The Plant Cell, Vol. 11, 2203–2216, November 1999, www.plantcell.org

Modification of Expansin Protein Abundance in Tomato Fruit Alters Softening and Cell Wall Polymer Metabolism during Ripening

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The role of the ripening-specific expansin Exp1 protein in fruit softening and cell wall metabolism was investigated by suppression and overexpression of *Exp1* in transgenic tomato plants. Fruit in which Exp1 protein accumulation was suppressed to 3% that of wild-type levels were firmer than controls throughout ripening. Suppression of Exp1 protein also substantially inhibited polyuronide depolymerization late in ripening but did not prevent the breakdown of structurally important hemicelluloses, a major contributor to softening. In contrast, fruit overexpressing high levels of recombinant Exp1 protein were much softer than controls, even in mature green fruit before ripening commenced. This softening was correlated with the precocious and extensive depolymerization of structural hemicelluloses, whereas polyuronide depolymerization was not altered. These data are consistent with there being at least three components to fruit softening and textural changes. One component is a relaxation of the wall directly mediated by Exp1, which indirectly limits part of a second component due to polyuronide depolymerization late in ripening, perhaps by controlling access of a pectinase to its substrate. The third component is caused by depolymerization of hemicelluloses, which occurs independently of or requires only very small amounts of Exp1 protein.

INTRODUCTION

Plant cell growth is constrained by the cell wall, whose rigid cellulose microfibrils are held together by three structurally independent but interacting matrices (Carpita and Gibeaut, 1993). The matrix glycans, or hemicelluloses, are polysaccharides composed of neutral sugars, a major constituent of which in dicotyledonous species is xyloglucan. Xyloglucan molecules coat and cross-link cellulose microfibrils and, by extensive noncovalent bonding, anchor the microfibrils relative to one another. The xyloglucan–cellulose framework is itself embedded in a pectin matrix composed of polyuronide molecules, together with a domain of structural glycoproteins. Precisely how cell wall components and their intermolecular interactions are changed to allow wall loosening during growth or remodeling during cell development is not known, but a group of highly conserved cell wall proteins called expansins have been implicated in many aspects (Cosgrove, 1998).

Expansins are encoded by large multigene families (Shcherban et al., 1995; Cho and Kende, 1997; Cosgrove et al., 1997; Brummell et al., 1999a), and the expression of expansin mRNA and protein is correlated with growth in many tissues of the plant, including hypocotyls (McQueen-Mason et al., 1992), coleoptiles (Li et al., 1993), internodes (Cho and Kende, 1997), leaves (Keller and Cosgrove, 1995), roots (Wu et al., 1996), and green fruit (Brummell et al., 1999a). However, expansins also may play a role in the cell wall modification involved in other aspects of plant development. In tomato, expansin *Exp1* mRNA is expressed at high levels specifically during fruit ripening (Rose et al., 1997) and is the major expansin gene family member expressed at this time (Brummell et al., 1999a). Ripening occurs toward the end of fruit development after fruit growth is complete (Gillaspy et al., 1993) and is a complex developmental program that includes changes in color, aroma, metabolism of sugars and organic acids, texture, and softening (Brady, 1987). These latter two processes are believed to be due to alterations in turgor and cell wall metabolism, which in tomato includes the extensive depolymerization of both pectins and

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hemicelluloses and slight depolymerization of cellulose (Huber and O'Donoghue, 1993; Maclachlan and Brady, 1994).

Little is known about the mechanism of expansin action in cell walls in vivo. Expansins lack significant hydrolase or transglycosylase activity (McQueen-Mason et al., 1992, 1993; McQueen-Mason and Cosgrove, 1995) but share structural features with polysaccharide binding proteins (Shcherban et al., 1995) and have been hypothesized to act at the matrix–microfibril interface (McQueen-Mason and Cosgrove, 1995). In vitro, active expansin protein causes cell wall loosening in isolated cell walls maintained under tension (McQueen-Mason et al., 1992; Li et al., 1993) and brings about mechanical weakening of paper (McQueen-Mason and Cosgrove, 1994), which is consistent with expansins acting through a disruption of noncovalent interactions, such as hydrogen bonding. The goal of this project was to suppress and overexpress the Exp1 protein in transgenic tomato plants, first to investigate the contribution of Exp1 protein to the fruit softening process during ripening, and second to gain some insight into the role of expansin protein in fruit cell wall metabolism in vivo.

RESULTS

Suppression and Overexpression of Exp1 in Tomato Plants

Two constructs containing the cDNA of the tomato fruit ripening–specific *Exp1* gene in the sense orientation driven by the constitutive cauliflower mosaic virus 35S promoter were assembled (Figure 1A) and used to transform tomato plants. One of these constructs contained the full-length coding region of the cDNA, and the other contained a truncated out-of-frame version in which the region encoding the first 77 amino acids of the deduced Exp1 protein was deleted. Transformation with the full-length construct was intended to result in both silencing of the endogenous *Exp1* gene by sense suppression (Napoli et al., 1990) and overexpression of functional Exp1 protein, whereas the transcript of the truncated construct could not produce functional Exp1 protein and was intended to result only in sense suppression of the endogenous gene. Plants transformed with the full-length coding *Exp1* construct were designated by the prefix 17, whereas the prefix 18 was used to designate plants transformed with the truncated *Exp1* construct.

Young leaves and red ripe fruit were collected from 136 primary transformants of the 17-series plants and from 49 primary transformants of the 18-series plants. *Exp1* mRNA abundance in these tissues was determined by RNA gel blot analysis, as shown for some representative samples in Figures 1B and 1C. Because the endogenous *Exp1* gene is not expressed in leaves (Rose et al., 1997), *Exp1* transcript detected in leaves results from expression of the transgene. In fruit from plants transformed with the full-length coding construct (Figure 1B, bottom), transcripts derived from the endogenous *Exp1* gene or from the *Exp1* transgene could not be separated by size, but because both transcripts may encode functional Exp1 protein, total *Exp1* transcript abundance was determined. In fruit from plants transformed with the truncated construct (Figure 1C, bottom), the transcript derived from the truncated transgene is slightly smaller than mRNA derived from the endogenous *Exp1* gene, and the abundance of only the latter was determined. A wide range of *Exp1* expression levels in fruit was found, with 8% of the 17-series plants and 18% of the 18-series plants showing

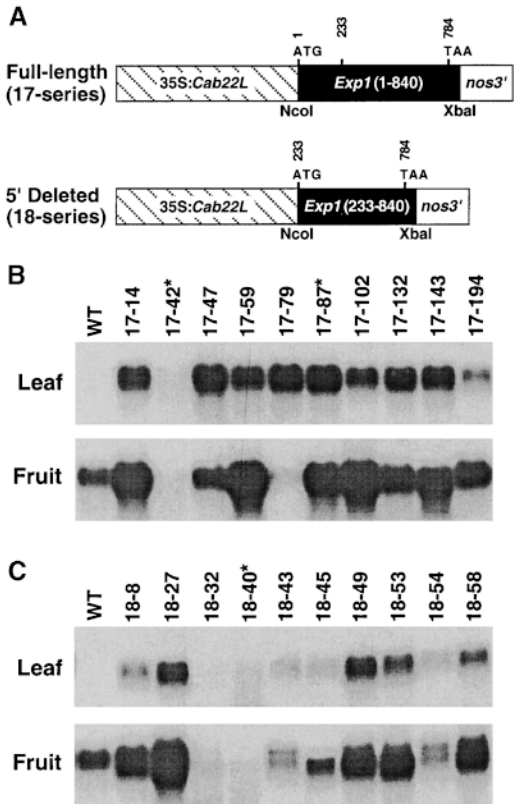

Figure 1. Constructs and Screening of Primary Transformants.

