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Molecular cloning and characterization of the $\beta$-subunit of tomato fruit polygalacturonase isoenzyme 1

Zheng, Liansheng, Ph.D.
The University of Arizona, 1994
MOLECULAR CLONING AND CHARACTERIZATION OF THE β-SUBUNIT OF
TOMATO FRUIT POLYGALACTURONASE ISOENZYME 1

by

Liansheng Zheng

A Dissertation Submitted to the Faculty of the
DEPARTMENT OF PLANT SCIENCES
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In the Graduate College
THE UNIVERSITY OF ARIZONA

1993
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Liangsheng Zheng entitled MOLECULAR CLONING AND CHARACTERIZATION OF THE β-SUBUNIT OF TOMATO FRUIT POLYGALACTURONASE I and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director

Dr. Dean Della Penna
STATEMENT BY AUTHOR

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Dedicated to

Hong Guo and Lily
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ABSTRACT

The β-subunit of PG1 was purified and cloned from tomato fruit. It is immunologically distinct from the catalytic PG2 subunit and heterogeneous in size (37 to 39 kD). DNA sequencing analysis of a full length β-subunit cDNA indicates that the β-subunit is encoded as a 69-kD precursor consisting of four distinct domains. The mature protein domain contains six N-linked core glycosylation sites and a novel 14-amino acid motif, FTNYGxxGNGGxxx, in which many of the phenylalanine residues are post-translationally modified. We propose that the β-subunit represents a class of bifunctional plant proteins that interact both with structural components of cell walls and catalytic proteins to localize and/or regulate metabolic activities within the cell walls.

β-subunit and PG2 expression patterns were investigated in both wild-type and ripening inhibitor mutant (rin) fruit. The results show that (1) β-subunit expression is ethylene-independent and unrelated to catalytic PG2 expression, and (2) β-subunit expression is regulated primarily by developmental cues. In addition, β-subunit antigen and mRNA were detected in root, leaf and flower tissues.

Ethylene treatment of immature wild-type and rin fruit resulted in accumulation of PG2 transcript but not PG2 protein. These results contrast with previous reports suggesting PG2 mRNA accumulation is ethylene independent and indicate that PG2 expression is a complex process involving integration of developmental and hormonal signal cues at the transcriptional and post-transcriptional levels.
Several β-subunit antisense plants were successfully obtained from transformation of wild-type plants with a β-subunit antisense construction. Western analysis showed that β-subunit expression was inhibited in transgenic fruit by up to 96% relative to wild-type controls. Preliminary analysis indicates that the transgenic antisense fruit developed and ripened normally. These plants will allow investigation of the \textit{in vivo} functions of the β-subunit in the future.
CHAPTER ONE
INTRODUCTION

The Problem and Its Context

Tomato fruit polygalacturonase (PG) is a cell wall endo-hydrolase catalyzing pectin degradation. Three PG isoenzymes, PG1, PG2A and PG2B can be isolated from ripening tomato fruit. During the past 20 years, a great deal of work has focused on various aspects of polygalacturonase physiology, biochemistry and molecular biology in an effort to understand the production and activity of the three PG isoenzymes during ripening. cDNA clones encoding the catalytic polygalacturonase polypeptide have been isolated by several research groups and extensive studies of the regulation of PG expression have been carried out in wild-type and ripening mutant tomato genetic backgrounds (Giovannoni et al., 1990; Gray et al., 1992). The catalytic polygalacturonase polypeptide of all three isoenzymes is encoded by a single PG2 gene that is transcriptionally active during tomato fruit ripening (DellaPenna et al., 1989; Knapp et al., 1989). The PG2A and PG2B isoenzymes are composed of a single catalytic PG polypeptide and differ only in their extent of glycosylation during post-translational processing. The PG1 isoenzyme is a complex formed by the association of at least one PG2 polypeptide with another glycoprotein found in tomato fruit cell walls, the β-subunit
protein. The association of the β-subunit with PG2 to form PG1 alters several biochemical parameters of the catalytic PG2 protein, such as increasing its binding affinity for cell walls and substantially increasing its heat-stability relative to the PG2 isoenzymes.

The β-subunit can be isolated from the cell walls of both green and ripe tomato fruit by high salt buffers and in the latter case is associated with PG2 polypeptides in the form of PG1 (Tucker et al., 1981; Pressey, 1984). β-subunit levels increase in developing fruit, well before the appearance of catalytic PG2 protein (Pogson and Brady, 1993a). It has been proposed that the β-subunit functions to immobilize, regulate or activate the catalytic PG2 protein in vivo (Knegt et al., 1988; 1991) and that PG1 is a physiologically active complex in vivo (Giovannoni et al., 1989; Smith et al., 1990; DellaPenna et al., 1990). This proposal is supported by the following lines of evidence: (1) the sequential appearance of PG1 and PG2 isoenzymes during fruit ripening (Tucker et al., 1980; Brady et al., 1982), (2) the observation that only PG1 can be extracted when maximal pectin degradation and solubilization are observed in wild-type and ripening inhibitor (rin) plants expressing an inducible PG2 transgene (DellaPenna et al., 1990), (3) lack of pectin degradation in transgenic tobacco plants expressing a tomato PG transgene and accumulating only PG2 isoenzymes (Osteryoung et al., 1990), and (4) the in vivo biphasic loss of PG activity during heat-treatment of intact fruit tissue that mimics the in vitro heat inactivation profile of mixtures of PG1 and PG2 isoenzymes (Pogson and Brady, 1993b).

