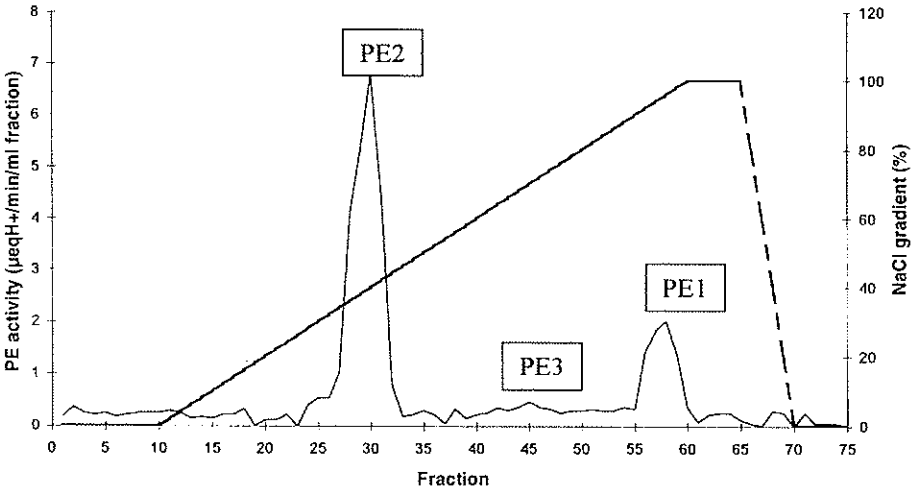

Figure 1. Heparin affinity column profile of pectinesterase isoforms from normal tomato fruit. This is a rate assay to show the relative proportions of activity in each isoform.

activity remains relatively constant throughout both development and ripening. The PE2 isoform represents the major form of the enzyme at all stages of development.

The PE2 isoform has been purified and sequenced (Markovic and Journval, 1986). Using this sequence data Ray *et al.* (1988) succeeded in isolating a clone from a tomato fruit cDNA library.

Characterisation of two further clones with homology to PE2 was later described by Hall *et al.*, 1994. It thus appears that PE2 may be encoded for by a small multi gene family. Two groups have carried out analysis of genomic clones (Harriman *et al.*, 1991; Hall *et al.*, 1994) in both cases they have shown that there are 3 PE2 like genes organised in tandem within the genome. There is some evidence that PE2 in tomato fruit may exist in multiple forms. Gaffe *et al.* (1994) identified five PE isoforms in tomato fruit using isoelectric focusing. Three of these isoforms cross reacted with an antibody raised against an equivalent of PE2 and all three isoforms disappeared in transgenic fruit produced by antisense RNA technology targeted against the PE2. It is possible that these isoforms arise from post translational modification of a single PE2 gene product, as for PG. It is also possible that these three PE2 sub forms may arise from the transcription and translation of two or more of the established PE2 multi gene family.

Several groups have down regulated the expression of the PE2 gene(s) using antisense RNA technology (Tieman *et al.*, 1992; Hall *et al.*, 1993). In all cases, total PE activity in the fruit was reduced to around 10% of normal. The levels of enzyme activities in the plants produced at Nottingham are shown in *Table 2*. The isoform levels during development for these antisense PE2 fruit is shown in *Figure 3*. In comparison to the normal levels shown in *Figure 2*, it can be seen that whilst the activity of PE2 has been reduced to almost zero, that of PE1 and PE3 is unaffected. This result would suggest that the genes for both PE1 and PE3 have insufficient sequence homology to the PE2 antisense gene to result in silencing. This in turn would suggest that these then represent completely different genes. Putative PE1 (Warrilow *et al.*,