(A) Schematic representation of *Exp1* constructs. For the full-length coding construct (17-series plants), an NcoI site was introduced at the first Met of the *Exp1* cDNA by site-directed mutagenesis, before a translationally in-frame fusion to the cauliflower mosaic virus 35S promoter with *Cab22L* leader. For the out-of-frame truncated construct (18-series plants), an NcoI site was introduced 233 bp downstream of the first Met. *nos*, nopaline synthase.

(B) *Exp1* mRNA abundance in leaves and fruit of plants transformed with the full-length construct (17 series).

(C) *Exp1* mRNA abundance in leaves and fruit of plants transformed with the truncated construct (18 series).

In **(B)** and **(C)**, the three lines selected for further study are indicated with asterisks. WT, wild type.

suppression of *Exp1* mRNA to 10% or less than that of wild-type levels. It was noted that in general, silencing of *Exp1* transgene expression in the leaf was correlated with silencing of endogenous *Exp1* expression in the fruit; conversely, high expression of the transgene in the leaf was correlated with high endogenous and transgene expression in the fruit. However, in ~10% of the population, high expression in the leaf accompanied low expression in the fruit (e.g., plant number 17-79), or vice versa.

Several lines showing a high level of suppression or overexpression were selected for further study. Segregation analysis (data not shown) was used to determine that strongly suppressed plants 17-42 and 18-40, and overexpressing plant 17-87 possessed single-locus insertions of the selectable marker gene. The T-DNA insertions of these plants were mapped by DNA gel blot analysis. Figure 2 shows that the T-DNA insertions of suppressants 17-42 and 18-40 produced inverted repeats of the transgene, the presence of which frequently has been correlated with post-transcriptional gene silencing (Stam et al., 1997; Waterhouse et al., 1998). In both lines, the inverted T-DNA repeats were stabilized by asymmetric deletions, which for line 17-42 was of ~900 bp and included the right borders of both T-DNA elements, and for line 18-40 was of ~300 bp, which included the right border of one element. Line 18-40 also had deletions of both T-DNA left borders and part of the selectable marker gene of one element. The overexpressing line 17-87 possessed a single, complete insertion of the T-DNA.

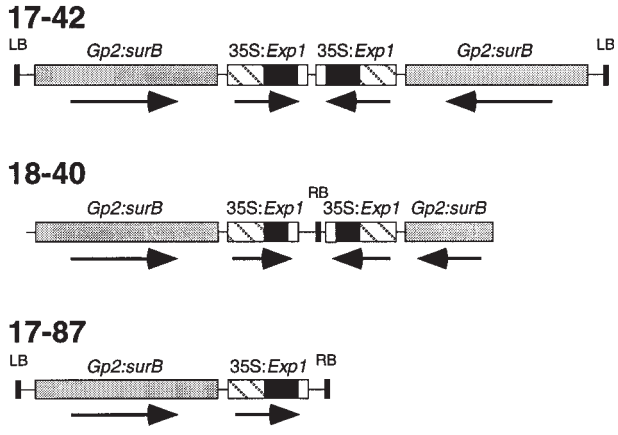

Figure 2. Schematic Representation of T-DNA Insertions.

T-DNA insertions of three single locus transformants were mapped by DNA gel blot analysis. Transformants 17-42 and 18-40 were suppressants, and transformant 17-87 was an overexpresser. The *Gp2:surB* chlorsulfuron resistance selectable marker gene is shown as shaded boxes, the 35S promoter as striped boxes, the *Exp1* transgene coding region as black boxes, and the nopaline synthase transcription terminator as white boxes. Arrows beneath each gene indicate direction of transcription. LB, T-DNA left border; RB, T-DNA right border.

For the above-mentioned three transgenic lines, *Exp1* mRNA and protein abundance were determined during fruit ripening in the succeeding T_2 generation. Figure 3A shows that in azygous control plants of each line, from which the *Exp1* transgene had been eliminated by segregation, *Exp1* mRNA was not present at the mature green 2 stage but had appeared by the turning stage of ripening, reaching maximum accumulation in red ripe fruit. In homozygous fruit of lines 17-42 and 18-40, *Exp1* mRNA accumulation was strongly suppressed throughout ripening, and in homozygous fruit of line 17-87, it was substantially increased. At the red ripe stage, *Exp1* mRNA abundance was suppressed to ~5% that of the wild type in line 17-42, ~1% that of the wild type in line 18-40, and increased to ~500% of the wild-type levels in line 17-87. The mRNA accumulation of three related expansin genes expressed predominantly in green fruit, *Exp3*, *Exp4*, and *Exp5* (Brummell et al., 1999a), was not altered in *Exp1*-suppressed fruit (data not shown).

Exp1 protein also was absent in cell walls of control fruit at the mature green 2 stage, although a cross-reacting band of slightly higher molecular mass was visible. Exp1 protein accumulation correlated with ripening in cell walls of control fruit, achieving highest levels at the red ripe or overripe stage. Exp1 protein accumulation was strongly suppressed in lines 17-42 and 18-40, although a cross-reacting band of slightly higher molecular mass was seen in some lanes and strongly increased in line 17-87. The relative abundance of Exp1 protein in red ripe fruit was estimated by comparing dilutions of cell wall proteins on an immunoblot. Figure 3B shows that Exp1 protein was present at ~5% of the wild-type levels in line 17-42, 3% of the wild-type levels in line 18-40, and 300% of the wild-type levels in line 17-87. Note that in line 17-87, because overexpression of *Exp1* was driven by the 35S promoter, constitutive high-level expression of *Exp1* mRNA and protein was detected during fruit development, including at the mature green 2 stage before the onset of ripening.

Suppression of Exp1 Is Correlated with Increased Fruit Firmness and Overexpression with Enhanced Softening

Figure 4 shows the changes in fruit firmness during ripening in T_2 generation azygous controls and transgenic lines in which *Exp1* mRNA and protein levels were suppressed or overexpressed. In controls of all three lines, fruit firmness declined by approximately one-third between harvest at the midmature green stage and 3 days later. The decline in fruit firmness and the accumulation of *Exp1* mRNA both begin late in the mature green stage, immediately before the beginning of ripening (Maclachlan and Brady, 1994; Brummell et al., 1999a). After this, fruit firmness decreased progressively during ripening, and from the breaker stage (defined as the first visible exterior red color at the blossom end of the fruit) declined by approximately one-half at the red ripe stage (6 days postbreaker) and by two-thirds at the overripe

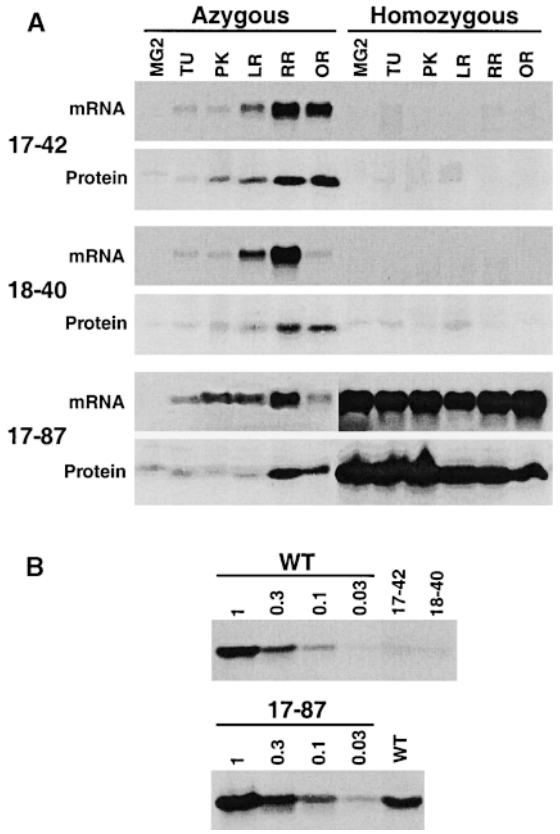

Figure 3. RNA Gel Blot and Protein Immunoblot Analyses of *Exp1* in Azygous and Homozygous Fruit during Ripening.