However, other studies suggest that PG1 is an extraction artifact (Pressey 1986; Knegt et al., 1991), since PG2 and the β-subunit protein can be recovered separately from ripe fruit
cell walls by differential extraction at extremes of ionic strengths and of pH (Pressey, 1986).

In order to gain insight into the function of PG1 and PG2, it is necessary to understand the structure of the β-subunit protein and the regulation of its expression during tomato fruit development and ripening. In order to further our understanding of the regulation of polyuronide degradation during ripening and clarify the controversy over the existence of PG1, the β-subunit was isolated and studied. The major goal of my research was to clone and characterize the β-subunit from tomato plants, and design experiments to test the function of the β-subunit in vivo, under the direction of my advisor, Dr. DellaPenna.
Literature Review

1. Plant Cell Walls

Definition

"What is a plant cell? Does its boundary start with the outer cell wall or with the plasmalemma?" Stafford presented these questions to plant scientists at the beginning of her letter to the editor in the journal, Plant Cell (Stafford, 1991). The letter initiated a controversy over distinctively differing definitions of a plant cell and its crux is whether or not a plant cell wall can be considered an organelle of the cell. In her letter, she pointed out that a plant cell wall is one of the intracellular compartments of a plant cell, i.e. that the wall is intracellular but not extracytoplasmic.

Later, Staehelin disagreed with Stafford's opinion, based on several reference books that gave a general definition of a cell: "A cell is a small, self-propagating compartment that is bound by a semipermeable membrane and is filled with cytoplasm, a concentrated aqueous solution of chemicals" (Staehelin, 1991). He concluded that a plant cell wall is a special form of extracellular matrix which cannot be considered an essential part of a plant cell, because the plant cell will not be killed when its cell wall is removed. This was followed by an evolutionary definition of the plant cell wall from Sack, who suggested that a protoplast and a cell were identical, according to the logic of the unifying definition of a cell (Sack, 1991).
In my opinion, I agree with Robinson and Stafford in that a plant cell wall is a vital, necessary part of the plant cell. The cell walls of plants are fundamentally involved in many aspects of the plants, including the morphology, growth and development of plant cells and tissues. For example, a protoplast must produce a new cell wall after cell division for regeneration take place. This new wall is initially constructed intracellularly in the form of the centrifugally growing cell plate. When it fuses with the plasma membrane of the maternal cell, the forming cell wall becomes an extracellular entity (Robinson, 1991).

Functions

The plant cell wall is a layer of structural material found external to the protoplast. Cellulose, hemicellulose, pectic polysaccharides, lignin and structural proteins are the major components of the plant cell wall. Because of the presence of walls, the distention of protoplasts by the osmotically active vacuole is restricted and the size and shape of a cell becomes fixed at maturity. Cell walls function as "the skin and skeleton" of plants and also form a physical and biological barrier that protects the cells from invasion by viral, bacterial, and fungal pathogens. For instance, plant cell walls contain proteins that can specifically inhibit pathogen produced cell wall-degrading enzymes (Albersheim and Andersom, 1971), and other which can degrade the walls of invading microbes (Esquerre-Tugaye et al., 1979; Boller, 1987). In addition, cell walls, particularly primary
cell walls, are not simply a static, protective structure but actively participate in growth, differentiation, cellular recognition systems, and other metabolic processes of the plant (McNeil et al., 1984).

Polysaccharides

In higher plants, two types of cell walls can be found: a thin primary wall and a thicker secondary wall. The primary wall is laid down by relative small, young, undifferentiated cells that are still actively growing. Secondary walls are produced after cells stop growing. Primary cell walls of a variety of higher plants appear to have many features and similar chemical structures in common. However, the composition and ultrastructure of secondary cell walls vary tremendously from one cell type to another and are poorly characterized compared to primary cell walls. Thus, this review will only be concerned with the composition, structure and architecture of primary cell walls.

A typical primary cell wall consists of relatively rigid cellulose microfibrils embedded in a gel-like matrix composed of non-cellulosic polysaccharides and glycoproteins. Primary cell walls are generally composed of 90% polysaccharide and 10% protein (McNeil et al., 1984). The polysaccharides are classified into three major groups: celluloses, hemicelluloses and pectic polysaccharides. Celluloses usually account for 20-30% of the dry weight of a primary cell wall and largely determine the architecture of the cell wall. Celluloses consist of an unbranched polymer of D-glucose residues joined by
β-1,4-glycosidic bond linkages. Several dozen linear chains aggregate via inter- and intra-molecular hydrogen bonds to form a structure called a microfibril, which are generally 5 - 15 nm wide and spaced 20 - 40 nm apart in the wall (Delmer, 1983). Many microfibrils together form a framework interpenetrated by two other matrix polysaccharides --- hemicellulose and pectins. The biological function of the microfibrils is presumed to be structural, providing shape and strength to plant cell walls based on experiments in which after cellulose synthesis was chemically inhibited, cells burst under their own turgor pressure (Richmond, 1984).

Hemicelluloses are the second class of cell wall polymers and defined as those polysaccharides non-covalently associated with cellulosics, namely xyloglucans and glucurono-arabinoxylans. All hemicelluloses possess a 1,4-β-glucan backbone with α-xylosyl residues attached to the 6-position of β-galactosyl residues, and terminal galactose attached to the 2-position of the xylosyl residues by a β-linkage (Hayashi, 1989). Xyloglucans contain D-glucose, D-xylose, and D-galactose and are present both in dicot and monocot cell walls, but are generally present in larger amounts in the walls of dicots (20 - 25%) relative to those of monocots (about 1 - 5%) (Darvill et al., 1980). Xyloglucans appear to be virtually absent from most secondary walls of dicots. The second major hemicellulose group, glucurono-arabinoxylans have a backbone of β-1,4-linked D-xylosyl residues, some of which carry single α-L-arabinose and/or α-D-glucuronic acid residues, attached to the 2- and/or 3-o-position. A β-1,4-linked xylan chain can hydrogen bond to cellulose, and this explains the strong association
observed between glucurono-arabinoxylans and cellulos. Glucurono-arabinoxylans typically make up roughly 5% of the primary cell walls in dicots, 20% of the primary walls of grasses, and 20% of the secondary walls of both dicots and monocots (McNeil et al., 1980).

