Reduction of Tomato Polygalacturonase β Subunit Expression Affects Pectin Solubilization and Degradation during Fruit Ripening

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The developmental changes that accompany tomato fruit ripening include increased solubilization and depolymerization of pectins due to the action of polygalacturonase (PG). Two PG isoenzymes can be extracted from ripe fruit: PG2, which is a single catalytic PG polypeptide, and PG1, which is composed of PG2 tightly associated with a second noncatalytic protein, the β subunit. Previous studies have correlated ripening-associated increases in pectin solubilization and depolymerization with the presence of extractable PG1 activity, prior to the appearance of PG2, suggesting a functional role for the β subunit and PG1 in pectin metabolism. To assess the function of the β subunit, we produced and characterized transgenic tomatoes constitutively expressing a β subunit antisense gene. Fruit from antisense lines had greatly reduced levels of β subunit mRNA and protein and accumulated <1% of their total extractable PG activity in ripe fruit as PG1, as compared with 25% for wild type. Inhibition of β subunit expression resulted in significantly elevated levels of EDTA-soluble polyuronides at all stages of fruit ripening and a significantly higher degree of depolymerization at later ripening stages. Decreased β subunit protein and extractable PG1 enzyme activity and increased pectin solubility and depolymerization all cosegregated with the β subunit antisense transgene in T2 progeny. These results indicate (1) that PG2 is responsible for pectin solubilization and depolymerization in vivo and (2) that the β subunit protein is not required for PG2 activity in vivo but (3) does play a significant role in regulating pectin metabolism in wild-type fruit by limiting the extent of pectin solubilization and depolymerization that can occur during ripening. Whether this occurs by direct interaction of the β subunit with PG2 or indirectly by interaction of the β subunit with the pectic substrate remains to be determined.

INTRODUCTION

During the ripening of many climacteric fruit, homogalacturonan and rhamnogalacturonan polymers of the cell wall pectin matrix undergo a series of structural and chemical changes. These changes include (1) the cleavage of methyl ester groups from the pectin backbone and the initiation of calcium binding to form interionic calcium bridges between adjacent pectin chains (Carpita and Gibeaut, 1993); (2) an increase in pectin solubilization and a corresponding decrease in the average ular size of these pectins (Seymour et al., 1987; DellaPenna et al., 1990; Smith et al., 1990); and (3) the loss of neutral sugar residues, such as arabinosyl and galactosyl from pectin side chains (Gross and Wallner, 1979). These ultrastructural and chemical changes within the pectin fraction of fruit cell walls are temporally correlated with the de novo synthesis of several cell wall hydrolases including polygalacturonase (PG) and pectin methylesterase (PME) (reviewed by Fischer and Bennett, 1991).

The down-regulation of genes encoding tomato fruit PG and PME in transgenic plants by antisense RNA has led to a greater understanding of the role each catalytic enzyme plays in pectin metabolism during fruit ripening (Smith et al., 1988, 1990; Tieman et al., 1992; Hall et al., 1993). Decreased PME activity is associated with a higher degree of methyl esterification, decreased chelator solubility, and decreased depolymerization of polyuronides during ripening (Tieman et al., 1992). The down-regulation of PG expression and the resultant accumulation of less than 1% of normal PG activity during ripening also greatly inhibits pectin depolymerization, but has little effect on solubilization (Smith et al., 1990). Conversely, overexpression of the catalytic PG protein in the ripening inhibitor (rin) background, which does not normally accumulate appreciable amounts of PG (Tucker et al., 1980; DellaPenna et al., 1987), results in pectin solubilization and depolymerization similar to ripening wild-type fruit (Giovannoni et al., 1989; DellaPenna et al., 1990). These latter two sets of experiments conclusively demonstrate that production of the catalytic PG polypeptide during ripening is sufficient and necessary for pectin solubilization and depolymerization.

The catalytic PG protein produced during tomato fruit ripening is encoded by a single gene (Bird et al., 1988) that is

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transcriptionally activated at the onset of ripening (DellaPenna et al., 1989). Although only a single PG polypeptide is produced, two PG isoenzymes are predominantly extracted from ripe tomato cell walls, PG1 and PG2, which are both capable of degrading cell wall pectin in vitro (Pressey and Avants, 1973; Tucker et al., 1980). The PG2 isozyme is composed of a single catalytic PG polypeptide, whereas PG1 is thought to consist of at least one catalytic PG2 protein and an ancillary glycoprotein, the β subunit (Tucker et al., 1981; Moshrefi and Luh, 1983; Pogson et al., 1991). High concentrations of chaotropic agents are required to dissociate the PG1 heterodimer in vitro, indicating that the association between the β subunit and PG2 is very stable (Pressey, 1988; Pogson et al., 1991; Pogson and Brady, 1993a). The formation of PG1 confers characteristic physical and biochemical properties to the catalytic PG2 polypeptide, including greatly increased heat stability, which can be readily used to determine the relative amounts of the two isoenzymes in complex mixtures (Tucker et al., 1980, 1981; Knegt et al., 1988).