(A) Total RNA (10 μg per lane) isolated from fruit of the indicated genotype and developmental stage was separated electrophoretically, blotted to a nylon membrane, and *Exp1* mRNA detected by hybridizing with radiolabeled *Exp1* cDNA. Total cell wall protein (100 μg per lane) prepared from similar fruit tissue was separated by SDS-PAGE, blotted to a polyvinylidene fluoride membrane, and Exp1 protein detected by reaction with Exp1 antiserum. Fruit from azygous plants are shown on the left, and fruit from homozygous plants are on the right. Developmental stages are as follows: MG2, mature green stage 2; TU, turning; PK, pink; LR, light red; RR, red ripe; OR, overripe.

(B) Exp1 protein content in cell walls of red ripe fruit of suppressed and overexpressing lines was estimated relative to wild type (WT) by immunoblot analysis. Total cell wall protein was 100 μg per lane or a dilution of 0.3, 0.1, or 0.03 of 100 μg as indicated. Methods are as given in **(A)**. Gel at the top shows a dilution series of the wild type versus suppressants 17-42 and 18-40. Gel at the bottom shows a dilution series of overexpresser 17-87 versus the wild type.

stage (12 days postbreaker). All three control azygous lines showed very similar softening behavior during ripening.

In line 17-42, Figure 4A shows that suppression of Exp1 protein accumulation to ~5% that of wild-type levels resulted in an increase in fruit firmness early in ripening but that this was no longer apparent from the light red ripening stage onwards. In line 18-40, however, where accumulation of Exp1 protein was reduced to lower levels than in line 17-42 (to ~3% of wild-type levels), Figure 4B shows that firmness was increased at all ripening stages, including the overripe stage. The magnitude of the increase in firmness above azygous controls was similar at each time point, but due to the softening occurring during ripening, the relative

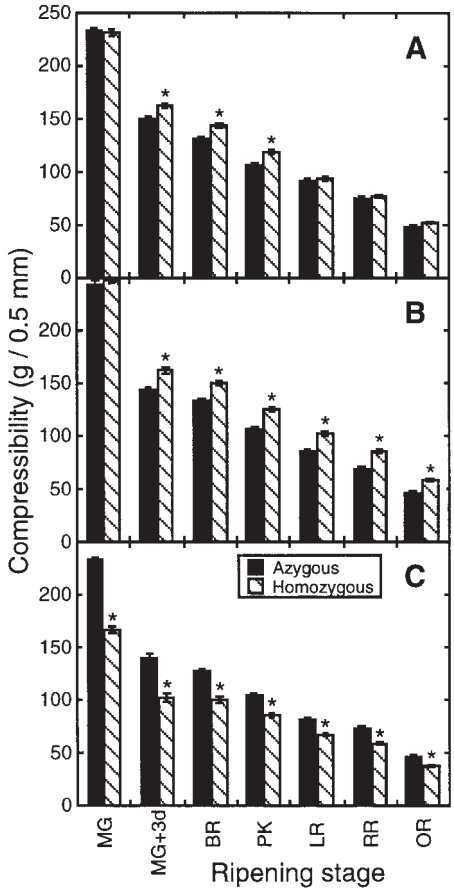

Figure 4. Effect of *Exp1* Suppression and Overexpression on Fruit Firmness during Ripening.

A minimum of 30 fruit was harvested from azygous control plants (black bars) and from homozygous plants (striped bars) at the mature green stage and ripened off the vine. Fruit compressibility was measured at the indicated intervals as the force required to compress the pericarp by a fixed distance of 0.5 mm (three readings per fruit per time point). Bars represent standard errors, and asterisks indicate that homozygotes were significantly different ($P < 0.05$) from azygous controls. Developmental stages are as follows: MG, mature green; MG+3d, late mature green; BR, breaker; PK, pink; LR, light red; RR, red ripe; OR, overripe.

(A) Firmness of suppressed line 17-42 relative to azygous controls.

(B) Firmness of suppressed line 18-40 relative to azygous controls.

(C) Firmness of overexpressing line 17-87 relative to azygous controls.

enhancement increased from 13% firmer than controls at breaker to 23% firmer at overripe.

Figure 4C shows that constitutive overexpression of recombinant Exp1 protein throughout fruit development, including at the mature green stage, resulted in substantially enhanced fruit compressibility at all stages of fruit development and ripening, particularly in mature green fruit. Mature green fruit of line 17-87 was ~30% softer than controls, and although the magnitude of the difference between homozygous overexpressers and controls declined as ripening progressed, it remained evident even in overripe fruit.

The softening behavior of transgenic T_2 fruit ripened on the vine and harvested at the same stages as above showed similar findings to fruit ripened off the vine. Examination of fruit from the succeeding T_3 generation also gave comparable results, confirming that the phenotype segregated with the transgene. No difference in ripening rate between transgenic and azygous fruit was noted in fruit ripened either on or off the vine (data not shown).

The average size of *Exp1*-suppressed fruit was not noticeably different from controls, but fruit overexpressing *Exp1* were on average approximately one-half the size of controls. In addition, the pericarp tissue possessed a rubbery texture, suggesting that the overexpression of Exp1 protein affected not only fruit growth but also the properties of the fruit cell wall. The height of plants constitutively overexpressing *Exp1* was not significantly reduced, implying that Exp1 may not affect cell expansion, although leaves were slightly smaller and showed some epinasty.

Effect of Modified Exp1 Levels on Fruit Cell Wall Pectins

To investigate the cell wall changes that may underly changes in fruit softening, we examined the depolymerization of cell wall polymers during ripening by using size exclusion chromatography. Cell wall extracts were prepared from control azygous and homozygous fruit of suppressed line 18-40 and overexpresser line 17-87 at four ripening stages. Pectins were extracted in two cell wall fractions: a *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA)–extractable fraction enriched in ionically bound polyuronides, and a Na_2CO_3–extractable fraction enriched in covalently bound polyuronides. The amounts of pectins solubilized by these two extracts changed with ripening essentially as described previously (Brummell and Labavitch, 1997), and no significant effects of Exp1 suppression or overexpression on pectin extractability were observed (data not shown).

Figure 5A shows that in control fruit at the mature green stage, CDTA-extractable polyuronides eluted as a major peak in or close to the void (V_o) of the column (molecular mass of 20×10^6 D or greater). During ripening (Figures 5B to 5D), the amount of polyuronide in this peak declined, concomitant with the increasing accumulation of lower molecular mass species nearer the total volume (V_t) of the column (100 kD). However, Figures 5A to 5D show that in fruit

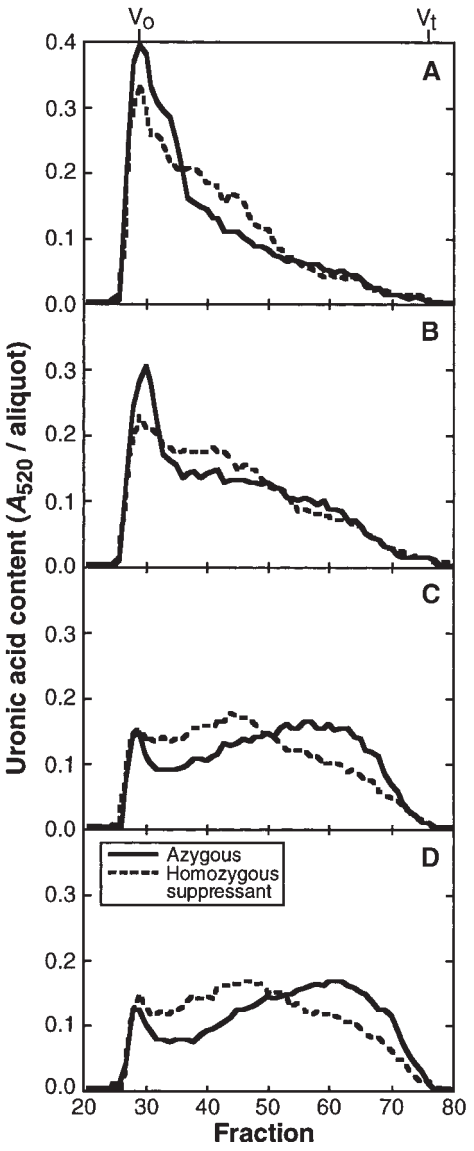

Figure 5. Size Distribution of CDTA-Extractable Polyuronides from Fruit of Suppressed 18-40 Relative to Controls.

