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Differential Expression of the Two Subunits of Tomato Polygalacturonase Isoenzyme 1 in Wild-Type and *rin* Tomato Fruit[1]

Liansheng Zheng, Colin F. Watson, and Dean DellaPenna*

Department of Plant Sciences, University of Tucson, Arizona 85721

The β subunit of tomato (*Lycopersicon esculentum* Mill.) fruit polygalacturonase 1 is a cell wall glycoprotein that binds to and apparently regulates the catalytic PG2 polypeptide in vivo. β Subunit and polygalacturonase 2 (PG2) expression have been investigated in both wild-type and *ripening inhibitor* (*rin*) mutant fruit. During fruit development and ripening, β subunit expression was unrelated to expression of the catalytic PG2 protein. In wild-type fruit, β subunit mRNA and protein were first detected early in development and increased to maximal levels before PG2 mRNA and protein were detected. At the onset of ripening β subunit mRNA decreased dramatically, but β subunit protein levels remained stable. In *rin* fruit, which fail to ripen, β subunit expression was similar to that in wild type, although PG2 mRNA and protein were not detected. These data suggest that β subunit expression is ethylene independent and regulated primarily by developmental cues. This conclusion is supported by results from ethylene-treated immature (20 days after pollination) wild-type and *rin* fruit in which no significant differences were observed in β subunit expression patterns in response to ethylene treatment. Surprisingly, RNA blot analysis indicated that catalytic PG2 mRNA was induced in immature *rin* fruit after 3 d of exogenous ethylene treatment. In addition, β subunit mRNA and protein were also detected at lower levels in root, leaf, and flower tissues of both genotypes, suggesting a broader functional role for the protein.

Tomato (*Lycopersicon esculentum* Mill.) fruit PG is a cell wall hydrolase catalyzing pectin solubilization and degradation during ripening. The expression, activity, isoenzyme composition, and function of PG during fruit ripening have been the focus of extensive studies in recent years (reviewed by Fischer and Bennett, 1991). Three PG isoenzymes are present in extracts of ripening tomato fruit, PG1, PG2A, and PG2B, all of which can degrade polyuronides in vitro (Ali and Brady, 1982; Themmen et al., 1982; Moshrefi and Luh, 1983). The catalytic polypeptides of the three PG isoenzymes are the product of a single PG gene (Bird et al., 1988). The PG2 isoforms are composed of single catalytic PG polypeptides and differ only in their degree of glycosylation (DellaPenna and Bennett, 1988). PG1 is a complex composed of at least one catalytic PG2 polypeptide tightly associated with a 38-kD noncatalytic glycoprotein, known as the converter or β subunit protein (Tucker et al., 1981; Pressey, 1984; Knegt et al., 1988, 1991; Pogson et al., 1991).

The β subunit of PG1 is a heat-stable glycoprotein found at high levels in fruit cell wall tissues and at lower levels in leaf tissue (Pressey, 1984). The amount of immunologically detectable β subunit protein increases in developing tomato fruit well before the appearance of catalytic PG2 protein (Pogson and Brady, 1993a). The β subunit can be extracted from the cell walls of both green and ripe tomato fruit by high salt buffers and in the latter case is associated with PG2 polypeptide(s) in the form of PG1 (Tucker et al., 1981; Pressey, 1984). Purified β subunit can also associate with and convert PG2 in vitro into an isoenzyme that closely resembles PG1 (Tucker et al., 1981; Pressey, 1984; Knegt et al., 1988). This association alters the kinetic parameters of the associated PG2 protein and increases its heat stability more than 25°C, a property that can be conveniently used to determine relative amounts of PG1 and PG2 isoenzymes in a mixture (Tucker et al., 1980, 1981; Knegt et al., 1988).