Several independent lines of physiological, biochemical, and molecular evidence have been put forth to support the hypothesis that PG1 is the active isoenzyme in vivo. These include the observation that only PG1 can be extracted when maximal pectin solubilization and depolymerization are observed in both wild-type fruit and rin fruit expressing an inducible PG2 transgene (DellaPenna et al., 1990). Subsequent accumulation of high levels of extractable PG2 activity in both tissues is not accompanied by further pectin solubilization or depolymerization (Giovannoni et al., 1989; DellaPenna et al., 1990). Furthermore, the in vivo biphasic loss of PG activity during heat treatment of intact fruit tissue mimics the in vitro heat inactivation profile of mixtures of PG1 and PG2 isoenzymes, suggesting that the PG1 complex exists in vivo (Pogson and Brady, 1993b). Finally, analysis of transgenic tomato plants constitutively expressing an antisense PG2 transgene has shown that the residual 1% PG enzyme activity extracted from ripe fruit is exclusively in the form of PG1 and is sufficient for wild-type levels of pectin solubilization to occur during ripening. Based on this result, it was proposed that only very low levels of PG1 were required for normal pectin solubilization (Smith et al., 1990). These combined studies suggest that the presence of extractable PG1 activity is correlated with pectin solubilization and in some cases with pectin depolymerization during tomato fruit ripening and therefore implicate the β subunit protein as an important factor in regulating or restricting the catalytic PG2 protein in vivo (Knegt et al., 1988, 1991; Giovannoni et al., 1989; DellaPenna et al., 1990).

To further understand the structure of the β subunit and its role in pectin metabolism and PG isozyme production, Zheng et al. (1992), using mixed oligonucleotides derived from β subunit protein sequence, isolated β subunit cDNAs from an immature green fruit cDNA library. RNA gel blot analysis of tomato fruit RNA demonstrated that β subunit expression is largely fruit specific as well as temporally separated and independent of expression of the catalytic PG2 gene (Zheng et al., 1992, 1994). Comparison of purified β subunit protein sequence with that deduced from the β subunit cDNA has revealed that, in addition to the mature β subunit protein domain, the cDNA encodes a hydrophobic signal sequence, an N-terminal propeptide, and a large (25-kD) C-terminal propeptide. The deduced mature protein domain is composed almost entirely of a novel repeating 14–amino acid consensus sequence, FTYGYxGNGGXxx (where “x” is most often a charged or uncharged polar amino acid). Although similar in some regard to shorter, more highly conserved repeating motifs in well-characterized cell wall structural proteins (e.g., hydroxyproline-rich glycoproteins and proline-rich proteins; for review, see Showalter, 1993), the size and variability of the β subunit motif clearly place it in a distinct and separate class. The repeating motif feature coupled with data suggesting that the β subunit restricts or targets the catalytic PG2 protein to specific sites within the cell wall has led us to hypothesize that the β subunit represents a new class of "regulatory" cell wall proteins that interacts with both catalytic proteins and structural components of the cell wall.

Despite extensive studies of the structure, regulation, and function of the catalytic PG protein during tomato fruit ripening, it is still unclear if PG1 isozyme formation occurs in vivo or whether PG1 and/or PG2 alone is sufficient for pectin solubilization and depolymerization in vivo. To test the hypothesis that the β subunit is involved in pectin depolymerization or solubilization in vivo and that the formation of PG1 is required for PG activity in vivo, several independently transformed tomato lines with substantially reduced levels of β subunit protein in mature green fruit were generated. Those lines with less than 1% of their total PG activity as PG1 in ripe fruit were analyzed in greater detail with respect to pectin solubilization and depolymerization during fruit ripening. These results are discussed in relation to the proposed function of the β subunit and the roles of PG1 and PG2 in modifying pectin chemistry during fruit ripening.

**RESULTS**

**Construction of the β Subunit Antisense Transgene and Transformation of Tomato Plants**

Figure 1 outlines the construction of the β subunit antisense gene (pBant1) used to transform tomato cotyledons. pBant1 contains nucleotides 1 to 1790 of the 2227-bp full-length β subunit cDNA described in Zheng et al. (1992) inserted in the antisense orientation in place of the β-glucuronidase (GusA) coding region of pBH121. Further details of the construction and transformation are provided in Methods. Two control lines were generated (controls 1 and 2) through tissue culture of tomato cotyledons and the subsequent transfer of mature plants into soil. Genomic DNA isolated from independently transformed antisense lines was digested, gel blotted, and probed with purified β subunit cDNA to confirm that those plants containing the kanamycin selection marker gene also had integrated an
**β Subunit Protein Levels and PG Isoenzymes in Ripe Fruit of Control 1 and Plants Transformed with an Antisense β Subunit Transgene**

Developing fruit were grouped into stages by days after pollination (DAP), e.g., 24, 33, 37, and 42 DAP, with 42-day-old fruit classified as mature green (MG). The visual onset of ripening, i.e., the first visible external coloration of the green fruit, was ~45 DAP and is referred to as the breaker (Br) stage. Later ripening stages were classified as days after breaker stage (e.g., Br+2, Br+5, Br+7, or Br+10). At least three fruit were collected at the Br+7 day ripening stage from individual transgenic lines and control 1, and the combined pericarp tissue from each was used for subsequent analysis. Total cell wall proteins isolated from several antisense transformed lines were assayed to determine their immunologically detectable levels of β subunit and PG2 proteins as well as the extractable PG1 intact copy of the transgene. Digestion with PstI, which flanks the β subunit antisense transgene of pBant1, was used to determine the presence of intact transgenes, whereas digestion with enzymes that cleave pBant1 only once, external to the antisense β subunit cDNA (e.g., HindIII), were used to generate restriction fragment length polymorphisms for determining gene copy number. Seventeen independently transformed lines containing intact transgenes were identified, nine contained one transgene, five contained two transgenes, and three lines contained three or more transgenes (data not shown). Figure 2 shows DNA gel blot analysis and the transgene copy number of the four independently derived antisense lines studied in the greatest detail, TA8, TA9, TA10, and TA42.