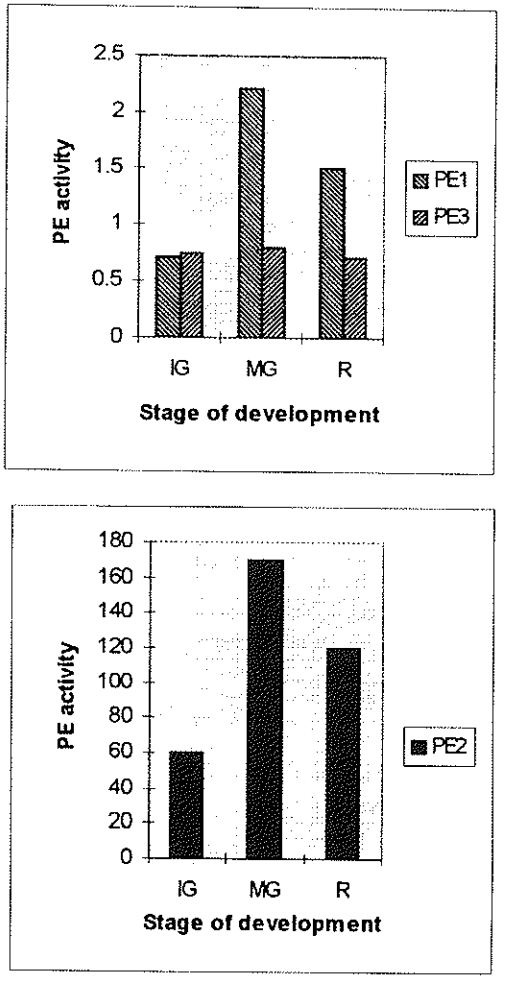

Figure 2. Pectinesterase activity in normal tomato fruit. Enzyme activity is expressed as (ueq\min\gfwt).

1994) and PE3 (Tucker and Zhang, unpublished data) isoforms have both been purified to homogeneity and their N-terminal amino acid sequences have been determined. These sequences, along with that for the PE2 isoform, are shown in *Figure 4*. It can be seen that all three sequences are very different, again confirming the possibility that the three isoforms are encoded for by separate genes or multi gene families. The identification and characterisation of these genes for PE1 and PE3 remains to be carried out. The study of these other two PE isoforms is particularly important since these appear to be the isoforms which are prevalent in vegetative tissue (Warrilow *et al.*, 1994). Similarly, antisense PE2 plants, whilst showing a marked decline in fruit PE levels, show no effect whatsoever on levels of PE activity in leaves or roots. The vegetative forms of PE appear, at least on the basis of isoelectric focusing and ion-exchange chromatography, to be similar to the PE1 and PE3 isoforms present in the fruit (Tucker, unpublished). This has led Gaffe *et al.* (1994) to describe the

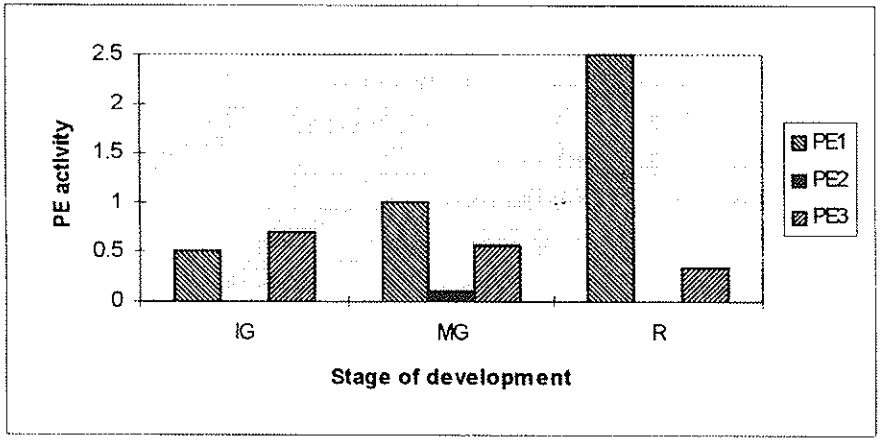

Figure 3. Pectinesterase activity in transgenic tomato fruit. Activity is expressed as (ueq\min\gfwt).

PE1	LSXGQVESG
PE2	HANAVVAQ
PE3	EDPYRVFDW

Figure 4. N-terminal amino acid sequences of putative pectinesterase isoforms from tomato fruit.

presence of two groups of isoforms in plant tissue. Group I are fruit specific isoforms, all of which appear to be related to PE2. Group II are isoforms present in both fruit and vegetative tissue and these presumably correspond to PE1 and PE3.

β-GALACTOSIDASE

In tomato three β–galactosidase isoforms have been separated by a combination of ion-exchange and gel permeation chromatography (Pressey, 1983). However, only one of these three isoforms was found to be capable of degrading a β(1–4)-galactan isolated from tomato cell walls (Pressey and Himmelsbach, 1984; Carey *et al.*, 1995). This isoform was thus recognised as an exo- acting β(1–4)-galactanase. Whilst levels of β-galactosidase activity remain fairly constant during tomato fruit ripening, the β(1–4)-galactanase activity increases dramatically (Carey *et al.*, 1995). This increase does not occur in the tomato ripening inhibitor (*rin*) mutant (Carey *et al.*, 1995).