The presence and function of PG1 and the role of the β subunit in regulating PG activity in vivo remain unresolved. It has been proposed that PG1 is an extraction artifact, since PG2 and β subunit protein can be recovered separately from ripe fruit cell walls; however, this differential extraction could only be accomplished in buffers of radically different ionic strengths and extremes of pH (Pressey, 1986; Knegt et al., 1991). Other lines of evidence from molecular and biochemical studies suggest that PG1 is the physiologically active complex in vivo and have implicated the β subunit as playing an important role in immobilizing or regulating the catalytic PG2 protein in vivo (Giovannoni et al., 1989; DellaPenna et al., 1990). Evidence supporting this hypothesis includes: (a) the sequential appearance of PG1 and PG2 during fruit ripening (Tucker et al., 1980; Brady et al., 1982), (b) the observation that only PG1 can be extracted when maximal pectin degradation and solubilization are observed in ripening wild-type fruit and in *rin* fruit expressing an inducible PG2 transgene (DellaPenna et al., 1990), (c) lack of pectin degradation in transgenic tobacco plants expressing a tomato PG transgene and accumulating only PG2 isoenzymes (Osteryoung et al., 1990), and (d) the in vivo biphasic loss of PG activity during heat treatment of intact fruit tissue that mimics

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* Corresponding author; fax 1–602–621–7186.

Abbreviations: DAP, days after pollination; PG, polygalacturonase [poly(1,4-α-D-galacturonide)glycanohydrolase, EC 3.2.1.15]; *rin*, *ripening inhibitor*.

the in vitro heat inactivation profile of mixtures of PG1 and PG2 isoenzymes (Pogson and Brady, 1993b).

A full-length β subunit cDNA clone has recently been isolated and characterized and encodes a 69-kD precursor protein that is subject to extensive posttranslational modification to yield the 38-kD mature β subunit protein. The primary sequence of the mature protein domain is composed almost entirely of a novel 14-amino acid motif, FTNYGxx-GNGGxxx. Expression of β subunit and PG2 mRNA were reported to be temporally separated, suggesting that expression of the two subunits of PG1 are controlled by different developmental or hormonal cues (Zheng et al., 1992).

To further our understanding of the factors controlling expression of the two subunits of PG1, we have analyzed β subunit and PG2 mRNA and protein levels in greater detail during wild-type fruit development and ripening. In addition, to address whether developmental or hormonal (i.e. ethylene) cues regulate the dramatic decrease in β subunit expression at the onset of ripening, we have investigated the accumulation of β subunit and PG2 mRNAs and proteins in wild-type and *rin* fruit in the presence or absence of exogenous ethylene. Wild-type and *rin* fruit grow and develop similarly prior to ripening; however, fruit homozygous for the *rin* mutation show a complete inhibition of ripening and produce only background levels of ethylene and trace amounts of PG2 mRNA and protein (Herner and Sink, 1973; McGlasson et al., 1975; DellaPenna et al., 1987). The genetic ripening deficiency of *rin* fruit, when compared with wild-type fruit, allows one to resolve developmental effects on gene expression from ripening or ethylene-related effects. Finally, we report the expression of β subunit mRNA and protein in nonfruit tissues known to contain other developmentally regulated PG activities.

MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum Mill.* cv Ailsa Craig and *rin*) were raised in 10-gallon pots in a controlled environment greenhouse with 22°C day and 18°C night temperatures under natural lighting and standard cultural practices. The *rin* mutant was near isogenic (backcrossed eight times to Ailsa Craig). Flowers were hand-pollinated and tagged at anthesis, and fruit were harvested at the indicated DAP. Twenty-DAP fruit (both wild-type and *rin*) were 50% full size, contained solid locule tissue, and were classified as immature green. Thirty-DAP fruit of both genotypes were full size and classified as mature green.

For measurement of endogenous ethylene levels, mature green wild-type fruit were harvested and placed individually in 500-mL glass jars at 20°C and sealed for 30 min, at which time ethylene in the jar's head space was determined by GC analysis. Ethylene production by individual fruit was monitored at 24-h intervals, and the fruit were collected at different ripening stages, based on endogenous ethylene production rates. For ethylene treatments, immature-green wild-type and *rin* fruit were harvested, randomly separated into two treatment groups, and placed in sealed 10-L plastic containers at 20°C. One group of fruit was treated with 15 $\mu L\ L^{-1}$ of ethylene in water-saturated air (continuous flow at 1.5 L min^{-1}), and the control group was treated with only water-saturated air (1.5 L min^{-1}). Fruit samples were collected at 0, 1, 3, and 5 d after treatment. Pericarp tissues were excised, frozen immediately in liquid nitrogen, and stored at −80°C.