Polyuronides extracted from cell walls of azygous control (solid lines) or homozygous line 18-40 (dashed lines) fruit at four ripening stages were subjected to size exclusion chromatography on a column of Sepharose CL-2B, and column fractions were assayed for uronic acid content. The void (V_o) and total volume (V_t) of the column are marked.

(A) Polyuronides from control and line 18-40 at the mature green 2 stage.

(B) Polyuronides from control and line 18-40 at the pink stage.

(C) Polyuronides from control and line 18-40 at the red ripe stage.

(D) Polyuronides from control and line 18-40 at the overripe stage.

suppressed in Exp1 protein accumulation, polyuronide depolymerization was substantially arrested later in ripening. At the red ripe and overripe stages, the largest proportion of the polyuronides eluted between the void and fraction 45, whereas in controls, the bulk of the polyuronides eluted between fraction 50 and the V_t of the column. Although it is not possible to calibrate columns of this separation range due to the lack of available standards, the difference in polyuronide degree of polymerization between control and Exp1-suppressed lines was substantial.

In contrast, Figures 6A to 6D show that when Exp1 protein was overexpressed, depolymerization of CDTA-extractable polyuronides was indistinguishable from controls at all fruit development stages, including mature green. This suggests that the reduction of cell wall polyuronide depolymerization seen in Exp1-suppressed fruit is most likely due to indirect effects of Exp1 protein on cell wall polyuronide metabolism.

Polyuronides extractable in Na_2CO_3 showed an elution profile quite different from those extracted in CDTA. Figure 7A shows that these polyuronides formed a predominant peak of relatively low molecular mass with a much lower abundance of high molecular mass species. During ripening, no substantial differences to this molecular mass profile were observed, and no effect of suppression or overexpression of Exp1 protein was detected (data not shown). However, this fraction is deesterified by the alkaline Na_2CO_3 used for extraction, and it is possible that ripening-related changes in intermolecular ester links are masked by this treatment.

Effect of Modified Exp1 Levels on Fruit Cell Wall Hemicelluloses

Cell wall hemicelluloses were extracted from the depectinated cell wall residues by sequential treatment with 4% (w/v) and 24% (w/v) KOH containing 0.1% (w/v) $NaBH_4$. The 4% KOH treatment extracted approximately one-third of the KOH-extractable neutral sugar polysaccharides, and the 24% KOH treatment extracted two-thirds. These proportions were not affected either by ripening stage or by suppression or overexpression of Exp1 protein. Xyloglucan was found predominantly in the latter extract.

Figure 7B shows that the 4% KOH-extractable hemicelluloses eluted from the column with a molecular mass profile composed of four main peaks, in the void (equivalent to dextran standards of 1000 kD or greater), fraction 47 (200 kD), fraction 58 (70 kD), and fraction 62 (40 kD), of which the most prominant peak was at 70 kD. Xyloglucan was present at low levels coincident with the 200-kD peak. No substantial changes to these molecular mass profiles occurred during ripening, and no effect of either suppression or overexpression of Exp1 protein was observed (data not shown).

For fruit suppressed in Exp1 protein and for corresponding controls, molecular mass profiles of 24% KOH-extractable hemicelluloses were determined for both all neutral

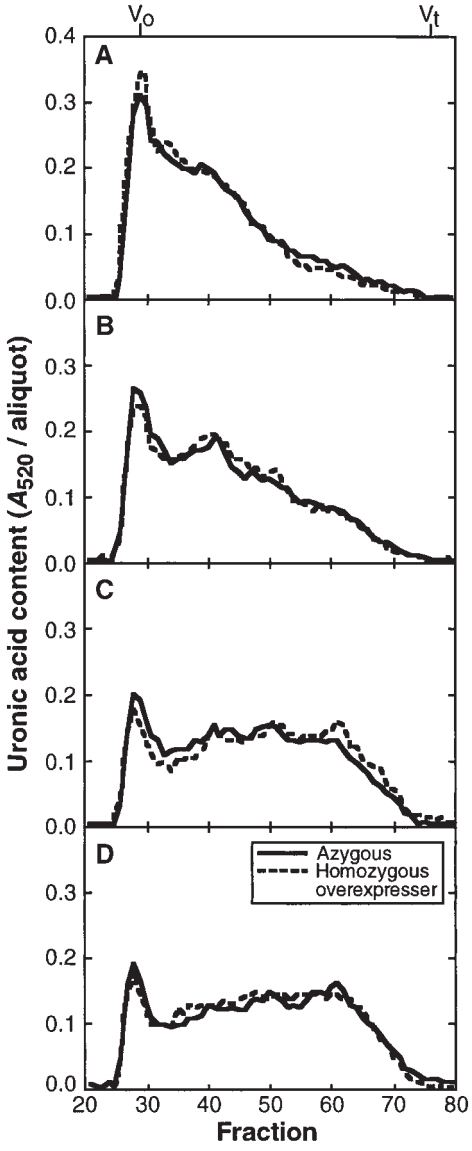

Figure 6. Size Distribution of CDTA-Extractable Polyuronides from Fruit of Overexpresser 17-87 Relative to Controls.

Polyuronides extracted from cell walls of azygous control (solid lines) or homozygous line 17-87 (dashed lines) fruit at four ripening stages were subjected to size exclusion chromatography on a column of Sepharose CL-2B, and column fractions were assayed for uronic acid content. The void (V_o) and total volume (V_t) of the column are marked.

(A) Polyuronides from control and line 17-87 at the mature green 2 stage.

(B) Polyuronides from control and line 17-87 at the pink stage.

(C) Polyuronides from control and line 17-87 at the red ripe stage.

(D) Polyuronides from control and line 17-87 at the overripe stage.

sugar-containing polysaccharides (Figures 8A to 8D) and xyloglucan (Figures 8E to 8H). Figure 8A shows that in mature green fruit of controls, total hemicellulosic polysaccharides eluted from the column with a molecular mass profile composed of four major peaks. The two predominant peaks overlapped and possessed maxima in the void of the column (1000 kD or greater) and in fraction 38 (350 kD). The two minor peaks possessed maxima of ~300 kD (fraction 42) and 70 kD (fraction 58). During ripening in control fruit (Figures 8A to 8D), there was a loss of hemicelluloses eluting in the void and a reduction in the amount of material eluting in the 350-kD peak, coupled with a concomitant increase in the amount of material in the 300-kD peak, which also showed depolymerization during ripening, and in overripe fruit had a maximum at 200 kD (fraction 46). The hemicellulose peak at ~70 kD showed little change during ripening.

Analysis of xyloglucan molecular mass profiles in controls (Figures 8E to 8H) showed that in mature green fruit, xyloglucan molecules formed a polydisperse peak with a broad maximum extending from the void of the column to ~350 kD. During ripening, xyloglucan showed substantial depolymerization and became less polydisperse, with the greatest change seen between the pink and red ripe ripening stages, and in overripe fruit became a sharp peak with a maximum at ~300 kD. The changes in the molecular mass profile of xyloglucan occurring during ripening were consistent with the changes seen in the molecular mass profile of total hemicelluloses. However, xyloglucan constitutes at most only one-third of the polysaccharides in this extract, and ripening-related changes in other hemicellulosic polysaccharides also must be occurring.