**Figure 1.** Construction and Structure of the β Subunit Antisense Transgene.

Details of pBant1 construction are provided in Methods. P, H, S, and N are PstI, HindIII, Sall, and NruI, respectively; CaMV 35S, cauliflower mosaic virus 35S promoter; Nos term, nopaline synthase termination site. The ligation that produces pBant1 destroys the Smal and NruI sites in pBI121 and pBsub2.2, respectively.

**Figure 2.** DNA Gel Blot Analysis of Control 1 and β Subunit Antisense Lines.

Genomic DNA (10 μg) was digested with PstI, separated in an 0.8% agarose gel, and blotted onto a nylon membrane. The blot was probed with the full-length β subunit cDNA clone pBsub2.2. The arrow marks the position of the 3.2-kb band from the introduced β subunit antisense gene. The number of antisense genes contained in each line (as determined by restriction fragment length polymorphism analysis; data not shown) is indicated in the lower box.
Expression of β Subunit and PG2 Proteins and mRNAs during Development and Ripening of Control 1 and TA8 Fruit

The antisense line TA8, in which <1% PG1 was detected, contains two copies of the antisense transgene, which segregate as a single locus (102 kanamycin resistant [KanR] to 33 kanamycin sensitive [KanS]). Due to these combined attributes, TA8 was selected for further analysis. Cell wall proteins for gel blot analysis were extracted from fruit 24, 33, and 37 DAP and from MG and Br fruit to Br+10 fruit. Protein gel blot analysis was performed to determine whether the reduction in PG1 activity in Br+7 TA8 fruit (Figure 3) was a result of a reduction in β subunit protein levels during fruit development and to determine if accumulation of the PG2 protein during ripening was affected by the expression of the β subunit transgene. Figure 4A shows the gel blot analysis of control 1 and TA8 protein samples using β subunit and PG2 antibodies (top and bottom two gels, respectively). β Subunit protein was

Figure 3. Immunoblot Analysis of β Subunit and PG2 Proteins in Control 1 and Antisense Lines.

One microgram of total cell wall proteins isolated from Br+7 pericarp tissue from the indicated lines was resolved by SDS-PAGE and blotted onto nitrocellulose; β subunit (top) and PG2 (bottom) polypeptides were detected by their reaction with their respective antibodies. The level of PG1 isoasyme as a percentage of total PG activity in total cell wall protein extracts from each line and the amount of lycopene per gram fresh weight of tissue are indicated in the boxed table.

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Figure 4. Immunoblot and RNA Blot Analysis of β Subunit and PG2 during Fruit Development and Ripening of Control 1 and TA8 Fruit.

(A) Immunoblot analysis. One microgram of total cell wall proteins isolated from pericarp tissue of the indicated plant line and developmental stage was separated by SDS-PAGE, blotted onto nitrocellulose, and detected with β subunit or PG2 antisera, as described in Methods. The top two gels show β subunit protein levels in control 1 and TA8, and the bottom two gels show PG2 protein levels in control 1 and TA8. The developmental stages are 24, 33, and 37 DAP, respectively; MG, mature green; Br, breaker stage (time of first external color development); and Br+2, Br+5, Br+7, and Br+10 are days after breaker.

(B) RNA gel blot analysis. Total RNA (10 µg) isolated from the indicated plant line and ripening stage was separated electrophoretically, blotted onto a nylon membrane, and probed with either the β subunit cDNA clone (pBsub2.2) or a PG2 cDNA clone (pPG1.9). The top two RNA blots show β subunit mRNA levels in control 1 and TA8, and the bottom two RNA blots show PG2 mRNA levels in control 1 and TA8. The developmental stages are as given in (A).
detectable at the earliest stage analyzed (24 DAP) and accumulated throughout control 1 fruit development, which is similar to that described in previous studies of wild-type tomato fruit (Zheng et al., 1992, 1994). However, in TA8 fruit β subunit protein was almost undetectable at all stages of development. PG2 protein was first detectable at the Br+2 stage in both control 1 and TA8 fruit, increasing to maximal levels in both at the Br+10 stage. PG2 protein levels were somewhat higher in TA8 at the Br+7 stage but were otherwise apparently unaffected by expression of the antisense β subunit transgene.