The β-galactosidase isoform exhibiting galactanase activity has been purified and the sequence data obtained used to isolate related cDNA clones (Carey *et al.*, 1995). Several cDNA clones with homology to the β-galactosidase isoform have been identified (Tucker, unpublished; Gross, personal communication) suggesting that, like PE2, this β-galactosidase isoform may be encoded by a multi gene family. One of

the related cDNA clones has been used to down regulate the corresponding enzyme using co-suppression. The resultant enzyme activities are shown in *Table 2*. From northern blot analysis it has been shown that the expression of the endogenous gene corresponding to the cDNA used for the co-suppression has been reduced to around 10% of normal (Carey, 1996). However, the resultant loss of galactanase activity in the transgenics was only marginal, suggesting that there may be other isoform(s) remaining which account for the majority of the activity in fruit.

SIMULTANEOUS SILENCING OF POLYGALACTURONASE AND PECTINESTERASE

Since PG will only cleave the polyuronide chain between two adjacent de-esterified galacturonic acid residues, this enzyme is thought to act synergistically with PE. Thus, the simultaneous down regulation of both genes for PG and PE could be beneficial in reducing pectin degradation in fruit. This could, and indeed, has been achieved by crossing lines of transgenic plants showing either reduced PG or PE activities as described above. However, this process is both time consuming and labour intensive in the selection required. A much simpler method is the use of chimeric constructs as described by Seymour *et al.* (1993). In this method, a 244 bp fragment of PG coding sequence was ligated to the complete 1320 bp coding fragment of PE2 to give a chimeric construct. This construct was then placed under the control of a constitutive promoter and then used to transform tomato plants. The resultant transgenic fruit had both enzyme activities reduced, the levels of activity are shown in *Table 2* alongside those of the individual transgenic lines. It can be seen that the chimeric construct has resulted in a down regulation of the two endogenous genes to a similar extent as in the two individual lines.

This phenomenon has been shown in several independently transformed tomato lines and in each case both target genes are silenced co-ordinately (Jones *et al.*, 1996). In addition, it has been shown that the structure of the chimeric construct can be very flexible. Thus, the chimeric gene will work if both sequences are in the sense orientation (co-supression) or in the antisense orientation (Jones *et al.*, 1998). Indeed, the chimeric gene will still function to silence both target genes if it contains one of the sequences in a sense and the other in an antisense orientation both within the same construct (Jones *et al.*, 1998).

The reduction in PG in these plants is similar to that in the individual lines in that both isoforms of the enzyme are reduced in activity. However, the effect on PE activity is different to that in the individual line. The PE isoform profile from the ripe chimeric transgenic fruit is shown in *Figure 5*. For comparison, a normal profile is also shown in this figure. In this instance, an end point assay has been used to emphasis the presence or absence of particular isoforms. The size of the peaks in this case is not proportional to the actual relative activities of the three isoforms. It is apparent from *Figure 5* that both PE2 and PE3 appear to be markedly reduced in activity whilst PE1 remains relatively active. In comparison, in the individual transgenic line targeted at PE2 (activities shown in *Figure 3*) only PE2 has been down regulated. This difference may be accounted for by the fact that the entire coding sequence for PE2 was used in the generation of the chimeric transgenic plants whereas a smaller fragment was used to produce the antisense PE2 lines. It is possible that the extra coding sequence employed in the chimeric construct contains sufficient homology to a sequence in the PE3 gene to bring about silencing.