In fruit suppressed in Exp1 protein, the molecular mass profiles of both total hemicelluloses (Figures 8A to 8D) and xyloglucan (Figures 8E to 8H) were very similar to the control molecular mass profiles at every ripening stage except pink. This probably was due to the difficulties of obtaining exactly matched fruit at the pink ripening stage (polyuronides from these same fruit also were slightly more depolymerized than controls at the pink stage; Figure 5B) rather than to an effect of Exp1 suppression, because the difference was not present at the red ripe and overripe stages. Thus, suppression of Exp1 protein accumulation to 3% of wild-type levels did not detectably affect cell wall hemicellulose molecular mass profiles during ripening, suggesting that the increased firmness of Exp1-suppressed fruit is not due to a modification of hemicellulose depolymerization.

The effect of overexpression of Exp1 protein on hemicellulose depolymerization is shown in Figure 9. During ripening in control fruit, the molecular mass profiles of both total hemicelluloses (Figures 9A to 9D) and xyloglucan (Figures 9E to 9H) were very similar to the control fruit shown in Figure 8. However, at the mature green stage, fruit overexpressing Exp1 (Figure 9A) showed a hemicellulose molecular mass profile more characteristic of control fruit at the overripe stage in that there was little material in the void or 350-kD peaks and there was a predominant peak spanning

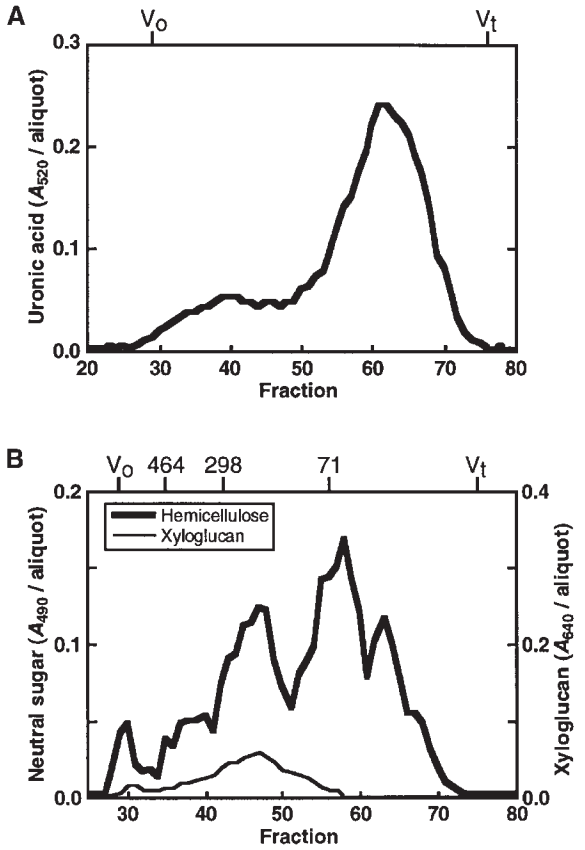

Figure 7. Size Distribution of Na_2CO_3–Extractable Polyuronides and 4% KOH-Extractable Hemicelluloses from Fruit Cell Walls.

(A) Shown are a representative molecular mass profile of Na_2CO_3–extractable cell wall polyuronides subjected to size exclusion chromatography on a column of Sepharose CL-2B and column fractions assayed for uronic acid content.

(B) Shown are representative molecular mass profiles of 4% KOH-extractable cell wall hemicelluloses subjected to size exclusion chromatography on a column of Sepharose CL-6B and column fractions assayed for contents of neutral sugars (thick line) and xyloglucan (thin line). Elution points of linear dextran standards are indicated at top in kilodaltons.

The void (V_o) and total volume (V_t) of the columns are indicated.

the 300- to 200-kD size range. The peak equivalent to ~70 kD also was slightly more pronounced than in controls. The molecular mass profile of xyloglucan showed corresponding changes and at the mature green stage (Figure 9E) was also already extensively depolymerized relative to controls. In overexpressing fruit, there was little further change in the molecular mass profiles of either total hemicelluloses or xyloglucan with ripening, whereas the molecular mass profiles of controls became more like those of the Exp1 overexpressers as ripening advanced. Thus, the overexpression of

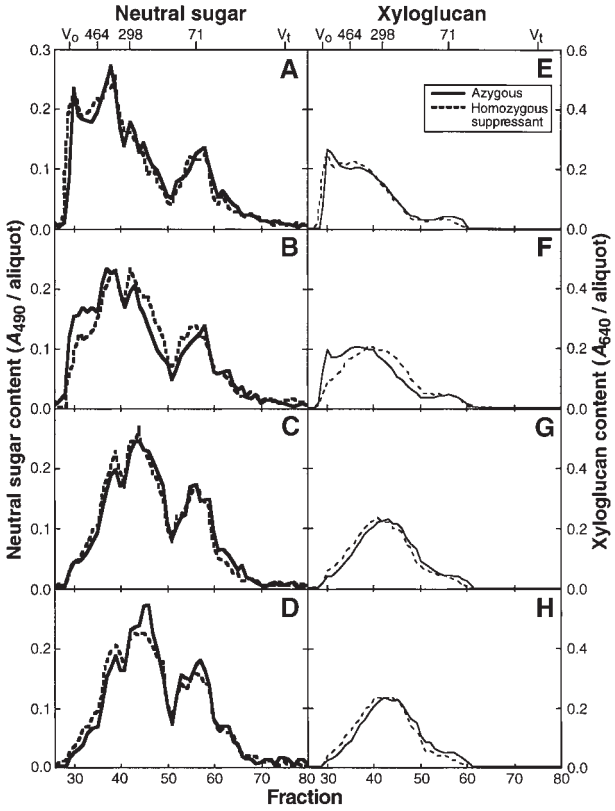

Figure 8. Size Distribution of Total Hemicelluloses and Xyloglucan from Fruit of Suppressant 18-40 Relative to Controls.

Hemicelluloses extracted from cell walls of azygous control (solid lines) or homozygous line 18-40 (dashed lines) fruit at four ripening stages were subjected to size exclusion chromatography on a column of Sepharose CL-6B, and column fractions were assayed for contents of neutral sugar (total hemicelluloses) (**[A]** to **[D]**) and xyloglucan (**[E]** to **[H]**). Void (V_o) and total volume (V_t) of the column and elution points of linear dextran standards are indicated in kilodaltons at top.

(A) to **(D)** Hemicelluloses from control and line 18-40 at the mature green 2 **(A)**, pink **(B)**, red ripe **(C)**, and overripe **(D)** ripening stages.

(E) to **(H)** Xyloglucan from control and line 18-40 at the mature green 2 **(E)**, pink **(F)**, red ripe **(G)**, and overripe **(H)** ripening stages.

Exp1 protein is correlated with cell wall hemicellulose depolymerization and fruit softening typical of ripe fruit, even in mature green fruit before the commencement of ripening.