RNA was extracted from the same tissue samples used for protein analysis to determine whether the reduction in β subunit protein levels was a result of reduced β subunit steady state mRNA levels during fruit development. Figure 4B (top two gels) shows that the pattern of β subunit RNA accumulation in control 1 was very similar to that previously reported by Zheng et al. (1992). β Subunit RNA was detectable at 24 DAP, reached maximal levels at 37 DAP, and declined to undetectable levels at the Br+2 stage. In contrast, β subunit RNA was almost undetectable throughout TA8 fruit development and ripening. PG2 gene expression in control 1 and TA8 fruit was also measured (Figure 4B, bottom two gels) and found to be very similar, except at the Br+7 stage, where steady state PG2 mRNA levels in TA8 fruit were higher than in control 1.

Extractable PG1 and PG2 Isoenzyme Levels during Ripening of Control 1 and TA8 Fruit

Total cell wall proteins isolated from developmental stages MG (42 DAP) through Br+10 fruit (≈53 DAP) were assayed by differential heat inactivation to determine the relative levels of PG1 and PG2 in control 1 and TA8 fruit during ripening. Figure 5A shows that PG1 activity in control 1 fruit was first detectable at the Br+2 stage where it constituted 80% of the total PG activity. As ripening progressed in control 1 fruit, PG1 activity increased very slightly, whereas PG2 activity increased almost fivefold. In contrast, Figure 5B shows that very little PG1 activity was detected at any TA8 ripening stage. The amount and pattern of accumulation of PG2 activity detected during ripening of TA8 fruit were similar to that seen in control 1 but clearly higher at the Br+2 stage. The pattern of total PG enzyme activity during ripening closely reflected immunologically detectable PG2 protein accumulation in control 1 and TA8 (Figure 4A, bottom two gels and Figures 5A and 5B). Figure 5C shows that the developmental accumulation of lycopene during ripening of control 1 and TA8 fruit was almost identical. The timing and levels of ethylene production in TA8 and control 1 fruit were also nearly identical (data not shown). These results indicate that the overall ripening process in TA8 was not adversely affected by the down-regulation of β subunit expression. Similarly, plants expressing an antisense PG2 transgene and accumulating less than 10% of normal PG levels have also been shown to ripen normally, as determined by lycopene accumulation and ethylene production rates (Smith et al., 1990).

![Figure 5. Polygalacturonase isoenzyme Activities and Lycopene Production during Control 1 and TA8 Fruit Ripening.](image-url)
Polyuronide Solubilization and Depolymerization in Control and Antisense Fruit

To address whether the β subunit regulates or restricts the action of PG in solubilizing or depolymerizing pectin, or whether modifying β subunit expression affects pectin chemistry at all, the physical characteristics of EDTA-soluble cell wall polyuronides were analyzed from transgenic and control fruit tissues. Figure 6 illustrates the solubilization of polyuronides as ripening progresses in TA8 and control 1 fruit. The yield of chelator-soluble polyuronides from ripening control 1 cell walls is consistent with previously published data using the same experimental protocol (DellaPenna et al., 1990; Smith et al., 1990). Similarly, as previously described by DellaPenna et al. (1990), the majority (~80%) of solubilization occurred by the Br+2 stage in control 1, where extractable PG activity was predominantly in the form of PG1 (see Figure 5A). However, while the pattern of polyuronide solubility during ripening of TA8 was similar to control 1, the absolute amount of polyuronides extracted from TA8 cell walls throughout ripening was significantly higher as compared to control 1. The histogram in Figure 7 shows that in three additional independent transgenic lines in which β subunit and hence PG1 activity were also greatly reduced (TA9, TA10, and TA42, and see Figure 3), the yield of EDTA-soluble polyuronides from Br+7 fruit was much greater compared to two independently generated control lines. The increase in soluble polyuronides was especially evident for the TA9 line (three or more transgenes) in which the yield was 171% that of control samples. The average EDTA-soluble polyuronide yield at Br+7 was 88 μg mg⁻¹ cell wall material for controls (9% of the cell wall dry weight), whereas that of the four transgenic lines was 140 μg mg⁻¹ cell wall material (14% of the cell wall dry weight).

The size profiles of EDTA-soluble polyuronides from control 1 and TA8 at three different stages of ripening are shown in Figure 8. Polyuronides isolated from MG control 1 and TA8 fruit eluted near the column void volume, indicating a high degree of polymerization (Figure 8A). As ripening progressed to the Br+2 and Br+7 stages in TA8 and control 1, an increasing proportion of the EDTA-soluble polyuronides showed a decrease in size, as evidenced by their elution in the included volume of the column (Figure 8, fractions 15 to 30). The size profiles of control 1 and TA8 were nearly identical at the MG and Br+2 stages (Figures 8A and 8B). However, at the later Br+7 ripening stage two way analysis of variance for eluted column fractions 15 to 30 established a significant difference (P < 0.001) between the size distribution of smaller EDTA-soluble polyuronides isolated from TA8 fruit and those from control 1. At this ripening stage (Figure 8C), 61% of the EDTA-soluble polyuronides are recovered in fractions 15 to 30 for TA8 versus 46% for control 1. Similar increases in the amount of smaller polyuronides were observed when TA8 profiles were compared to control 1 profiles at the Br+5 and Br+10 stages (data not shown). Furthermore, size fractionation profiles of Br+7 EDTA-soluble polyuronides from two additional transgenic lines with <1% of control 1 PG1 activity and β subunit protein levels showed a significant difference (P < 0.05) in the amounts of smaller uronides when compared to control 1. No significant difference was observed between the gel filtration profiles of
control 1 and an untransformed wild-type profile at the Br+7 stage.