Nucleic Acid Isolation

Total RNA was extracted from root, leaf, flower, and fruit pericarp tissues, following the method of Cathala et al. (1983) with modifications. Frozen tissue was ground to a powder in liquid nitrogen with a coffee mill. Lysis buffer (8 M guanidine hydrochloride, 10 mM EDTA, 300 mM Tris-HCl [pH 7.6], 8% [v/v] 2-mercaptoethanol) was mixed with the powder at a ratio of 2 mL buffer/g powdered tissue. The mixture was vortexed vigorously and extracted with an equal volume of phenol-chloroform. After the mixture was centrifuged at 12,000*g* for 10 min, the aqueous phase was removed and extracted again with an equal volume of phenol-chloroform and once with chloroform. RNA was precipitated by the addition of one-tenth volume of 3 M sodium acetate (pH 5.4) and 2.5 volumes of ethanol, followed by incubation for 2 h at −20°C. The precipitated RNA was recovered by centrifugation at 12,000*g* for 30 min, and the pellet was washed twice with ice-cold 3.0 M sodium acetate (pH 5.4). The total RNA pellet was dissolved in solubilization buffer (10 mM Tris [pH 7.6], 1 mM EDTA, 0.1% [w/v] SDS), and insoluble material was removed by centrifugation in a microcentrifuge for 5 min at maximum speed. Total RNA was used directly for RNA blot analysis.

RNA Blot Analysis

Total RNA (20 μg/sample) was denatured and separated in a 1.2% (w/v) agarose gel containing formaldehyde and blotted to nylon membranes according to the method of Sambrook et al. (1989). After immobilization by UV cross-linking, the blot was stained with methylene blue to ensure equal loading in each sample lane. Hybridization was carried out in buffer containing 50% (v/v) formamide, 2.5× Denhardt's reagent (1× Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.1% SDS (w/v), 5× sodium chloride-sodium phosphate-EDTA, and 100 μg/ml low mol wt denatured DNA at 42°C overnight. Probes were labeled by random priming (BRL) with [^{32}P]dCTP (Amersham). The probes used were a 2.2-kb full-length tomato fruit β subunit cDNA clone, pBsub2.2 (Zheng et al., 1992), a 1.9-kb full-length tomato fruit PG cDNA, pPG1.9 (DellaPenna et al., 1987), and a 1.0-kb constitutively expressed tomato fruit cDNA clone, D21 (Lincoln et al., 1987). D21 hybridizes to a mRNA of unknown function that is expressed at relatively constant levels, varying less than 2-fold during tomato fruit development and ripening (Lincoln et al., 1987).

Extraction of Tomato Fruit Cell Wall Proteins

Frozen powdered tissue (500 mg for fruit or root or 200 mg for leaf or flower) was transferred into an Eppendorf

tube, and 1 mL of ice-cold water (pH 3.0) was added (Pressey, 1986; Pogson and Brady, 1993a). The tube was then vortexed for 1 min and centrifuged at maximum speed in a microcentrifuge for 5 min at 4°C, and the supernatant was discarded. The cell wall pellet was again suspended in 1 mL of ice-cold water (pH 3.0), washed, and pelleted as before. The supernatant was again discarded, and the cell wall debris was extracted with an equal volume per weight of 3.0 M NaCl, 20 mM sodium acetate (pH 6.0) to approximate a final concentration of 1.5 M NaCl, 10 mM sodium acetate. The mixture was incubated with shaking at 4°C for at least 4 h. The resulting slurry was centrifuged at maximum speed in a microcentrifuge at 4°C for 5 min. The supernatant, containing total cell wall proteins, was collected, and the protein content was determined by a bicinchoninic acid assay following the manufacturer's protocol (Pierce, Rockford, IL). The protein concentration of all samples was diluted to 1 $\mu g\ \mu L^{-1}$ with 1.5 M NaCl, 10 mM sodium acetate (pH 6.0) and stored at −80°C until used.