Pectins are the third major class of plant cell wall polysaccharides and are defined as those found in covalent association with galacturonosyl-containing polysaccharides, such as rhamnogalacturonan I and II, homogalacturonan, arabans, galactans and arabinogalactans. Pectins have been described as "hairy" or "smooth" block polymers (Jarvis, 1984). Rhamnogalacturonan I and II (RG-I and RG-II) are found in the "hairy blocks" (Darvill et al., 1978), and are characterized by their resistance to pectinases and numerous sugars other than galacturonic acids (especially rhamnose, galactose and arabinose). Pectins containing "smooth" blocks (homogalacturonans) consist mainly of contiguous unbranched α-D-galacturonic acid residues with occasional rhamnose residues which are suggested to act as molecular "punctuation marks", with intervening homo-galacturonic acid blocks being in some cases fully methylesterified and in others completely unesterified. The rhamnose residues may also act as anchorage points for neutral arabinose- and/or galactose-rich side chains (Jarvis, 1984). Because of their high percentage of galacturonic acid residues, pectins are negatively charged and avidly bind cations, such as Ca$^{2+}$ (Fry, 1986).

Pectins likely have multiple functions in plants. They are present in higher percentages in primary walls than in secondary walls, suggesting a role for the polymer in
growth. They are also highly hydrophilic polysaccharides and are thought to introduce water into the matrix and loosen the wall (Carpita and Gibeaut, 1993). Finally, specific pectic fragments have been shown to act as signaling molecules in response to pathogen attacks (reviewed in Ryan and Farmer, 1991).

Structural Proteins

In addition to a wide variety of polysaccharides, plant cell walls contain structural proteins, which can be classified into at least three major groups, hydroxyproline-rich glycoproteins, glycine-rich proteins and proline-rich proteins (Varner and Lin, 1989). All hydroxyproline-rich glycoproteins (HRGPs) share several characteristics, such as a high percentage composition of hydroxyproline residues, high pI (usually >10) and unique protein motifs (Varner and Lin, 1989). Extensins are the best characterized HRGPs and are important components of the cell wall matrix, accounting for as much as 18% of the wall's mass in some species (Lamport, 1965). Extensins have been found and characterized in a wide variety of plant species (reviewed in Varner and Lin, 1989; Showalter, 1993 and Keller, 1993).

Two kinds of the HRGP motifs are most common in dicot extensins, Ser-Hyp-Hyp-Hyp-Hyp and Ser-Pro-Pro-Pro-Pro (Lamport, 1977). The length and number of these repeating units differ among various extensins (Showalter, 1993). Like dicot extensins, monocot extensins are also highly basic proteins, rich in hydroxyproline
residues and proline, and O-glycosylated through hydroxyproline residues with one to four arabinosyl residues. However, different versions of extensins have been characterized in monocots. For instance, Kieliszewski and Lamport (1987) reported that two monocot extensins, a histidine-hydroxyproline-rich protein (HHRGP) and threonine-hydroxyproline-rich (THRGP), were found in the graminaceous monocot Zea may. These monocot extensins are related to but distinct from typical dicot extensins and possess two unique motifs, Thr-Pro-Lys-Pro-Thr-Hyp-Hyp-Thr-Tyr-Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp and Ala-Thr/Ser-Lys-Pro-Pro (Kieliszewski and Lamport, 1987; Kieliszewski et al., 1990). In contrast to the typical dicot extensins, THRGPs are extremely rich in threonine, contains less valine and tyrosine, and are more lightly glycosylated than their dicot counterparts.

Extensins have major functions in cell wall architecture, growth and development in part through interactions with nonproteinaceous cell wall components. It has been demonstrated by immunocytochemical studies that extensins are involved in the plant's defense against pathogen attack and wounding. For example, extensins accumulated in the walls of living, uninfected cells close to sites where fungal and bacterial growth is restricted by the plant defense responses (Mazau et al., 1987). Extensins also accumulate in melon plants infected with the fungus Colletotrichum lagenarium (Esquerre-Tugaye et al., 1979) and there is a rapid accumulation of extisin mRNAs in response to fungal infection and elicitor treatment (Showalter et al., 1985).
Glycine-rich proteins (GRPs) are another major class of cell wall structural proteins that have been found in many plant species (reviewed in Showalter, 1993). Several GRP cDNAs and genomic clones have been isolated and characterized from tomato, tobacco, carrot, *chenopodium rubrum* and French bean. Gly-X is a noticeable and specific motif structure found in these proteins, in which the X position is most frequently Gly but can be Ala or Ser. Generally, GRPs contain 47% to 70% glycine arranged in the Gly-X motif units. Results from analyzing GRP cDNA and genomic DNA sequences reveal they contain a signal sequence, indicating that they are indeed secreted proteins (Lei and Wu, 1991). The function of glycine-rich proteins is unclear.