Inheritance of the β Subunit Antisense Transgene

DNA gel blot analysis of TA8 and TA42 had shown that two antisense transgenes were integrated into the nuclear genome of both lines (Figure 2 and results not shown). Segregation analysis for the T-DNA-encoded kanamycin resistance marker in T2 progeny seed indicated that the two integrated transgenes in TA42 were inherited as unlinked loci (84 Kanr:5 Kanr), whereas the two transgenes in TA8 were inherited as a single dominant Mendelian locus (102 Kanr:33 Kanr). Additionally, in more than 60 T2 TA lines analyzed by DNA gel blotting, the two transgene insertions were always inherited together (data not shown). TA8 was therefore chosen for inheritance analysis of β subunit antisense transgenes, for its reduced PG1 levels, and for alterations in pectin solubility and depolymerization in homozygous progeny plants and in those in which the transgene locus had segregated away. TA8 T2 seedlings were germinated, and those homozygous for both copies of the transgene were identified by comparison of the hybridization intensity of DNA fragments from the endogenous β subunit gene with those from the added transgenes. In addition, T3 seedlings from putative homozygous T2 lines were germinated on kanamycin-containing media to genetically confirm their homozygosity. “Wild-type sibling” TA8 T2 lines in which the transgene locus had segregated away were similarly identified and confirmed.

MG and Br+7 fruit were collected from TA8 T2 progeny plants homozygous for both transgene copies and TA8 T2 wild-type siblings. Cell wall proteins were extracted from MG and Br+7 fruit and EDTA-soluble polyuronides isolated from Br+7 fruit. In six homozygous TA8 T2 lines, the levels of immunologically detectable β subunit protein were reduced to near the limits of detection, whereas in six TA8 “wild-type sibling” lines the levels of immunologically detectable β subunit protein were restored to that of control 1 fruit (data not shown). Similarly, elevated EDTA-soluble polyuronide levels at the Br+7 stage also cosegregated with the antisense transgene in these lines (data not shown). The inserted histogram in Figure 9 shows the levels of soluble polyuronides and corresponding chromatographic size profiles from Br+7 fruit of a representative homozygous TA8 T2 antisense line, TA8-48, and a wild-type sibling line, TA8-23. TA8-48 shows greatly elevated levels (200%) of EDTA-soluble polyuronides compared to TA8-23. In addition, increased polyuronide depolymerization at the Br+7 stage also cosegregated with the antisense transgene as evident in TA8-48, where 47% of the soluble polyuronides eluted in the included volume (Figure 9, fractions 15 to 30), compared to 31% in TA8-23. Two-way analysis of variance for fractions 15 to 30 established a significant difference (P < 0.001) between the size distribution of EDTA-soluble polyuronides isolated from TA8-48 fruit and those from TA8-23.

DISCUSSION

Tomato fruit ripening leads to the ordered disruption of the plant cell wall architecture and integrity. This complex developmental process presumably occurs as a result of the coordinated synthesis of a diverse class of catalytic enzymes involved in cell
to play a role in the degree of pectin depolymerization during fruit PG isoenzymes, PG1 and PG2, raise the possibility that probably work cooperatively on a substrate polymer by inter-wall metabolism. Many of these newly synthesized enzymes targeting and regulation of the catalytic PG2 polypeptide once fruit development presents interesting possibilities about the described

Effects of β Subunit Antisense Gene Expression on β Subunit Levels, PG Isoenzyme Composition, and Pectin Metabolism

Antisense technology has been used successfully to manipulate the expression of several tomato ripening-associated genes including the genes encoding PG (Sheehy et al., 1988; Smith et al., 1988, 1990), PME (Tieman et al., 1992; Hall et al., 1993), 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (Oeller et al., 1991), ACC oxidase (Hamilton et al., 1990), and phytoene synthase (Bird et al., 1991). The dramatic reduction in steady state levels of β subunit RNA and protein throughout TA8 fruit development (Figure 4), as a result of the integration of an antisense β subunit gene, is therefore similar to previously published data. The lack of β subunit protein during the ripening of TA8 fruit results in the virtual absence of extractable PG1 activity at all ripening stages compared with control fruit (Figures 5A and 5B). Significantly, lycopene accumulation, expression of PG2 mRNA, and protein and ethylene production during ripening of control 1 and TA8 fruit were similar (Figures 4C and 5B, respectively, and data not shown). These results demonstrate that expression of the β subunit antisense gene does not affect ripening in general or the expression of the catalytic PG2 protein and that fruit collected from control 1 or TA8 at any given time point are at an equivalent ripening stage and can be directly compared. Antisense inhibition of β subunit gene expression in TA8 fruit therefore allows a direct test of the functional role of the β subunit, and hence of PG1, in pectin metabolism during the normal ripening process.