SDS-PAGE and Immunoblot Analysis

We have found that β subunit protein resolves well in an SDS-PAGE gel containing 8 M urea, whereas catalytic PG2 protein resolves better in SDS-PAGE without urea. The SDS-PAGE system described by Laemmli et al. (1970) was used for all analysis in a 1.5-mm Mini-Protein II electrophoresis cell (Bio-Rad). For β subunit polypeptide analysis, both the stacking gel and the 10% polyacrylamide running gel contained 8 M urea, and for catalytic PG polypeptide analysis, a standard 10% SDS-PAGE gel was used. Cell wall extracts (1 $\mu g\ \mu L^{-1}$) were diluted with an equal volume of water, and 2 volumes of 2× loading buffer (for standard gel analysis, 0.125 M Tris-Cl [pH 8.0], 4% [w/v] SDS, 20% [v/v] glycerol, and 10% [v/v] 2-mercaptoethanol; for urea gel analysis the buffer also contained 4 M urea). Samples were boiled in a water bath for 5 min and subjected to electrophoresis at 100 V for 2 h. Separated polypeptides were electroblotted onto a nitrocellulose membrane in 100 mM Gly, 20 mM Tris, 0.01% SDS, and 20% methanol at 100 V for 45 min, using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad).

Blots were blocked with 1× PBS containing 2% (w/v) nonfat dry milk and 0.01% (v/v) Tween-20 for 1 h 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 h. Polyclonal β subunit antiserum, raised in rabbits against purified β subunit protein, was the generous gift of Dr. A.B. Bennett (Davis, CA). Polyclonal antibody to PG2 was generated by Della-Penna et al. (1987). After three 10-min washes with 1× PBS and 0.1% (v/v) Nonidet P-40, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000 dilution, Bio-Rad) in 1× PBS, 0.2% (w/v) nonfat dry milk, and 0.01% (v/v) Tween-20 for 1 h 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.

RESULTS

β Subunit and PG2 Expression during the Onset of Wild-Type Fruit Ripening

After 2 to 3 d at 20°C, detached 30-DAP wild-type fruit began to produce endogenous ethylene and ripen with the first external color change visible at 0.5 nL g^{-1} h^{-1}. RNA blot analysis indicated that expression of the two subunits of PG1 overlapped during the initial stages of wild-type fruit ripening (Fig. 1A). PG2 mRNA levels increased gradually as ethylene evolution increased, whereas β subunit mRNA decreased from maximal to undetectable levels during the same period. β Subunit mRNA levels exhibited a transient decrease as ethylene levels increased to 0.5 nL g^{-1} h^{-1}. This transient decrease was observed in multiple RNA blot analyses and multiple RNA extractions. D21 mRNA levels, a constitutively expressed mRNA control, remained relatively constant during this period, indicating that this decrease was not due to uneven loading of RNA. It should also be noted that ethylene treatment of immature wild-type fruit resulted in a similar transient decrease in β subunit mRNA levels (see below).