Proline-rich proteins (PRPs) are the third general class of cell wall structural proteins and are characterized by their high proline and hydroxyproline content and a repeating motif that differs from other cell wall proteins, Pro-Pro-Val-X-Lys in which the X is often His, Tyr, or Glu (Cassab and Varner, 1988). PRPs have been identified in carrot, tomato fruit, and soybean (reviewed in Showalter, 1993). The first monocot proline-rich protein was cloned from maize (Jose-Estanyol *et al.*, 1992). This proline-rich protein contains two kinds of motifs, Pro-Pro-Tyr-Val and Pro-Pro-Thr-Pro-Arg-Pro-Ser.

Proline-rich protein genes, like GRPs and HGRPs, show developmentally regulated, cell-specific expression and can be induced by hormones, such as auxins (Wyatt *et al.*, 1992; McClure *et al.*, 1988; Hong *et al.*, 1989; Kleis-San Francisco and Tierney, 1990). Unlike the two other groups of cell wall structural proteins (HHRGPs and GRPs), PRPs are not wounding-inducible (Kleis-San Francisco and Tiemey, 1990). However,
elicitor application and wounding have been demonstrated to induce rapid oxidative cross-linking of existing proline-rich proteins in the walls (Baradley et al., 1992), and treatment of bean or soybean cells with fungal elicitor or glutathione causes a rapid insolubilization of preexisting proline-rich proteins. These results suggest that although PRP genes are not wound inducible, their protein products nonetheless function in plant defense.

Pectic Enzymes

In addition to structural proteins, many enzymatically active proteins are present in primary cell walls of higher plants. They are usually glycoproteins and can be extracted from plant cell walls. Most well-characterized cell wall enzymes are either hydrolases or oxido-reductases, including glycosidases, peroxidases and pectic enzymes (Cassab and Varner, 1988). Enzymes active in cell wall assembly/synthesis must also be present, but have not been as well characterized as cell wall degrading enzymes.

A variety of glycosidases have been identified in cell walls of living tissues and in isolated wall preparations (Pierrot and van Wielink, 1977). Generally they are exoglycanases and hydrolyze polysaccharides from the non-reducing end, releasing monosaccharides (van Der Wilden and Chrispeels, 1987). Peroxidases are another general class of cell wall enzymes that are widely distributed among higher plants and probably occur in all primary cell walls (Clarke and Shannon, 1976; Mazz and Welinder, 1980).
These enzymes are capable of utilizing hydrogen peroxide to oxidize a wide variety of hydrogen donors, such as phenolic substances, nitrite, indole, amines, and certain inorganic ions.

Pectic enzymes degrade the galacturonan chain of pectic substances and are generally divided into two groups, pectinesterases and depolymerases (Chesson, 1980). Pectinesterases are responsible for saponification of esterified regions of pectic substances, while depolymerases hydrolyze glycosidic α-1,4 bonds of the D-galacturonan chain and include hydrolases and lyases.

Pectinesterases catalyze the de-esterification of pectins and are highly specific for the galacturonan structure of the substrates. They fail to de-esterify methyl esters of the structurally related polysaccharides alginic acid and gum tragacanth, and have a lower specificity for the alcohol reside of the ester. They do not hydrolyze the methyl ester of D-galacturonic acid, the mono- and di-methyl esters of its dimer, and the triester of its trimer. They hydrolyze all ethyl, propyl and allyl esters of pectic acid (Manabe, 1973).

Previous work suggested that pectinesterases were present and involved in fruit ripening. Much attention has been devoted to their study in tomato fruit. Tomato pectinesterases de-esterify pectins with the formation of blocks of free carboxyl groups. Pectinesterase activity is present in green tomato fruit and increases several-fold during fruit ripening (Tucker et al., 1982). The first primary structure of a tomato pectinesterase was published by Markovic and Jornvall (1986). Two pectinesterase isoenzymes have been identified in tomato fruit (Pressey and Woods, 1992) which differ in molecular
weights (23.8 kD and 24.3 kD), pIs (9.3 and 8.9) and amino acid composition. However, they have similar N-terminal amino acid sequences and cross-react with antibodies raised against each other.

Pectin lyases catalyze the cleavage of α-D-1,4- glycosidic bonds of D-galacturonans by β-elimination and specifically split highly esterified pectin (Kiss, 1974). There are four kinds of lyases which are differentiated by their substrate preference and mechanism of hydrolysis, endo-pectate lyase (EC.4.2.2.2), exo-pectate lyase (EC.4.2.2.9), endo-pectin lyase (EC.4.2.2.10) and oligogalacturonate lyase (EC.4.2.2.6). Unlike the other endo-pectic depolymerases, pectin lyases show considerably reduced activity for de-esterified pectic acid. Pectate lyases preferentially hydrolyze substrates of de-esterified and non-esterified D-galacturonan. Endo-pectate lyases are characterized by their alkaline pH optima (pH 8 - 9.6) and absolute dependence on presence of divalent cations for enzyme activity. Exo-pectate lyases are specific for the penultimate bond of the reducing end of a galacturonan chain. The hydrolytic product is an unsaturated digalactosiduronate. Unfortunately, there is little information on the functional importance of pectate lyases in tomato plants.

Pectin hydrolases can be divided into exo- and endo-polygalacturonases, based on their action patterns and preferred substrates. Exo-polygalacturonases catalyze the hydrolytic cleavage of the terminal α-1,4- bonds of the galacturonan chain, degrading pectic acid from the non-reducing end ultimately resulting in complete hydrolysis to
galacturonic acid. In contrast to endo-polygalacturonases, they also act as glycosidases, hydrolyzing digalactosiduronate.