Inhibiting β subunit production resulted in significantly increased levels of extractable EDTA-soluble polyuronides at all stages of ripening in TA8 as compared to control 1 (Figure 6). Similar increases in the level of EDTA-soluble polyuronides were observed in multiple independent antisense transgenic lines at the Br+7 ripening stage (Figure 7). These results indicate that in wild-type fruit the β subunit plays an important role in limiting the amount of cell wall pectins that are solubilized during ripening. In addition to increasing pectin solubilization, inhibition of β subunit expression in antisense lines was associated with a statistically significant increase in the depolymerization of EDTA-soluble polyuronides at later ripening stages (Figure 8C). Increased depolymerization of EDTA-soluble polyuronides was also observed in multiple independently derived antisense lines (data not shown) and suggests that in addition to restricting pectin solubilization the β subunit

![ EDTA-Soluble Polyuronides and Gel Filtration Analysis of EDTA-Soluble Polyuronides Isolated from TA8-23 and TA8-48 Br+7 Fruits.](figure9.png)

**Figure 9.** EDTA-Soluble Polyuronides and Gel Filtration Analysis of EDTA-Soluble Polyuronides isolated from TA8-23 and TA8-48 Br+7 Fruits.

The panel inset shows the levels of EDTA-soluble polyuronides isolated from fruit cell walls of TA8-23 ("wild-type" sibling) and TA8-48 (homozygous for antisense gene) at the Br+7 ripening stage. Bars indicate the standard deviation from the mean. The graph shows the chromatographic profile of 1 mg of EDTA-soluble polyuronides isolated from TA8-23 and TA8-48 resolved on a Sepharose CL4B column, as described in Methods. A single representative gel filtration profile is shown.
also plays a role in limiting pectin depolymerization during wild-
type fruit ripening. In the β subunit antisense line studied in
greatest detail (TA8), both phenotypes (increased polyuronide
solubility and increased depolymerization) cosegregated with
the antisense β subunit transgene in T2 progeny plants (Fig-
ure 9). These combined results clearly demonstrate that
elevated levels of EDTA-soluble polyuronides during ripening
and the increased depolymerization of these polyuronides later
in ripening are specific, genetically transmittable phenotypes
associated with reduced β subunit protein levels brought about
by antisense inhibition of β subunit expression during fruit
development.

Increased levels of pectin solubilization and depolymeriza-
tion in β subunit antisense lines were correlated with changes in
three distinct but associated aspects of PG protein and iso-
enzyme activity as a result of the expression of the antisense
β subunit gene: (1) dramatic reduction in β subunit protein lev-
els throughout ripening in TA8 as compared to control 1, (2)
greatly reduced percentage of extractable PG1 enzyme activity
as a component of total activity in antisense fruit as compared
to control fruit (<1% versus 25%, respectively), and (3) detection
of almost all PG activity in antisense fruit in the form of
PG2 (>99% in antisense lines versus 75% for control lines).
It is conceivable that the increased relative amount of PG2
in β subunit antisense lines plays a role in the observed modifi-
cations to pectin metabolism in ripening antisense fruit;
however, several lines of evidence argue against this possi-
ibility. First, Smith et al. (1990) have shown that ripe fruit expressing
a PG antisense gene accumulated less than 1% of normal PG
activity, all in the form of PG1, and exhibited normal levels of
EDTA-soluble pectins compared to control plants. This would
make it extremely unlikely that the relative increase in PG2
isoenzyme activity alone in TA8 could account for the increase
in pectin solubilization. Second, in transgenic experiments with
the tomato ripening mutant rin, which accumulates normal lev-
els of β subunit protein (Zheng et al., 1994) but insufficient
PG2 for pectin solubilization and depolymerization (Seymour
et al., 1987; DellaPenna et al., 1990), expression of an induc-
able PG2 transgene caused rin fruit to undergo near normal
levels of pectin solubilization and depolymerization. This oc-
curred in transgenic rin fruit when only 30% of wild-type PG
activity was present and was extracted exclusively in the form
of PG1 (Giovannoni et al., 1989; DellaPenna et al., 1990). These
studies strongly support our conclusion that it is the absence
of the β subunit rather than the presence of additional PG2
that is responsible for elevated levels of EDTA-soluble poly-
uronides and increased depolymerization in β subunit
antisense fruit.

In their studies of PG antisense fruit, Smith et al. (1990) sug-
gested that because the 1% residual PG protein in ripe
antisense fruit was extracted in the form of PG1, only minimal
levels of PG1 were required for pectin solubilization in vivo.
If this hypothesis was correct, then we would have expected
unaltered or lowered levels of EDTA-soluble polyuronides in
transgenic β subunit antisense lines with reduced levels of PG1.
Because we observed both elevated levels of polyuronide
solubilization throughout TA8 fruit ripening and near maximal
levels of solubilization by the Br+2 stage in the virtual absence
of extractable PG1 (Figures 5 and 6), we concluded that the
formation of PG1 is not required for this PG-mediated activity
in vivo. As with solubilization, the increased depolymerization
of pectins in TA8 fruit during later ripening stages in the ab-
sence of β subunit protein suggests that the β subunit, and
hence PG1 formation, is also not required for pectin depoly-
merization in vivo. Thus, experiments reported in this study,
PG2 antisense experiments (Smith et al., 1988, 1990), and
transgenic rin experiments (Giovannoni et al., 1989; DellaPenna
et al., 1990) all indicate that (1) low levels of PG2 activity rela-
tive to the total produced during ripening are sufficient for near
maximal solubilization of polyuronides and (2) polyuronide
depolymerization occurs over a much longer period of time and
appears to require higher levels of PG2. The differing rates
at which PG2-mediated pectin solubilization and depolymer-
ization occur during ripening suggest that at least two classes
of PG-accessible sites exist within the pectin substrate: those
that are cleaved early in the ripening process during pectin
solubilization that are very labile to PG-mediated hydrolysis,
and others that are initially more recalcitrant to PG-mediated
hydrolysis and require higher levels of PG2 protein over an
extended period of time for pectin depolymerization to be
completed.