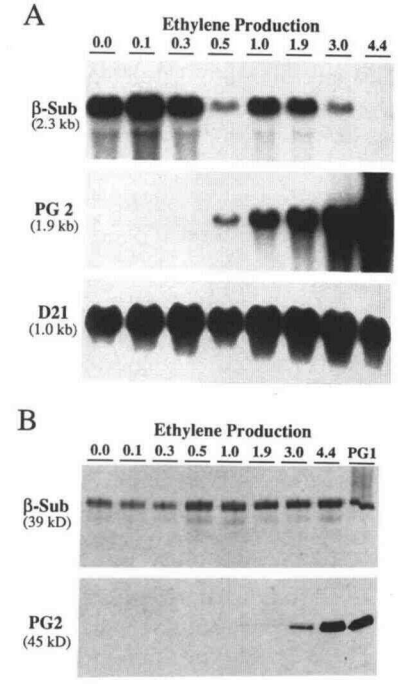

Figure 1. RNA blot analysis of β subunit, PG2, and D21 mRNA and immunoblot analysis of β subunit and PG2 protein levels during wild-type fruit ripening. Proteins and RNA were isolated from the same tissue. The onset and progress of ripening was staged by measuring endogenous ethylene production rates of individual fruit (nL ethylene g^{-1} h^{-1}, indicated above each lane). A, RNA blot analysis: 20 μg of total RNA was electrophoretically separated, transferred to a nylon membrane, and probed sequentially with the indicated cDNA clones. B, Immunoblot analysis: 5 μg of total cell wall protein were separated by SDS-PAGE, blotted to nitrocellulose, and detected with β subunit or PG2 antiserum as described in "Materials and Methods." PG1 lane, 1 μg of purified PG1 protein.

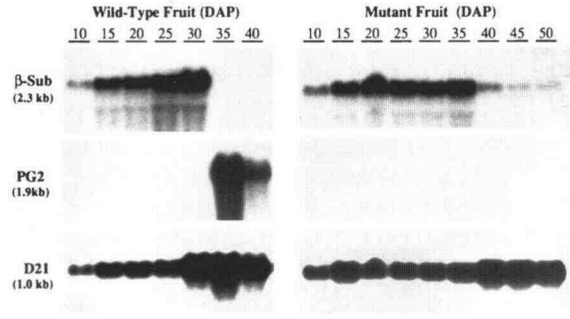

Figure 2. RNA blot analysis of β subunit, PG2, and D21 RNA levels during wild-type and *rin* fruit development. Total RNA (20 μg) of each sample was electrophoretically separated, transferred to a nylon membrane, and probed sequentially with the indicated cDNA clones. Equal specific activities were used in each hybridization, and all blots were exposed overnight.

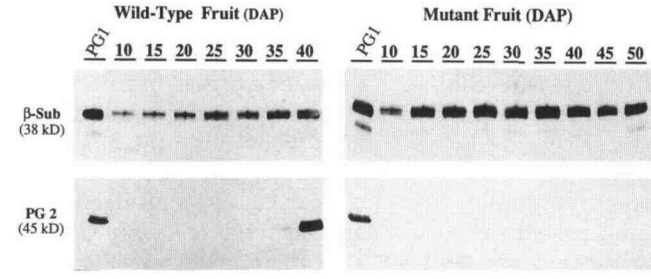

Figure 3. Immunoblot analysis of β subunit and PG2 protein during wild-type and *rin* fruit development. Total cell wall protein (5 μg) from each sample was separated by SDS-PAGE, blotted to nitrocellulose, and detected with β subunit or PG2 antiserum as described in "Materials and Methods." PG1 lane, 1 μg of purified PG1 isozyme. The minor band in the PG1 standard lanes of the top panel is a degradation product from storage.

Immunoblot analysis of cell wall protein extracts isolated from the same tissue samples (Fig. 1B) showed that β subunit protein levels were relatively constant during ripening and apparently unaffected by increasing ethylene evolution. The PG2 polypeptide first became detectable in cell wall protein extracts of fruit producing 1.0 nL g^{-1} h^{-1} ethylene but did not accumulate to appreciable levels until 3.0 nL g^{-1} h^{-1} ethylene. The appearance of PG2 protein lagged behind that of its mRNA, which became detectable at a much earlier ripening stage (0.5 nL g^{-1} h^{-1} ethylene).

β Subunit and PG2 Expression during Wild-type and *rin* Fruit Development

RNA blot analysis (Fig. 2) showed that β subunit mRNA was detectable as early as 10 DAP in both wild-type and mutant fruit. In wild-type fruit, the amount of β subunit mRNA gradually increased during fruit development and reached peak levels 30 DAP (Fig. 2). During the onset of ripening (30–35 DAP), β subunit mRNA decreased below levels of detection, whereas PG2 mRNA reached its maximal level 35 DAP and decreased by 40 DAP. These results agree with previous studies (Zheng et al., 1992). In *rin* fruit (Fig. 2), the pattern of β subunit mRNA accumulation was similar to that during wild-type fruit development (10–30 DAP), although it peaked somewhat earlier (20 DAP) and accumulated to slightly lower maximal levels than in wild-type fruit. However, unlike in wild-type fruit, β subunit mRNA remained at a relatively high level in 35-DAP *rin* fruit and was still detectable up to 50 DAP. PG2 mRNA was not detected in *rin* fruit at any developmental stage. The control mRNA, D21, showed very similar expression patterns during wild-type and *rin* fruit development.