Endo-polygalacturonases are the most commonly encountered pectic enzymes and catalyze the hydrolytic cleavage of the glycosidic α-D-1,4- bonds of non-esterified D-galacturonic acid residues (Chesson, 1980). Endo-polygalacturonases from a variety of sources demonstrate remarkably similar optimum temperature and pH profiles (40 - 45°C, pH 3.5 - 5.5). They also have a similar preference for high molecular weight galacturonans. Tomato fruit endo-polygalacturonase is one of the best studied pectin hydrolases and has been the focus of extensive studies for the past two decades. The focus of my work during the past three years has been tomato fruit PG1, one of three polygalacturonase isoenzymes found in ripening tomato fruit. I will discuss this specific endo-polygalacturonase isoenzyme in later sections.

2. Tomato Fruit

Advantages

Fruit generally can be classified into climacteric and non-climacteric fruit based on their respiration behavior during ripening (Biale, 1960). The respiratory rate of the climacteric fruit declines to a low value prior to ripening, then sharply increases at the
onset of ripening. The climacteric rise terminates late in ripening at the climacteric peak. The non-climacteric fruit do not have such a marked change in respiration.

Tomato is an excellent model system for studying the fundamental mechanisms of climacteric fruit ripening. First, the conversion of tomato fruit from a mature green to fully ripe state is characterized by dramatic changes in color, composition, aroma and texture. Color change is the first visible sign of the onset of tomato fruit ripening and continues throughout ripening. Color is easily and widely used to classify fruit ripening stages. Second, because tomato is a self-pollination diploid crop, the arduous work of emasculation and pollination can be avoided. Third, tomato has a relatively small genome, well defined genetic map (Mutschler et al., 1987) and rich germplasm sources (Tigchelaar et al., 1978). In particular, several naturally-occurring ripening-impaired mutants are available to assist studies of fruit ripening (Seymour et al., 1987). Finally, tomato is one of a few important crops that can be easily transformed.

Fruit Cell Walls

A typical tomato fruit cell wall is similar to other higher plant cell walls, consisting of celluloses, glycoproteins, hemicelluloses and pectins (Fisher and Bennett, 1991). Pectins, or polyuronides, are particularly abundant in the middle lamella region joining adjacent cell walls. Mature-green fruit are composed mostly of pericarp parenchyma cells which have relatively thick cell walls. The pectin content of fruit cell walls is substantially
higher and protein content lower than cell walls of other tissues. During ripening, the cell walls of tomato fruit undergo dramatic changes, the most notable being the solubilization and degradation of polyuronides (Giovannoni et al., 1990). During the past two decades, cell wall solubilization, degradation and other changes associated with tomato fruit ripening have been extensively investigated by many research groups. This research has focused on isolating and characterizing ripening-related proteins and genes, studying the expression and regulation of these genes during the ripening process and their hormonal and developmental regulation (Fischer and Bennett, 1991). Ultimately, functional questions about these proteins are addressed.

Ripening Mutants

Ripening-impaired mutants differ dramatically from the sequence of ripening events observed in normal tomato varieties. *Never-ripe (Nr), ripening inhibitor (rin)* and *non-ripening (nor)* mutants are the best studied tomato fruit ripening mutants. Their major physiological and biochemical changes associated with normal ripening are either lacking or greatly reduced as a result of their genetic deficiency (McGlasson and Franklin, 1979; Knapp et al., 1989). The *Nr* mutant is a dominant mutation that is located on chromosome 9 and retards the onset of fruit ripening. *Nr* fruit soften slowly, contain very low PG activity (Hobson, 1967) and have reduced and delayed ethylene evolution to a maximum of 50% of normal wild-type fruit (Tigchelaar et al., 1978). The *rin* mutant is a
spontaneous recessive mutation, which in the homozygous condition results in non-climacteric fruit (Robinson and Tomes, 1968). The rin gene is located on chromosome 5. Normal ripening processes are almost totally inhibited in the rin mutant, including chlorophyll degradation, carotenoid biosynthesis, ethylene production and fruit softening. Only trace levels of polygalacturonase activity can be found in rin fruit during or after the period when normal fruit ripen (Tigchelaar et al., 1978). rin fruit also remain firm for very long periods and are resistant to post-harvest pathogens (Buescher and Tigchelaar, 1975). nor is also a recessive mutation and its effects on fruit ripening are similar to the rin gene (Ng and Tigchelaar, 1977). The nor gene is located on chromosome 10, 3.5 map units away from the uniform ripening (u) gene (Strand and Barman, 1978). All of these ripening mutants have been widely used to study the mechanism of tomato fruit ripening and the rin mutant in particular has been extensively utilized in this research.

Ethylene and Fruit Ripening

Fruit ripening is the characteristic final step of the reproductive cycle in a climacteric fruit and is under genetic control (Brady, 1987). The ripening process is not a simple senescence phenomenon but rather involves many biochemical and physiological reactions. These reactions are highly coordinated and regulated by plant hormones, developmental cues, genetic and environmental factors. Theologis (1992) has proposed
that at least two signal transduction pathways are operating during fruit ripening, developmental and ethylene-dependent pathways. The existence of the latter in fruit has been demonstrated by physiological and molecular studies of ACC-synthase antisense RNA transgenic tomato plants (Oeller et al., 1991).