DISCUSSION

The softening of tomato fruit during ripening is accompanied by alterations both in the architecture and physicochemical properties of the cell wall, and in the polymers of which it is composed. The most prominant changes are the solubilization and depolymerization of polyuronides (Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997), the depolymerization of hemicelluloses (Tong and Gross, 1988; Maclachlan and Brady, 1994), and the loss of galactose (Gross, 1984). Both the pectins and hemicelluloses are composed of a diverse range of polysaccharides; the hemicelluloses, including xyloglucan, glucomannans, xylans, arabinans, and galactans; and the pectins, homogalacturonans, and smaller amounts of heterogeneous rhamnogalacturonans (Seymour et al., 1990; Carpita and Gibeaut, 1993). During ripening, coordinated increases occur in the mRNA

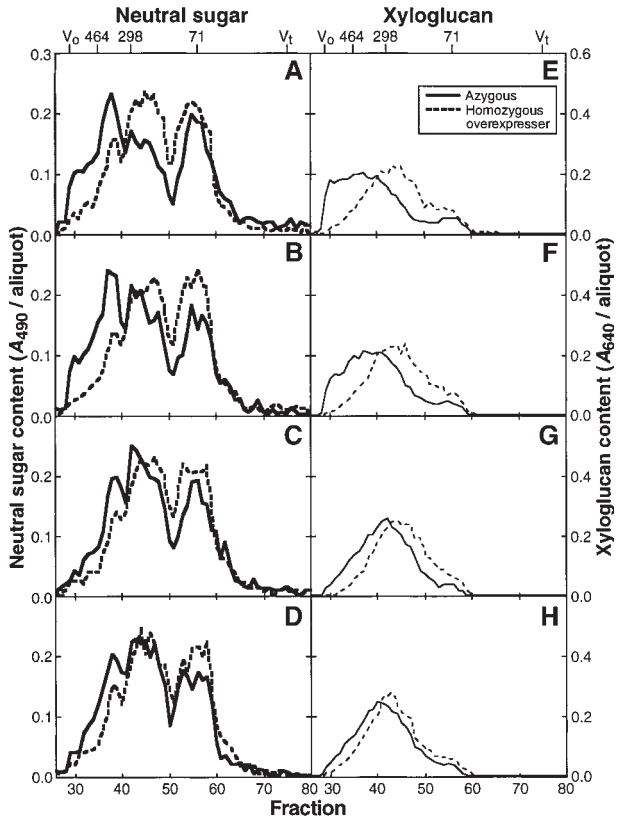

Figure 9. Size Distribution of Total Hemicelluloses and Xyloglucan from Fruit of Overexpresser 17-87 Relative to Controls.

Hemicelluloses extracted from cell walls of azygous control (solid lines) or homozygous line 17-87 (dashed lines) fruit at four ripening stages were subjected to size exclusion chromatography on a column of Sepharose CL-6B, and column fractions were assayed for contents of neutral sugar (total hemicelluloses) (**[A]** to **[D]**) and xyloglucan (**[E]** to **[H]**). Void (V_o) and total volume (V_t) of the column and elution points of linear dextran standards are indicated in kilodaltons at top.

(A) to **(D)** Hemicelluloses from control and line 17-87 at the mature green 2 **(A)**, pink **(B)**, red ripe **(C)**, and overripe **(D)** ripening stages.

(E) to **(H)** Xyloglucan from control and line 17-87 at the mature green 2 **(E)**, pink **(F)**, red ripe **(G)**, and overripe **(H)** ripening stages.

abundance and activity of a range of enzymes that may contribute to cell wall polymer modification or depolymerization, including endopolygalacturonase (PG; Brady et al., 1982; DellaPenna et al., 1986), pectin methylesterase (PME; Harriman et al., 1991), endo-1,4-β-glucanase (EGase; Hobson, 1968; Lashbrook et al., 1994), xyloglucan endotransglycosylase (XET; Arrowsmith and de Silva, 1995), β-galactosidase (Carey et al., 1995; Smith et al., 1998), and expansin (Rose et al., 1997). How these various enzymes act cooperatively or independently to achieve the precise disassembly of particular cell wall components or complexes during fruit softening is not known.

Role of Exp1 Protein in Pectin Depolymerization

Figure 5 shows that in control fruit, considerable polyuronide depolymerization occurs in a CDTA-extractable pectin fraction during ripening but that this is substantially arrested when Exp1 protein is suppressed to ~3% of wild-type levels. Although pectin disassembly may be brought about by several enzymes, PG is believed to be responsible for a large part of polyuronide depolymerization, and its mRNA, protein, and activity accumulate to very high levels late in the ripening of tomato fruit (Tucker et al., 1980; Brady et al., 1982; DellaPenna et al., 1986; Spiers et al., 1989). However, PG-mediated polyuronide depolymerization does not appear to be the primary determinant of fruit softening. Antisense suppression of PG activity to 0.5% of wild-type levels (Sheehy et al., 1988; Smith et al., 1988) resulted in only a modest reduction of polyuronide depolymerization (Brummell and Labavitch, 1997) and a very small increase in firmness later in ripening (Kramer et al., 1992; Langley et al., 1994). Furthermore, expression of a chimeric PG transgene in a nonsoftening mutant tomato fruit that normally lacks this activity resulted in polyuronide solubilization but did not restore softening (Giovannoni et al., 1989).

PG catalyzes massive polyuronide degradation in vitro, but in the cell wall its activity appears to be limited by some chemical or physical restriction (Seymour et al., 1987; Huber and O'Donoghue, 1993; Brummell and Labavitch, 1997). Pectins secreted to the cell wall possess methylester side chains, which are removed by PME as a necessary prerequisite for PG action (Carpita and Gibeaut, 1993). Also, in the wall, PG may exist in association with the β subunit, a small protein that could restrict PG activity by binding either to the enzyme or to its substrate (Watson et al., 1994). Antisense suppression of PME or β-subunit protein accumulation reduced or slightly increased polyuronide depolymerization, respectively, but with little effect on fruit softening during ripening (Tieman et al., 1992; Tieman and Handa, 1994; Watson et al., 1994).

In contrast, strong suppression of Exp1 protein accumulation in line 18-40 resulted in a moderate increase in fruit firmness throughout ripening (Figure 4B), part of which may be due to reduced pectin breakdown. The reduction of polyuronide depolymerization in Exp1-suppressed fruit was evident late in ripening; in tomato, accumulation of high levels of PG also occurs relatively late in ripening (Tucker et al., 1980; Spiers et al., 1989), indicating that PG may be the pectinase whose activity is restricted by Exp1 action. Indeed, the reduction of polyuronide depolymerization by suppression of Exp1 was greater than that due to suppression of PG activity itself (Brummell and Labavitch, 1997) but is presumably an indirect effect. Figure 6A shows that overexpression of Exp1 protein in mature green fruit, where Exp1 is not normally present, does not alter the polyuronide molecular mass profile, suggesting that Exp1 does not itself cause pectin hydrolysis. Later in ripening, when PG appears, Figures 6B to 6D show that pectin depolymerization occurs equivalently in control and overexpressing fruit, and Exp1 overexpression did not significantly increase this process.

Examination of *PG* mRNA abundance in *Exp1*-suppressed fruit found that the accumulation of *PG* mRNA was reduced relative to controls, by three- to fivefold late in ripening (data not shown). However, *PG* mRNA abundance was still substantial in these fruit, suggesting that although there may be some interaction between Exp1 action and PG protein accumulation, this was not sufficient to cause the observed reduction in polyuronide depolymerization. Overall, the data suggest that the action of Exp1 protein on cell wall structure may be necessary to allow some component of polyuronide disassembly to occur, perhaps by controlling access of PG to its substrate.

Role of Exp1 Protein in Hemicellulose Depolymerization

Figures 7B and 8 show that the 24% KOH-extractable hemicelluloses, which are tightly bound to microfibrils, were generally of higher molecular mass than the less tightly associated 4% KOH-extractable hemicelluloses and contained most of the xyloglucan. Ripening was accompanied by a depolymerization of high molecular mass hemicelluloses, including xyloglucan (Sakurai and Nevins, 1993; Maclachlan and Brady, 1994), which was limited to polymers tightly bound to cellulose, as previously found (Tong and Gross, 1988). Tomato fruit contain several enzymic activities that may contribute to hemicellulose depolymerization, notably EGases and XETs. At least two EGases, Cel1 and Cel2, show increases in mRNA abundance coincident with ripening (Lashbrook et al., 1994), but antisense suppression of each of these genes separately did not detectably alter fruit softening (Lashbrook et al., 1998; Brummell et al., 1999b). XET activity may be involved specifically in xyloglucan modification, and although total XET activity was much higher in green rather than in red fruit (Faik et al., 1998), the mRNA of one XET gene increased in abundance during ripening (Arrowsmith and de Silva, 1995).