Immunoblot analysis of these tissues showed that β subunit protein was detectable throughout wild-type and mutant fruit development, increased gradually during wild-type fruit development until 25 DAP, and remained relatively constant thereafter (Fig. 3). In mutant fruit, β subunit protein levels increased up to 15 DAP and then remained constant. Catalytic PG2 polypeptides became detectable 35 DAP in the wild-type fruit but were not detected at any developmental stage in *rin* fruit (Fig. 3). The amount of cell wall protein extracted per gram fresh weight did not differ significantly in identically aged *rin* and wild-type fruit (data not shown).

β Subunit and PG2 Expression in Ethylene-Treated Fruit

Differential responses of the two subunits of PG1 to ethylene treatment were observed at the level of mRNA accumulation in immature (20 DAP) fruit. RNA blot analysis indicated a transient decrease in β subunit mRNA levels in wild-type and *rin* fruit after 1- and 3-d air treatments (Fig. 4), possibly an effect of removing the fruit from the plants. In air-treated wild-type and mutant fruit, β subunit mRNA levels recovered to near that in 0-d fruit after 5 d. However, in ethylene-treated wild-type and mutant fruit, β subunit mRNA levels decreased more sharply after 1 d of ethylene treatment compared to air treatment. In ethylene-treated wild-type fruit, β subunit mRNA levels had recovered by 5 d, whereas in ethylene-treated *rin* fruit, β subunit mRNA levels remained somewhat depressed (Fig. 4).

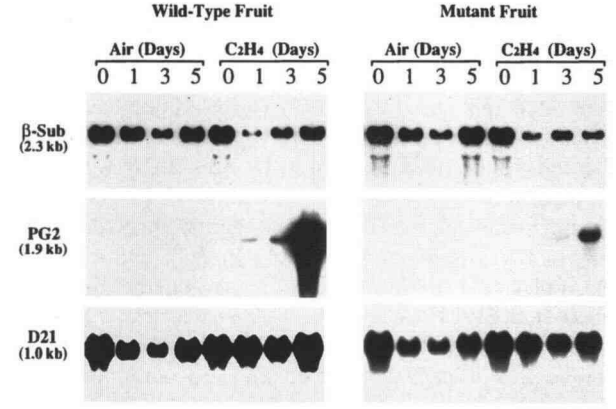

Figure 4. RNA blot analysis of β subunit, PG2, and D21 RNA levels in air- and ethylene-treated immature wild-type and *rin* fruit. Total RNA (20 μg) of each sample was electrophoretically separated, transferred to a nylon membrane, and probed sequentially with the indicated cDNA clones.

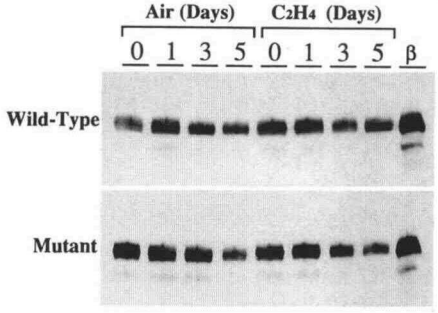

Figure 5. Immunoblot analysis of β subunit protein levels in air- and ethylene-treated immature wild-type and *rin* fruit. Total cell wall protein (5 μg) from each sample was separated by SDS-PAGE, blotted to nitrocellulose, and detected with β subunit antiserum as described in "Materials and Methods." β lane, 500 ng of purified β subunit protein. The minor band in the β lane is a degradation product from storage.