Ethylene, one of the simplest organic molecules with biological activity, is an important phytohormone and exists in a gaseous form. Ethylene can regulate ripening, dormancy, senescence, abscission, epinasty, leaf expansion, flower induction, swelling and elongation (Abeles et al., 1993). The ethylene biosynthetic pathway in higher plants has been determined by using inhibitors, precursors, and labeled compounds (Yang and Hoffman, 1984). The acronym MSAE is used to describe the pathway from L-methionine (M), S-adenosylmethionine (S), 1-aminocyclopropane-1-carboxylic acid (A) to ethylene (E). Oxygen is absolutely required for ethylene biosynthesis. There are two key enzymes in the MSAE pathway, ACC synthase and ACC oxidase (also called EFE, the ethylene-forming enzyme). ACC synthase catalyzes the elimination of 5'-methylthioadenosine (MTA) from SAM to ACC (Adams and Yang, 1979) and has been isolated and cloned from wounded tomato tissue (Bleecker et al., 1986). ACC oxidase catalyzes the last step in the MSAE pathway, the conversion of ACC to ethylene. It is a bisubstrate enzyme and requires both molecular oxygen and ACC as substrates. A cDNA clone (pTOM13), isolated from ripening and wounded tomato tissue (Smith et al., 1986), has been identified as ACC oxidase by the use of antisense RNA (Hamilton et al., 1990).
As mentioned above, ethylene can induce morphological and metabolic changes in adjacent tissues and nearby plants. Changes caused by ethylene include flower and leaf senescence, leaf abscission, flower initiation, breaking of seed dormancy and fruit ripening (Abeles et al., 1992). Ethylene effects on fruit ripening are probably the best-known response of plants to ethylene. It has been reported that immature 17- and 25-day old tomato fruits could respond to ethylene by exhibiting increased reddening and respiration (Lyons and Pratt, 1964). Internal levels of ethylene in ripening tomato fruits usually are about 3 μL/L (Burg and Burg, 1962) but the internal distribution of ethylene may not be uniform in tomato fruits, since significant differences in ACC content, ACC synthase, and EFE activity were noted among different tissues in ripening tomato fruit (Brecht, 1987).

During the past 10 years, the tomato has played a major role in ripening research and allowed the regulation of gene expression by ethylene during ripening to be studied in great detail at the molecular level. Like other climacteric fruit, endogenous ethylene was considered to be the ripening trigger in tomato fruit (Brady, 1987) and endogenous ethylene production is the best molecular marker for the onset of fruit ripening. A similar natural ripening process can be induced in mature green fruit by treatment with ethylene at concentrations above 0.1 μL/L for a suitable period. Ripening induced by exogenous ethylene is considered to be qualitatively identical with that occurs naturally.

It has been suggested that ethylene involvement in fruit ripening is largely at the level of gene regulation (Speirs et al., 1984). Mature green tomato fruit were found to contain about 40% of their ribosomal RNA in polyribosomes. At the breaker stage of
tomato fruit ripening, 50% of rRNA was in polyribosomes, and this distribution was maintained until fruits were fully ripe. Treatment of mature green tomato fruit with ethylene also promotes changes in \textit{in vitro} translation products similar in some cases to those observed during fruit ripening (Lincoln \textit{et al.}, 1987). The hypothesis that ethylene controls fruit ripening has been proven by antisense RNA experiments in which expression of the two key enzymes of the ethylene biosynthetic pathway, ACC synthase and ACC oxidase were dramatically inhibited (Hamilton \textit{et al.}, 1990; Oeller \textit{et al.}, 1991). The expression of an ACC-synthase antisense gene inhibits tomato fruit ripening and the inhibitory effect can be reversed by the addition of exogenous ethylene or propylene (Oeller \textit{et al.}, 1991).

The precise interaction between ethylene and the genes involved in fruit ripening is poorly understood. Recent work by Chang \textit{et al.} (1993) appears to be a promising direction for further studying the regulation of gene expression by ethylene. They reported that an Arabidopsis ethylene-response ETR1 protein contained a two-component regulator in the carboxyl-terminal portion that has a high degree of sequence identity with the large family of prokaryotic signal transducers known as two-component systems. ETR1 acts early in the ethylene signal transduction pathway, possibly as an ethylene receptor, or as a regulator of the pathway. Investigation of ETR1 and the proteins or genes that ETR1 interacts with may be a key for understanding the mechanism of ethylene regulation and extensive studies in this field will help us understand the mechanism of tomato fruit ripening.
3. Antisense Technology

Definition

Since Ecker and Davis (1986) first reported a successful antisense experiment in protoplasts, this new technology has been used in the study of gene expression during tomato fruit ripening. Although the mechanism of antisense inhibition remains unclear, the basic idea is to block the genetic information flow from DNA to protein by introduction of a modified gene which can produce a RNA strand complementary to the sequence of the target mRNA. When the two complementary RNA strands are synthesized and interact in vivo, hybridization is thought to occur between them, and this impairs mRNA maturation and/or translation. In theory, this technology can produce a genetic mutant lacking functional expression of the target gene.

Since a single plant cell is capable of regenerating a whole plant, plants were the first multicellular organisms in which endogenous genes were successfully down-regulated by antisense counterparts (Ecker and Davis, 1986). Antisense technology has a number of obvious advantages in plant research. First, antisense technology can be used in any genetic background. Second, the role of a previously identified gene sequence can be determined in different tissues or related species. Third, it can help to elucidate the function(s) of a gene without the prerequisite of identifying, isolating, or characterizing the protein product. Therefore, antisense technology was quickly applied to study the mechanism of tomato fruit ripening.
PG Antisense Research

It is not surprising that expression of the tomato polygalacturonase gene was one of first plant genes successfully inhibited by antisense technology. The enzyme had been extensively studied at the genetic, biochemical and physiological levels making it an ideal candidate target gene. After Smith et al. (1988) and Sheehy et al. (1988) first demonstrated the effectiveness of PG antisense RNA in tomato plants, numerous groups have shown down-regulation by antisense RNA of other target genes associated with tomato fruit ripening (Hamilton et al., 1990; Smith et al., 1990; Oeller et al., 1991; Kramer et al., 1992; Tieman et al., 1992; Picton et al., 1993; Carrington et al., 1993; Hall et al., 1993; Seymour et al., 1993). Several characteristics of PG antisense transgenic tomato plants are summarized below. (1) PG antisense RNA in transgenic tomato plants can effectively inhibit the accumulation of PG mRNA and enzymatic activity during fruit ripening. (2) The inhibition is specific. (3) Wide ranges of remaining PG enzyme activity are present in different primary antisense PG transformants. (4) PG antisense transgenic fruit exhibit normal ripening, at least as judged by color development. (5) PG antisense genes are stable and inherited in a Mendelian fashion.