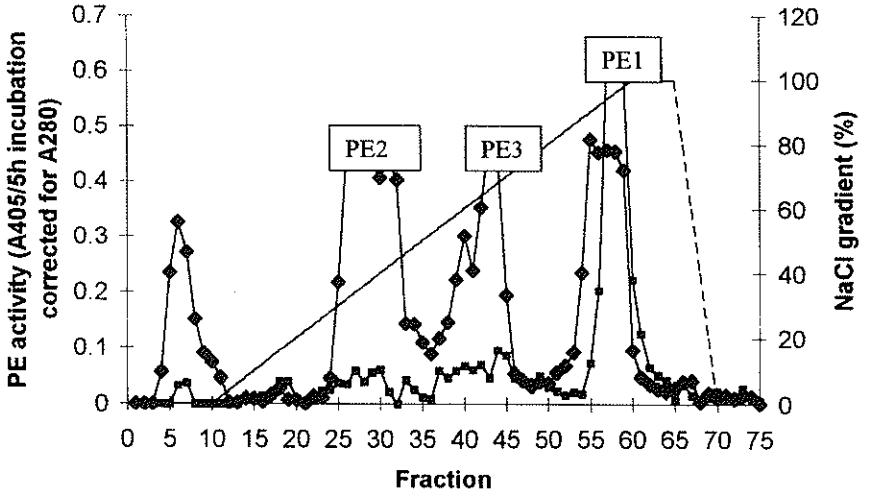

Figure 5. Heparin affinity column profile of pectinesterase isoforms from normal (filled diamonds) or PGPE (filled squares) tomato fruit. This is the result of an end point assay to emphasis the presence or absence of isoforms. The size of the peaks is not proportional to their relative activities.

Effects of gene silencing on cell wall degradation

The effect of silencing PG activity on cell wall degradation has been described in a previous volume (Tucker, 1990). The effects on pectin solubility, de-esterification and depolymerisation are summarised in *Table 3*.

It can be seen that whilst the level of de-esterification has been unaffected the degree of depolymerisation of the polyuronide has been markedly reduced. This observation was as expected given the mechanism of action of PG. Perhaps an unexpected observation was that the level of chelator solube polyuronide was not effected by the down regulation of PG. This does not mean that the solubility of the pectin has not been effected, however. These experiments used a strong chelating agent to solubilize the pectin and this may have masked more subtle solubility effects. Indeed, Carrington *et al.* (1993) have shown that the level of water soluble pectin in these transgenics is reduced compared to normal.

The effect of reduced PE2 on cell wall metabolism is shown in *Table 3*. In this case, as expected, there was a reduction, although not a complete cessation, of de-esterification with no effect on either depolymerisation or solubility of the polyuronide. This effect on de-esterification has been studied in more detail and the degree of esterification of the pectin in normal and transgenic fruit determined throughout

Table 3. Effect of down regulation of either PG or PE activity on wall meatabolism in intact tomato fruit

Line	Degree of esterification	Solubility (%)	Average mol.wt (KD)
Normal (green)	75	36	294
Normal (ripe)	56	59	111
PGas (ripe)	53	52	248
PE2as (ripe)	67	66	85

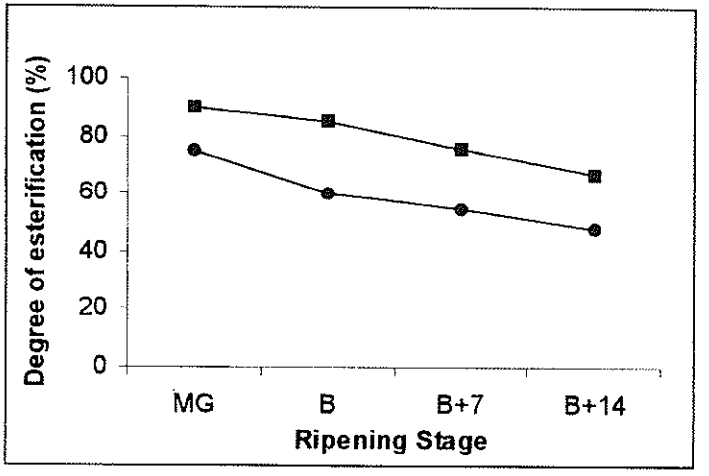

Figure 6. Changes in degree of esterification of pectin during ripening of normal (filled circles) or PE2 as (filled squares) tomato fruit. MG = Mature green, B = Breaker, B+7 = Breaker plus 7 days, B+14 = Breaker plus 14 days.

development and ripening. The results are shown in *Figure 6*. It can be seen that the antisense PE2 plants have a higher degree of esterification of their pectin at all stages of development. However, the de-esterification accompanying fruit ripening appears to proceed in the transgenic to the same extent as in normal fruit.

No differences have yet been detected between normal fruit and those from transgenics down regulated for β-galactosidase activity. In this case no change in the galactose content of the wall fractions could be seen (Seymour, personal communication). The precise effect on cell wall metabolism of the double down regulation of PG and PE remains to be determined, although preliminary experiments would suggest that depolymerisation of the pectin is reduced, solubility in green fruit enhanced but total solubility in ripe fruit unaffected (Simons, 1998).