PG2 mRNA was consistently detected by RNA blot analysis in immature-green wild-type fruit after 1 d of ethylene treatment (Fig. 4) and increased dramatically during the course of ethylene treatment. Surprisingly, PG2 mRNA was also detected in immature *rin* fruit after 3 d of ethylene treatment and continued to increase after 5 d of treatment (Fig. 4). However, in comparison with identically treated wild-type fruit, PG mRNA levels in *rin* fruit after 5 d of ethylene treatment were much lower. Similar results were obtained in multiple independent experiments. Immunoblot analysis of the same tissues (Fig. 5) showed that, although the amounts of β subunit protein isolated from cell walls of both genotypes decreased slightly during the treatment period, the relative patterns of expression were very similar between treatments and genotypes. Immunologically detectable PG2 protein was not detected in either wild-type or *rin* fruit cell wall extracts (results not shown), despite the induction of PG2 mRNA in both genotypes by ethylene treatment.

β Subunit Expression in Other Tissues

After an overnight exposure to x-ray film, β subunit mRNA could not be detected by northern blot analysis in root, leaf, and flower tissues of either wild-type or *rin* plants, although it was easily detected in fruit tissues after the same exposure time (Fig. 2). However, after exposure for 6 d, low levels of β subunit mRNA were detected in root, leaf, and flower tissues of both genotypes (Fig. 6). The nonfruit β subunit mRNA was similar in size to that in fruit. PG2 mRNA was not detected in any nonfruit tissues of either wild type or *rin* (data not shown). Immunoblot analysis revealed that a protein similar in size to the fruit β subunit protein was also present in cell wall protein extracts of roots, leaves, and flowers of both wild-type and *rin* plants (Fig. 6). The amount of protein detected was highest in floral tissue, whereas root tissue contained the lowest amount. In addition, a second, larger band was also consistently detected at variable levels in root, leaf, and flower tissues. Immunoreactive proteins were not detected in these tissues with preimmune serum (results not shown). PG2 protein was not detected in nonfruit tissues of either wild-type or *rin* plants (results not shown).

In addition to the major β subunit mRNA species (2.3 kb) detected in Figures 1, 2, and 4, a second, smaller band (2.0 kb) consistently hybridized to the β subunit cDNA probe. The signal intensity of this smaller mRNA band was always weaker and paralleled changes in levels of the major 2.3-kb β subunit mRNA. The expression pattern of the smaller mRNA showed no significant difference between the two genotypes. It is not known whether this smaller band represents differential processing, a separate gene product, or a specific degradation product. The latter is unlikely to be a result of extraction because PG2 and D21 mRNAs in the same samples were intact (Figs. 1, 2, and 4).

DISCUSSION

The ripening of wild-type fruit is due in large part to hormonally and developmentally mediated alterations in gene expression. The factors regulating a specific fruit or ripening-associated gene can be determined by studying its expression in wild-type fruit, in mutants genetically blocked from ripening (e.g. *rin*), and in response to exogenous application or removal of ethylene in both genotypes (Giovannoni et al., 1990; Gray et al., 1992; Theologis 1992). We have previously shown that during wild-type fruit ripening β subunit mRNA levels decrease dramatically, whereas PG2 mRNA levels increase rapidly (Zheng et al., 1992). These results raise several possibilities: (a) that the β subunit and PG2 respond coordinately but oppositely to the same developmental signal (ripening), (b) that they respond independently of each other, or (c) that β subunit expression responds negatively to elevated ethylene concentrations produced at the onset of ripening. To further understand the factors

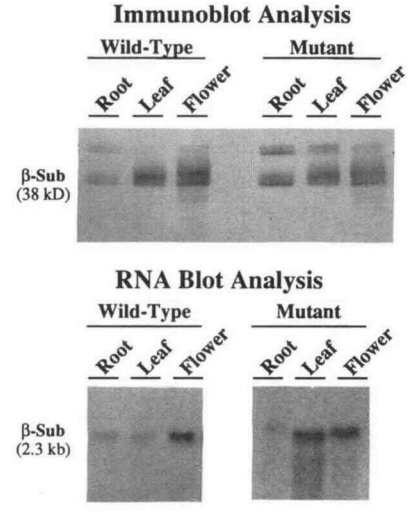

Figure 6. RNA blot and immunoblot analysis of β subunit RNA and protein levels in wild-type and *rin* root, leaf, and flower tissues. Immunoblot analysis: 5 μg of total cell wall protein from the indicated tissues were separated by SDS-PAGE, blotted to nitrocellulose, and detected with β subunit antibody as described in "Materials and Methods." RNA analysis: 20 μg of total RNA from the indicated tissues were electrophoretically separated, transferred to a nylon membrane, and probed with the β subunit cDNA insert. RNA blots were exposed for 6 d.

regulating β subunit expression at the onset of ripening, we have studied β subunit expression in fruit from wild type and the ripening-impaired mutant *rin* during development and ripening and in immature fruit of both genotypes treated with ethylene.