Figure 8 shows that suppression of Exp1 protein accumulation to 3% of wild-type levels does not prevent depolymerization of hemicelluloses during ripening, whereas Figure 9 shows that overexpression of high levels of Exp1 can

potentiate hemicellulose depolymerization. The relationship between Exp1 action and hemicellulose depolymerization can be explained by three basic mechanisms. One possibility is that the modification of the cell wall brought about by Exp1 and the depolymerization of hemicelluloses are independent events in vivo. Suppression of Exp1 protein to very low levels thus would be without effect on hemicellulose metabolism. If Exp1 is not normally involved in hemicellulose metabolism, its overexpression in green fruit then may cause the observed hemicellulose depolymerization through some aberrant mechanism. Green fruit possess high levels of hydrolases, including EGases and XETs (Maclachlan and Brady, 1994; Faik et al., 1998), and the presence of abnormally high amounts of Exp1 protein may increase the accessibility of substrate sites in hemicellulose molecules to these nonripening-related enzymes that normally are unable to attack them. The consequence of this would be a degradation of hemicelluloses, similar but perhaps not identical to that occurring during ripening but nevertheless resulting in fruit softening. In green fruit, overexpression of Exp1 to 300% of the levels found in wild-type ripe fruit potentiates hemicellulose breakdown, but it is not known if 3% of wild-type levels can evoke the same effect.

A second possibility is that Exp1 protein is necessary for hemicellulase action, as it is for some pectinase activities (Figure 5). Exp1 may normally function by exposing previously unavailable structural hemicellulose molecules to other degradative enzymes (Rose et al., 1997). The observation that constitutive overexpression of Exp1 promotes depolymerization of hemicelluloses in green fruit appears to support this suggestion. However, for this to be the mechanism, 3% of Exp1 protein would need to be sufficient to cause complete hemicellulose availability during ripening, although not enough for 100% of wild-type function in other aspects. For example, the indirect inhibition of polyuronide depolymerization shown in Figure 5 and the increase in fruit firmness shown in Figure 4B show that some components of cell wall metabolism are affected substantially by a 97% suppression of Exp1 protein. Suppression of Exp1 protein abundance to below 3% of wild-type levels, or preferably gene inactivation, is necessary to test this hypothesis. If, however, this mechanism proves to be the case, the regulation by Exp1 of both wall relaxation and the access of particular hemicellulases and pectinases to their substrates would imply a crucial role for this protein in fruit softening.

A third possibility is that hemicellulases are necessary for Exp1 action. Although an interaction at some level should not be excluded, this seems unlikely because, in vitro, expansins can cause extensive and prolonged cell wall loosening in the absence of active cell wall hydrolases (McQueen-Mason et al., 1992; McQueen-Mason and Cosgrove 1994, 1995). Thus, the role of Exp1 protein in hemicellulose metabolism remains equivocal, and further data are necessary to discriminate between the first two models. If Exp1 action is the trigger for hemicellulose breakdown during ripening, then hydrolytic activities already present in green fruit before ripening begins may make significant contributions to ripening-related cell wall changes. On the other hand, if Exp1 action and hemicellulose breakdown are independent, the appearance of ripening-related hemicellulases may be the primary determinant of hemicellulose depolymerization.

Expansin, Fruit Cell Wall Metabolism, and Softening

Fruit softening is probably caused by the cumulative effect of a range of modifications occurring in the networks of polymers making up the primary cell wall, all of which contribute in different ways to a loss of firmness and changes in textural qualities. During ripening, fruit softening is associated with depolymerizations of high molecular mass hemicelluloses, including xyloglucan, which are tightly associated with cellulose, and of ionically bound polyuronides. Suppression and overexpression of Exp1 protein have opposite effects on fruit softening, indicating a role for Exp1 in this process, but unexpectedly they also cause indirect changes in different cell wall components.

Overexpression of recombinant Exp1 protein in mature green fruit evokes extensive hemicellulose depolymerization and considerable softening, in the absence of polyuronide depolymerization. Thus, polyuronide depolymerization is not essential for at least one major component of softening. Figure 4C shows that fruit overexpressing Exp1 continue to soften during ripening, even though changes to the hemicellulose molecular mass profile are complete at the mature green stage, implying that mechanisms of softening other than hemicellulose breakdown occur as these fruit ripen. The magnitude of the difference in softness between overexpressing fruit and controls diminishes progressively with ripening, presumably as hemicelluloses of control cell walls also become increasingly depolymerized. In contrast, strong suppression of Exp1 protein accumulation increases fruit firmness to a similar extent throughout ripening (Figure 4B). Suppression of Exp1 protein, however, does not prevent hemicellulose depolymerization, which proceeds at wild-type levels, even though indirect effects on polyuronide disassembly are evident later in ripening. These findings suggest that the increased firmness of suppressed fruit is not due to reduced hemicellulose breakdown but may be due to the suppression of a direct effect of Exp1 protein on cell wall properties throughout ripening and to reduced polyuronide breakdown later in ripening.

Overall, the data are consistent with at least three processes contributing to textural changes and the loss of fruit firmness during ripening. Exp1 protein is responsible for one component, perhaps by a loosening of noncovalent linkages between unidentified polymers at the hemicellulose–microfibril interface, as suggested by McQueen-Mason and Cosgrove (1995). This is accompanied by the second and major component of softening, consisting of the depolymerization of structurally important hemicelluloses brought about by an independent mechanism that does not require

Exp1 protein or for which only very small amounts of Exp1 protein are sufficient. A third aspect of firmness loss is polyuronide depolymerization, the later stages of which are dependent on the Exp1-mediated relaxation of the wall structure necessary to allow PG or other enzymes access to polyuronide substrate sites. This would have the effect of ensuring that cell wall loosening and the complete fruit softening process precede the final component of polyuronide disassembly, which is involved in fruit deterioration. The data also suggest that at least some parts of the softening program occur in sequential steps that are dependent on the completion of previous steps.

METHODS

Production of Transgenic Plants

Using site-directed mutagenesis with mismatched oligonucleotides followed by polymerase chain reaction amplification, we introduced an NcoI restriction site into the *Exp1* cDNA at the ATG codon of the translational start and an XbaI site in the 3′ untranslated region 51 nucleotides after the translational stop codon. An out-of-frame truncated version of the *Exp1* cDNA was produced similarly, with the NcoI site introduced 233 nucleotides into the coding region. Both the in-frame full-length coding and the out-of-frame truncated versions of the mutagenized *Exp1* cDNA were ligated into the expression cassette of plasmid pJJ2104 (Harpster et al., 1988) between the cauliflower mosaic virus 35S promoter:*Cab22L* leader and the nopaline synthase 3′ transcription terminator (Figure 1A). These constructs were transferred to the binary vector pWTT2179, a derivative of pWTT2084 (Firoozabady et al., 1995) in which the *surB* chlorsulfuron resistance gene was instead driven by the pepper *Gp2* promoter (Baden et al., 1997). *Agrobacterium tumefaciens* LBA4404 harboring these constructs was used to transform cotyledons of tomato (*Lycopersicon esculentum* cv Ailsa Craig) essentially as described previously (Yoder et al., 1988). Regenerated transformed seedlings were selected on chlorsulfuron-containing media and rooted and grown to maturity in a greenhouse.

Screening Transgenic Plants by RNA Gel Blot Analysis

RNA was prepared from young leaves and red ripe fruit of primary transformants as described by Dunsmuir et al. (1987), and aliquots of 10 μg of total RNA were subjected to electophoresis in denaturing gels and then blotted to Duralon-UV membranes (Stratagene, La Jolla, CA). RNA gel blots were hybridized with a 500-bp fragment of the *Exp1* cDNA (composed of nucleotides 151 to 648 of the coding sequence) radiolabeled with α-^{32}P-dCTP, random hexamers, and the Klenow fragment of DNA polymerase I and then washed in 0.1 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS at 65°C.