Effect of gene silencing on fruit softening and processing

EFFECT ON INTACT FRUIT

The effect of down regulation of PG has also been described in a previous volume (Tucker, 1990). The effect on fruit softening, as determined by compression (Langley *et al.*, 1994) or stress relaxation experiments (Errington *et al.*, 1997), is marginal. The transgenic fruit may be slightly firmer but this is not really significant. The genetic modification does, however, seem to influence the cracking and resistance to transport damage of the fruit. Normal fruit harvested ripe and then transported suffer extensive (up to 50% of the fruit) cracking. Ripe fruit with reduced PG activity, however, only exhibit about 10% losses. This has lead to the introduction in the USA of the '*Flavr Savr*' tomato. This is a commercialised antisense PG tomato which can be harvested ripe, and hence has more flavour.

The down regulation of PE2 in the PE antisense fruit has little or no effect on softening. Similarily, no effect on softening was seen with the simultaneous down

regulation of both PG and PE with the chimeric construct. The down regulation of β-galactosidase again had little effect on the compressibility of the fruit but cracking during transport was, as with the reduced PG fruit, markedly reduced. In this case control ripe fruit exhibited 40% cracking compared to zero cracking in the transgenics (Tucker *et al.*, unpublished).

EFFECT ON PROCESSING

A large proportion of the annual tomato crop is processed into products such as paste and ketchup. A major quality attribute in this case is viscosity. A standard industrial measure of viscosity involves the use of a Bostwick tray. This is a perspex box about 1 metre long with a removable trap door sealing off a small reservoir at one end of the tank. A tomato paste is poured into the reservoir, the trap door removed and the distance that the paste flows down the tank in 30 seconds is then recorded. Pastes with highest viscosity thus move the shortest distance. Bostwick values have been obtained for tomato pastes made from fruit from normal, antisense PG (PGas), antisense PE2 (PE2as) and lines in which a chimeric construct has been used to down regulate both PG and PE (PGPEas). These pastes were made by homogenising fruit directly, i.e with no pretreatment, and as such are so called 'cold break' pastes. These values are shown in *Table 4*.

It can be seen that reduction in PE alone (PE2as) has had no effect on the viscosity of the resultant paste. Reduction in PG activity (PGas) results in a clear increase in viscosity, the Bostwick value being almost half that of the control normal paste. Interestingly, the fruit in which both PG and PE have been reduced (PGPEas) show no increase in paste viscosity. It would thus seem that the reduction in PE in these fruit negates the advantage of reduced PG activity. *Table 4* also shows the Bostwick values from these same fruit lines but after the fruit had been subjected to heating prior to being converted to paste. These are so called 'hot break pastes'. The heating has the effect of denaturing the PG and PE enzymes and results in total loss of activity in all fruit lines. In this case it can be seen that the Bostwick values for all lines are about equal. This result shows that in the PGas line the reduced Bostwick value is the result of reduced pectin degradation within the paste and not due to any intrinsic differences in the pectin within the fruit prior to its being converted to paste. This result is supported by the finding that pectin degradation in the fruit of even normal fruit is strictly controlled. It is not until fruit are homogenised (as in paste production) that PG and PE are released and allowed to fully degrade the pectin (Seymour *et al.*, 1987).

The results shown above for the Bostwick tests would indicate that whilst PG activity in tomato homogenates would seem to be disadvantageous for high

Table 4. Viscosity of pastes from normal and genetically modified tomato fruit as measured by the Bostwick method

Line	Bostwick value (cold break)	Bostwick value (hot break)
Normal	235	202
PGas	78	196
PE2as	232	172
PGPE chimeric	220	180
β-galactanase	153	ND

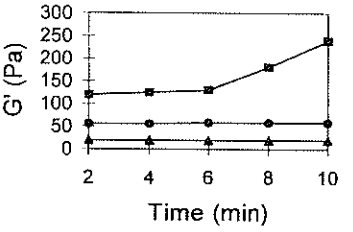

Figure 7. Effect of time on the viscosity of pastes from normal (filled circles), PG (filled squares) or PE2 (filled triangles) tomato fruit.

viscosity the presence of PE, at least at high levels, would seem to be an advantage. Also, since the enzyme activity appears to be most important in the paste, post homogenisation, rather than in the intact fruit, it was decided to examine the effect of time on paste viscosity. This was achieved by preparing tomato paste, placing a sample in a Bohlin rheometer and recording G' with time. The results are shown in *Figure 7*. It can be seen that the viscosity of paste from normal fruit does not change with time. This is also true of paste made from the PE2as line. However, the viscosity of pastes made from the PGas fruit showed a steady increase with time. The suggestion is that it is the action of PE in the PGas paste that is resulting in the increased viscosity with time.