However, different results have been reported in regard to the effect of PG antisense on tomato fruit softening. Smith et al. (1988) reported no significant differences in fruit softening between wild-type and antisense PG RNA fruits, despite the dramatic decrease in PG expression in the transgenic fruit. On the other hand, studies on the post-storage evaluation of firmness by Kramer et al. (1992) showed significant retardation
of softening in antisense fruit. Recently, Carrington et al. (1993) observed that PG antisense fruit showed significant retardation of softening as the antisense fruit changed from a turning stage to red, but little difference in softening as fruits change from green to the turning stage. In addition, antisense PG fruit also showed depressed levels of \( \alpha \)-arabinosidase and \( \beta \)-galactosidase. This effect might contribute to the retardation of antisense fruit softening later in ripening. In spite of the differing opinions regarding PG antisense effects on fruit softening, it is commonly accepted that PG antisense fruit are more resistant to cracking or mechanical damage, secondary fungal infection, and remain intact in the field for a longer period.

Antisense Inhibition of Other Ripening-related Enzymes

The most striking results of antisense technology have been obtained from ACC-synthase antisense RNA experiments by Oeller et al. (1991). Ethylene is an important gaseous hormone that controls climacteric fruit ripening. ACC-synthase antisense RNA was found to completely inhibit fruit ripening in transgenic tomato plants, via inhibition of ACC synthase and hence ethylene production. Production of the ethylene precursor ACC was almost completely inhibited (99.5%) by ACC-synthase antisense RNA in transgenic plants and the resulting effect of ripening inhibition could be reversed by application of exogenous ethylene. Tomato fruit ACC oxidase, another key enzyme in ethylene biosynthesis, was the first protein whose function was unknown and assigned by
antisense technology (Hamilton et al. 1990). A ripening-related cDNA clone of unknown function, pTOM13, was isolated from a ripe tomato fruit cDNA library by differential hybridization (Slater et al., 1985), and inserted in the reverse orientation into a vector under control of the CaMV 35S promoter. The chimeric pTOM13 antisense gene was expressed in transgenic tomato plants and reduced ACC-oxidase activity by 93% and ethylene production by 97% in fruit of homozygous transgenic lines (Hamilton et al. 1990). The ACC-oxidase antisense fruit were also greatly resistant to over-ripening and shrivelling, compared to control fruit. The results from ACC-synthase and oxidase antisense experiments demonstrated the function of ethylene as a regulator or trigger in controlling fruit ripening. E8, an ethylene responsive cDNA clone, was believed to be a dioxygenase related to ACC oxidase (53% nucleotide and 34% amino acid sequence identities). However, antisense inhibition of E8 expression stimulated ethylene production during tomato fruit ripening approximately 6-fold higher than that in control fruit (Pennarrubia et al., 1992). This surprising effect on ethylene synthesis might be explained by negative feedback regulation by E8 on ethylene production.

It is interesting to note that accumulation of PG mRNA during ripening is not affected by expression of ACC-synthase or oxidase antisense genes (Hamilton et al., 1990; Oeller et al., 1991; Picton et al., 1993). ACC-synthase antisense fruit accumulated PG2 mRNA to normal levels, but failed to produce PG2 polypeptides. After treatment with exogenous ethylene, the transgenic fruit ripened and accumulated PG2 protein, suggesting translational control of PG expression by ethylene. However, the expression and activity
levels of polygalacturonase in ACC-oxidase antisense fruit were similar to that in wild-type control fruit.

Pectinesterase (PE) is another pectin modifying enzyme found in tomato fruit whose function has been studied by antisense technology (Tieman et al., 1992). Their results indicate that antisense PME RNA did not interfere with the overall ripening process, though the antisense fruits had >90% reduction in PME enzyme activity and increased pectin esterification throughout ripening. Hall et al. (1993) demonstrated by pectinesterase antisense RNA that multiple pectinesterase isoenzymes resulted from differential expression of multiple genes and were immunologically unrelated. The accumulation of pectinesterase was inhibited up to 93% in antisense transgenic fruit, while PE enzyme activity was unaffected in leaves or roots of transformants in which antisense transcripts were detected. Like the results reported by Tieman et al. (1992), there was no major difference in fruit development and ripening between wild-type and transgenic plants. Recently, Seymour et al. (1993) reported the down-regulation of two non-homologous endogenous genes using a single gene construct. A chimeric gene was constructed by fusing a PE cDNA sequence encoding the PE mature protein to a PG cDNA sequence encoding the mature PG protein, and inserted in a sense orientation between a CaMV 35S promoter and terminator. The expression of this PGPE gene was observed in tomato transformants and found to inhibit the expression and activities of both endogenous PG and PE genes in transgenic fruit. The transcript of the chimeric gene was
detected in leaves of the transgenic plants, but no protein product was identified.
Unfortunately, there is no data describing the ripening behavior of these transgenic fruit.