Previous studies have correlated pectin solubilization and
in some cases depolymerization during ripening with the pres-
ence of extractable PG1 activity, implicating an important role
for the β subunit in pectin chemistry (Giovannoni et al., 1989;
DellaPenna et al., 1990; Smith et al., 1990). We have previ-
ously proposed that the β subunit interacts with structural
components of the cell wall and PG2 to target its activity or
acts in some other fashion to sterically or enzymatically re-
strict the PG2 polypeptide in vivo (Zheng et al., 1992, 1994).
The current study further defines β subunit function in vivo
by showing that the presence of the β subunit protein in the
cell wall during ripening limits, but is not required for, PG2 ac-
tivity in vivo. The data presented is most consistent with (1)
the β subunit protecting or limiting access of the catalytic PG
protein to pectin sites within the cell wall susceptible to its action
and (2) PG2 alone being responsible for pectin solubilization
and depolymerization in vivo. Two mechanisms for limiting PG2
activity can be proposed that are not necessarily mutually ex-
clusive: an indirect mechanism where interaction of the β
subunit with pectin would exclude PG2 from those regions of
its pectic substrate, or a direct mechanism where interaction of
β subunit with PG2 would restrict PG2 mobility to other areas
of the cell wall. Bearing in mind the strong affinity of PG2 for
the β subunit in vitro, we would predict that the latter mecha-
nism would be irreversible in vivo. Our experiments have not
excluded the possibility of PG1 formation in vivo but suggest
that, if formed, such a complex would not possess catalytic
activity as judged by solubilization and depolymerization. It
is important to stress that the formation of such an inactive
complex may still represent an important control point in the
regulation of pectin metabolism.
METHODS

Plant Material

Tomato (*Lycopersicon esculentum* cv Ailsa Craig) plants were grown under standard greenhouse conditions. Flowers were pollinated and tagged upon opening, and fruit were collected at the indicated days after pollination (DAP). Forty-two-day-old fruit were classified as mature green (MG), and the breaker stage (Br) (∼45 DAP) was considered to be the first visible external coloration of the green fruit. Fruit were allowed to ripen naturally on the plant and were harvested at the indicated developmental stage; locule tissue and seed were removed, and the remaining pericarp tissue was frozen in liquid N₂ and held at −80°C until analysis. At least three fruit were collected for each time point, and the same fruit tissue was used for all subsequent RNA, protein, and cell wall polyuronide extractions.

Plant Transformation

The antisense β subunit DNA fragment was derived from the previously described pBsub2.2 cDNA (Zheng et al., 1992). An NruI-SstI fragment from the β subunit cDNA was subcloned in the antisense orientation into the plant binary vector pBI121 (Clontech, Palo Alto, CA), which had been previously digested with Smal and SstI (to remove the β-glucuronidase gene from pB1121). The resulting antisense transformation vector was named pBan1 (Figure 1). pBan1 was transferred into *Agrobacterium tumefaciens* LBA4404 by triparental mating (Bevan, 1984). Tomato cotyledons were transformed by cocultivation on tobacco feeder cells, as described by DellaPenna et al. (1987). Transformed plants were selected by growth on kanamycin-containing media and confirmed by gel blot analysis (Sambrook et al., 1989) of the plant genomic DNA, using a 32P-labeled Xhol-PstI 2.2-kb fragment of the β subunit cDNA clone as a probe. Segregation analysis of the T-DNA-encoded kanamycin resistance trait in T2 and T3 progeny seedlings was performed on sterile Murashige and Skoog (Sigma, St. Louis, MO) media containing 50 μg mL⁻¹ kanamycin.

Polygalacturonase Enzyme Extraction and Activity Assay

Cell wall proteins were extracted according to the method of DellaPenna et al. (1987), except that precipitated proteins were resuspended in a minimal volume of 150 mM NaCl, 5 mM sodium acetate, pH 6.0, and dialyzed overnight against the same buffer at 4°C using a Microdialysis System (Bethesda Research Laboratories). Protein concentration was measured using a Pierce (Rockford, IL) protein assay kit. All protein samples were then diluted to 0.25 μg μL⁻¹. Prior to the enzyme assays, 20 μL of the enzyme solution was added to 30 μL of dilution buffer (150 mM NaCl, 80 mM sodium acetate, pH 4.4). Total PG activity was determined by incubating the 50-μL protein mix in 1 mL of 150 mM NaCl, 50 mM sodium acetate, pH 4.4, 0.1% polygalacturonic acid (Sigma, St. Louis, MO) at 37°C and measuring reducing sugars by the arsennomolybdate method (Nelson, 1944) using galacturonic acid as a standard. PG1 activity was measured by first heat inactivating PG2 in the 50-μL protein sample by incubation at 65°C for 5 min and then proceeding as described above for measuring total PG activity. PG2 activity was calculated by subtracting PG1 activity from the total PG activity. Purified PG1 and PG2 (Zheng et al., 1992) were used as internal standards to ensure complete heat inactivation of PG2 and that less than 15% of PG1 activity was lost during the 5-min incubation at 65°C. PG1 activity measurements were corrected accordingly. All assays were repeated three times in duplicate.