β subunit expression was similar during wild-type and *rin* fruit development, although the PG expression patterns of the two genotypes were completely different (Figs. 2 and 3). Furthermore, when immature-green fruit of both genotypes were treated with exogenous ethylene, β subunit protein levels were unaffected (Fig. 5), and β subunit mRNA levels were only slightly affected (Fig. 4) when compared to air-treated controls. These results strongly suggest that β subunit expression is ethylene independent both prior to and during ripening, unrelated to PG2 expression, and regulated primarily by developmental factors during fruit development and at the onset of ripening.

Wild-type and *rin* plants exhibited similar β subunit expression patterns in nonfruit tissues. Root, leaf, and flower tissues of both genotypes produced significant amounts of immunologically detectable β subunit protein identical in size with that in fruit and in some cases also accumulated lower levels of a larger immunoreactive protein (Fig. 6). These results do not agree with those reported by Pogson et al. (1993a), who failed to detect β subunit antigens in tomato stem and leaf tissues. These contrasting results may be explained by differences in the antibodies used by the two groups.

PG activity has been most extensively studied in fruit tissues but has also been reported in nonfruit tissues, including tomato leaf abscission zones (Kalaitzis and Tucker, 1993), maize pollen (Niogret et al., 1991; Allen and Lonsdale, 1993), *Sambucus nigra* abscission zones (Taylor et al., 1993), and *Oenothera organensis* pollen (Brown and Crouch, 1990). Many of the PG cDNAs isolated from these systems show significant homology to tomato fruit PG, and we hypothesize that β subunit protein(s) present in nonfruit tissues perform functions similar to that in fruit tissues, namely to immobilize or regulate the activity of PG polypeptides. Although we did not detect PG2 mRNA or PG2 proteins in nonfruit tissues, it is possible that PG gene expression in these tissues is cell specific or developmentally regulated and, hence, undetectable in our analysis of bulk tissues. Additionally, other PGs expressed in nonfruit tissues may be sufficiently divergent both immunologically and at the DNA sequence level from fruit PG to be undetected by the fruit PG2 cDNA and antibodies.

To our knowledge the observation that PG mRNA can be rapidly induced by exogenous ethylene in detached immature-green wild-type fruit and to a lesser degree in *rin* fruit has not been made previously (Fig. 4). PG gene induction in immature fruit also contrasts with previous data indicating that transcriptional activation of the PG gene is ethylene independent in mature fruit (Oeller et al., 1991; Piston et al., 1993). It is possible that PG gene induction by ethylene in immature fruit is due to increased sensitivity of the tissue to the hormone at this developmental time; however, reduced induction in *rin* fruit suggests that other developmental factors may also be involved. Further experiments are required to address these points.

In conclusion, the present study has shown that the two subunits of tomato fruit PG1 (the β subunit and catalytic PG2 polypeptide) are independently regulated during fruit development and overlap slightly in their expression patterns in the earliest stages of ripening. β Subunit expression appears to be regulated primary by developmental cues, although ethylene may play a minor role in modulating β subunit mRNA levels. The identification of multiple β subunit proteins in nonfruit tissues raises the possibility that differential processing of a single gene product occurs in these tissues or that multiple β subunit genes are expressed. Our recent isolation of multiple genomic clones closely related to the fruit β subunit cDNA supports this latter possibility (unpublished results). Several β subunit antisense and sense transgenic tomato lines have been successfully established, and studies with these transgenic plants will help us to address β subunit function in developing and ripening fruit as well as in other nonfruit tissues.

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