Segregation Analysis

Selected primary transformants (T_1 generation) were allowed to self-fertilize, and 128 seeds per line of the next (segregating) T_2 generation were grown in chlorsulfuron selection to determine segregation patterns of the transgenes. Lines with segregation patterns consistent with a single locus T-DNA insertion were retained. Twelve survivors per line of this chlorsulfuron treatment, that is, hemizygous or homozygous T_2 plants, were grown to maturity in a greenhouse. The zygosity of these plants was determined by determining the segregation pattern in the following T_3 generation, and homozygous T_2 plants were retained. Corresponding azygous plants for each line, from which the transgene had been eliminated by segregation, were identified by polymerase chain reaction and confirmed by 100% chlorsulfuron susceptibility of the seeds. Azygous and homozygous T_2 sibling plants were propagated by cuttings and provided the source of all fruit used in this study.

Fruit Material

Fruit were collected from homozygous transgenic plants and corresponding azygous control plants as described above, at particular ripening stages defined by internal anatomy and color (Gonzalez-Bosch et al., 1996) or by days after the breaker stage. Breaker stage is the first visible exterior red color at the blossom end of the fruit. The stages harvested were as follows: mature green stage 2 (full-size green fruit, one or two locules liquified), turning (10% red, approximately breaker +0.5 days), pink (50% red, approximately breaker +2 days), light red (100% light red, approximately breaker +4 days), red ripe (breaker +6 days, full red and edibly soft), and overripe (breaker +14 days, very soft with evident deterioration). In wild-type fruit, mature green stage 2 is before the accumulation of *Exp1* mRNA, which appears by mature green stage 4 just before breaker (Brummell et al., 1999a). Three fruit per genotype and ripening stage were collected, locules and seeds were removed, and pooled pericarp material was frozen in liquid nitrogen and then stored at −80°C. Samples of the same fruit material were used for all subsequent RNA, protein, and cell wall analyses. RNA gel blot analysis was conducted as above, and relative mRNA abundances were determined using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Mapping T-DNA Insertions

Genomic DNA was prepared (Greene et al., 1994) from young leaves of wild-type and selected transformants, and aliquots of 10 μg were digested with a battery of restriction enzymes. Restriction fragments were separated by electrophoresis on 0.8% agarose gels and blotted to Duralon-UV membranes. Replicate gel blots were hybridized as above with radiolabeled riboprobes corresponding to the *Exp1* gene, the chlorsulfuron resistance *surB*-selectable marker gene, and the right and left T-DNA borders. T-DNA insertions were mapped by comparison to wild type of the number and size of restriction fragments binding to these probes.

Cell Wall Protein Extraction and Protein Gel Blot Analysis

Frozen fruit pericarp tissue was powdered in liquid nitrogen and then homogenized with 2 volumes of homogenization buffer (40 mM sodium phosphate, pH 7.0, 3 mM EDTA, 5 mM DTT, and a cocktail of protease inhibitors). Supernatants containing soluble proteins were discarded, and pellets were resuspended in SDS loading buffer (Laemmli, 1970), which was supplemented with 5% 2-mercaptoethanol but from which bromophenol blue was omitted. Samples were

heated in a boiling water bath for 10 min, mixed, heated for a further 10 min, and centrifuged, and the supernatants containing cell wall proteins were aspirated. This extraction procedure solubilized all immunodetectable Exp1 protein from crude cell wall preparations. Protein contents were quantified relative to dilutions of a BSA standard by spotting dilutions onto 3MM paper (Whatman, Maidstone, UK), washing with acetone, and staining with Coomassie Brilliant Blue R 250.

Exp1 antiserum was a generous gift of J. Rose (University of Georgia, Athens). Antiserum was purified before use as described previously (Harpster et al., 1998) by incubating with cell wall protein from immature green fruit, lacking *Exp1* expression, which had been covalently coupled to an *N*-hydroxysuccinimide–activated Sepharose column (Pharmacia, Piscataway, NJ) to form an affinity column. Unbound antiserum was enriched for antibodies specific to Exp1 protein.

Cell wall proteins (100 μg per lane) were separated by SDS-PAGE and electroblotted to Immobilon-P membrane (Millipore, Bedford, MA). Protein gel blots were blocked with nonfat dried milk, incubated with purified Exp1 antiserum at a 1:5000 dilution overnight, washed, reacted with goat anti–rabbit antibody coupled to alkaline phosphatase, and rewashed. Bound antibodies were detected using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as substrate.

Measurement of Fruit Firmness

Fruit were harvested from homozygous and corresponding azygous control plants at the mature green stage and ripened in air at 20°C. Pericarp firmness was determined using a model TA.XT2 Texture Analyzer (Stable Micro Systems, Godalming, UK) fitted with a flat probe 25 mm in diameter operated at 0.5 mm sec^{-1}, at the following intervals: mature green, mature green +3 days (equivalent to late mature green, i.e., full-size green fruit, all locules liquified, some internal coloration), breaker, breaker +2 days (pink), breaker +4 days (light red), breaker +6 days (red ripe), and breaker +12 days (overripe). At each time point, the force required to compress the pericarp by 0.5 mm was recorded at three different points on the equator of each fruit. Data were analyzed by ANOVA, and means were compared using the Least Significant Difference test at a significance level of 0.05.

Cell Wall Fractionation and Size-Exclusion Chromatography

Acetone-insoluble cell wall material was prepared from frozen fruit pericarp by using the Tris-buffered phenol method (Huber and O'Donoghue, 1993). Aliquots of 100 mg were sequentially extracted with *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid (CDTA) and Na_2CO_3 to extract pectins, as described previously (Brummell and Labavitch, 1997), and then with 4% and 24% KOH to extract hemicelluloses, as described previously (Maclachlan and Brady, 1994). Equal amounts (1 mg) of dialyzed CDTA-extractable and Na_2CO_3–extractable polyuronides were applied to a column of Sepharose CL-2B (96 × 1.5 cm) eluted with 0.2 M ammonium acetate, pH 5.0, and fractions of 2 mL were collected. Uronic acid contents of aliquots of column fractions (0.4 mL) were determined by the method of Blumenkrantz and Asboe-Hansen (1973). KOH-extractable hemicelluloses were neutralized, precipitated, and treated with α-amylase (Maclachlan and Brady, 1994), and equal amounts (2 mg) were applied to a column of Sepharose CL-6B (97 × 1.5 cm) and eluted with 0.1 M NaOH, and fractions of 2 mL were collected. Column fractions were assayed for neutral sugar content (aliquot of 0.4 mL) by the phenol-sulfuric method (Dubois et al., 1956) and for xyloglucan content (aliquot of 0.8 mL) by the iodine binding method (Kooiman, 1960), an assay that detects polymeric xyloglucan greater than ~20 kD. The CL-6B column was calibrated with linear dextrans of known size, but because these may possess a different conformation to cell wall hemicelluloses, they should be used as an estimate of molecular mass only.

ACKNOWLEDGMENTS

We thank Joss Rose (University of Georgia) for mutagenesis and the generous gift of Exp1 antiserum, Kathy Lee (DNA Plant Technology [DNAP]) for assembly of constructs, Fereshteh Saghaei and Stacey Crocker (Seminis Vegetable Seeds) for assistance with plant transformations, Carol McGugin (DNAP) for segregation analysis, Jay Maddox and Vinnie Pirozzi (DNAP) for maintaining greenhouse plants, Kristen Hadfield and Adnan Sabehat (University of California at Davis) for assistance with fruit collection and firmness measurements, and Ed Ralston and Steve Scofield (DNAP) for comments on the manuscript. We are particularly indebted to Joss Rose for his contribution during the early stages of this project. This work was supported by Seminis Vegetable Seeds.

Received June 23, 1999; accepted September 10, 1999.

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