Nucleic Acid Extraction and Analysis

Fruit RNA was extracted according to the method of Hamilton et al. (1990), and total RNA used for RNA gel blot analysis was as previously described by DellaPenna et al. (1986). Genomic DNA was extracted from young tomato leaf tissue according to the method of Dellaporta et al. (1983). Total RNA (10 μg) was size fractionated on an 1.2% agarose gel containing 2.2 M formaldehyde and blotted onto nylon membranes (MSI, Westboro, MA) according to Sambrook et al. (1989). After immobilization by baking for 2 hr at 80°C, the blot was stained with methylene blue to ensure equal loading in each sample lane (Sambrook et al., 1989). Hybridization was performed according to the method of Smith et al. (1990) with 32P-dCTP (Amersham)-labeled random primed (Bethesda Research Laboratory) probes. Labeled probe (1 × 10⁶ cpm) was used per milliliter of hybridization buffer. The probes used were a 2.2-kb full-length tomato fruit β subunit cDNA clone, pBsub2.2 (Zheng et al., 1992), and a 1.9-kb full-length tomato fruit PG cDNA, pPG19 (DellaPenna et al., 1997). Genomic DNA was digested with the indicated restriction enzymes, fractionated on an 0.8% agarose gel, and transferred onto a nylon membrane. Prehybridization and hybridization were conducted as described by Balague et al. (1993) with a random primed β subunit cDNA probe (Zheng et al., 1992).

Isolation and Column Chromatography of Pectic Polysaccharides

Isolation of acetone insoluble cell wall materials from pericarp tissue and subsequent extraction of EDTA-soluble pectic polysaccharides were as described by DellaPenna et al. (1990). Uronic acids were measured by the metahydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973) using polygalacturonic acid as a standard. Gel filtration chromatography of chelator-soluble pectic polysaccharides was performed as described by DellaPenna et al. (1990) using a Sepharose CL4B column (70 × 1.5 cm), except that 3.8-ML fractions were collected and analyzed for uronic acid content. Yields from gel filtration columns were consistently greater than 90%.

Statistical Analysis of Chelator-Soluble Polyuronide Solubilization and Depolymerization

The standard deviation from the mean was calculated for the amount of chelator-soluble polyuronides extracted at the indicated ripening stage from each line. At least two independent extractions were performed from a minimum of three pooled fruit that were assayed twice in duplicate. A two-way analysis of variance was performed (Minitab for Windows, version 9.2; Minitab Inc., State College, PA) on TAB and control 1 at the Br+7 ripening stage of two independent column chromatographs whose eluted fractions were assayed twice. Only those polyuronides eluted in the included volume (fractions 15 to 30) were considered for statistical analysis. A two-way analysis of variance was also performed between TAB-23 and TAB-46, except that a single column chromatogram was performed whose eluted fraction was assayed two independent times.
Tomato Fruit β Subunit and Pectin Chemistry

 Extraction of Tomato Fruit Cell Wall Proteins for SDS-PAGE and Immunoblot Analysis

Frozen powdered fruit tissue (500 mg) was extracted according to the method of Zheng et al. (1994). We have found that β subunit protein resolved best in an SDS-PAGE gel containing 8 M urea, whereas catalytic PG2 protein resolved better in SDS-PAGE without urea. The SDS-PAGE system described by Laemmli (1970) was used for all analysis in a 15-mm mini-PROTEAN II electrophoresis cell (Bio-Rad, Hercules, CA), except for β subunit polypeptide analyses, in which case the modified system of Zheng et al. (1994) was followed.

Protein blots were blocked with 1 × PBS containing 2% (w/v) nonfat dry milk and 0.01% (v/v) Tween-20 for 1 hr and then incubated with a primary antibody (1:1000 dilution for both PG2 and β subunit antibodies) in 1 × PBS, 0.2% (w/v) nonfat dry milk, and 0.01% (v/v) Tween 20 for 1 hr. Polyclonal β subunit antiserum, raised in rabbits against purified β subunit protein, was the generous gift of A.B. Bennett (University of California, Davis, CA). Polyclonal antibody to PG2 was generated by DellaPenna et al. (1994). After three 10-min washes with 1 × PBS, 0.1% (v/v) Nonidet P-40, the blots were incubated with a 1:3000 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) in 1 × PBS, 0.2% (w/v) nonfat dry milk, and 0.01% (v/v) Tween 20 for 1 hr and then washed three times as described above. The bound antibodies were localized using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrate.

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