The availability of these tomato fruit lines showing variable paste viscosity and presumably variable pectin structure in the pastes allowed a study of the possible structure/function relationships of the pectin to be undertaken. It was thought likely that the viscosity of the paste, as determined by the Bostwick measurement, was dependent on the viscosity of the serum component of the paste. To test this, pastes from normal and transgenic fruit were centrifuged to separate the serum from the solid fraction and then the serum viscosity determined using an Ostwald viscometer. *Table 5* shows the viscosity of the serum for all the cold and hot break pastes. This is shown alongside the corresponding Bostwick values.

It is apparent that there is, in fact, no correlation whatsoever between the Bostwick value for the whole paste and the corresponding serum viscosity. The composition of these sera have been determined (Errington *et al.*, 1998) but this does not help to explain any effect of serum on total paste viscosity.

Table 6 shows the solid volume fraction (the percentage of solids in the paste) again for normal and transgenic pastes. In this case a weak correlation may be seen with the Bostwick values. It is likely that it is the nature of the solid, or colloidal, material which is important, thus solids from cold break pastes were examined by confocal

Table 5. Comparison of paste and serum viscosities in normal and genetically modified tomato lines

Line	Bostwick value	Relative serum viscosity
Normal (hot break)	202	14.1
Normal (cold break)	235	1.5
PG as (hot break)	196	31.5
PG as (cold break)	78	1.3
PE2 as (hot break)	172	17.5
PE2 as (cold break)	232	5.6

Table 6. Comparison of paste viscosity and volume fraction of solids in normal and genetically modified tomato lines

Line	Bostwick value	Volume fraction of solids (%)
Normal (hot break)	202	24
Normal (cold break)	235	29
PGas (hot break)	196	24
PGas (cold break)	78	35
PE2as (hot break)	172	37
PE2as (cold break)	232	23

microscopy (Errington *et al.*, 1998). Paste from normal fruit showed massive cell disruption with only wall fragments visible under the microscope. This is consistent with the combined action of PG and PE totally degrading the pectin, weakening the cell wall and thus allowing cells to fragment. In contrast, pastes from the cold break PGas paste showed mainly intact cell walls, although whether this represented intact cells was not determined. The paste from the PE2as fruit also showed mainly intact cell walls yet in this case the paste viscosity is not as high as for the PGas paste. These results do not demonstrate unequivocally what factors within a paste determine its final viscosity. However, a theory can be proposed. In order to have a high viscosity the cell walls must remain relatively intact. The prevention of PG activity, either by down regulation of the enzyme or by reduction in PE activity preventing the generation of de-esterified target sites for PG on the pectin, can both bring this about. However, the presence of intact walls is not sufficient in itself to give high viscosity. The continued action of PE in the paste, as in the PGas line, presumably results in an increased negative charge on the cell walls. This could in turn increase the interactions between either the individual cells or the cells and serum components and result in an increased viscosity.

Cold break pastes made the tomato fruit transformed with an antisense β-galactosidase gene also show a marked increase in their viscosity as determined by the Bostwick method (*Table 4*). This is despite the fact that total galactanase activity has hardly been reduced at all and that no detectable change in cell wall metabolism has been detected in these fruit. It remains to be seen whether these pastes contain intact cell walls.

Conclusion

Genetic modification of plants provides a valuable tool for both fundamental studies and commercial applications. The use of gene silencing techniques, as described in this paper, can provide valuable information concerning the role of specific enzymes in metabolism. It has in particular been used, as shown here, to investigate the role of polysaccharide hydrolases in the metabolism of cell walls during fruit ripening. This technique can also be used to probe structure-function relationships by allowing precise and controllable modifications to these polysaccharides and then assessing the effect on physiological factors such as fruit softening or commercially important factors such as paste viscosity.

The genetic modification of plants holds potential for a wide range of commercial applications. Its use to modify the structure of plant derived polysaccharides as described in this paper being only one such application. This technology is still,

relatively speaking, in its infancy. It is, however, a technology that is advancing with astonishing speed. It has nonetheless already been employed to modify several polysaccharide structures and in some cases has already lead to successful commercial applications. Currently, such modifications have been carried out on a small number of polymers in a limited range of species and employing the modification of only a minority of the enzymes involved in polysaccharide synthesis or degradation. The potential for using this technology to study the roles of other enzymes in other plant systems is thus enormous. Similarly, the potential for further commercial applications must also be immense.

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