By using PG or PE antisense transgenic tomato plants, it appears that these two pectin modifying enzymes do not significantly affect general aspects of fruit ripening, although they do modify pectin chemistry. It is obvious that antisense RNA technology is a powerful tool to study the functional role of enzymes in fruit ripening, and can also produce commercially desirable traits in food crops. In the future, I expect to see antisense technology used with increasing frequency to enhance crop traits.

4. Tomato Fruit Polygalacturonases

Research History

Tomato polygalacturonase or PG [poly(1,4-a-D-galacturonide) glycanohydrolase, EC3.2.1.15] is a tomato fruit cell wall enzyme that catalyzes pectin degradation during ripening (reviewed in, Giovannoni et al., 1990). In 1945, MacDonnell et al. (1945) first reported the presence of polygalacturonase in ripening tomato fruit. Three years later, McCulloch and Kertesz (1948) reported two isoenzymes of polygalacturonase existed in tomato fruit which could be distinguished by their heat-resistance. In 1973, these two polygalacturonase isoenzymes were subsequently separated and characterized by Pressey and Avants (1973). The two isoenzymes differed in their stability to heat and molecular
weight, with PG1 being 84 kD and PG2 being 44 kD, respectively, as judged by
gel-filtration analysis. During the 1980's, many scientists made significant contributions to
our understanding of tomato polygalacturonases. Tucker et al. (1980) discovered that
there is a sequential appearance of the two isoenzymes, with polygalacturonase 1
appearing first, followed by polygalacturonase 2 later in ripening. However, they only
found a single 46-kD polypeptide when the two isoenzymes were resolved by SDS-PAGE.
The 46-kD polypeptide from both PG1 or PG2 reacted to an antibody raised against pure
PG2 protein. Subsequently, a heat-stable, non-dialyzable factor was isolated from green
fruit and found to be capable of converting PG2 \textit{in vitro} into an isoenzyme similar to PG1,
although the PG1 and PG2 isoenzymes could only be isolated from ripe tomato fruit
(Tucker et al., 1981). They proposed that this convertor factor was carbohydrate in
nature. Ali and Brady (1982) reported that in addition to PG1, there were actually two
PG2 isoenzymes that differed slightly in size and referred to them as PG2A (43 kD) and
PG2B (46 kD). In 1983, Moshrefi and Luh (1983) demonstrated that PG1 is composed of
two polypeptides, PG2 ($M_r = 47,500$) and a glycoprotein of $M_r = 41,400$. In 1986,
DellaPenna et al. isolated a PG cDNA clone (pPG16) from a ripe tomato fruit cDNA
library and reported that PG mRNA was 1.9 kb in size, expressed in large amounts during
ripening and encoded as a 54-kD precursor protein. In the meantime, the first full-length
tomato fruit PG cDNA sequence was reported by Grierson et al. (1986).

successfully used PG antisense chimeric genes to transform wild-type tomato plants. The
expression of PG was greatly reduced, while normal ripening processes were unaffected in the transgenic fruit. Shortly thereafter, the *ripening inhibitor* mutant (*rin*) was transformed with an inducible chimeric PG gene (Giovannoni *et al.*, 1989). These transgenic *rin* fruit produced all three PG isoenzymes that are normally present in wild-type fruit. However, normal ripening changes, such as softening, ethylene evolution and color development, were not affected in the transgenic *rin* fruit, although wild-type levels of polyuronide solubilization and depolymerization were observed. These results indicate that the action of PG is sufficient for polyuronide degradation but is not sufficient for the induction of fruit softening and does not affect other aspects of ripening. Later, DellaPenna *et al.* (1990) reported that maximal levels of polyuronide solubilization and depolymerization in transgenic *rin* fruit occurred when only PG1 could be isolated, prior to the accumulation of PG2 isoenzymes. This result suggested that PG1 was the primary isoenzyme responsible for polyuronide solubilization and depolymerization *in vivo*.

In 1992, Pogson *et al.* (1991) isolated and partially characterized the subunits of PG1. They confirmed that PG1 could be separated into three polypeptides of 45, 43, and 38 kD on a SDS-PAGE gel. The 45 and 43 kD polypeptides were recognized by antiserum raised against PG2A and appeared to be the PG2A and PG2B isoenzymes. The smaller 38-kD protein was a glycoprotein, immunologically distinct from the catalytic PG proteins which they termed the β-subunit.
PG Isoenzymes

As described above, three polygalacturonase isoenzymes, PG1, PG2A, and PG2B, are present in extracts of ripening tomato fruit. All are capable of degrading polyuronides in vitro. The catalytic PG polypeptides of all three isoenzymes are encoded by a single PG gene (Bird et al., 1988; DellaPenna et al., 1989; Smith et al., 1990). PG2A or PG2B consist of a single catalytic PG2 polypeptide differing only in degree of glycosylation. Complete chemical deglycosylation of a mixture of mature, purified PG2A and PG2B with trifluoromethane sulfonic acid yields a single polypeptide of 42 kD (DellaPenna and Bennett, 1988). PG1 is a more complex isoenzyme composed of at least one catalytic PG2 polypeptide (PG2A or PG2B) tightly associated with a non-catalytic glycoprotein, the β-subunit (Pressey and Avants, 1973; Tucker et al., 1981; Pressey, 1984; Knegt et al., 1988; Knegt et al., 1991; Pogson et al., 1991).

The PG2 gene contains nine exons and is present in one copy per haploid genome (Bird et al., 1988). In Vitro translation of the 1.9 kb PG2 mRNA yields a polypeptide with a molecular weight of 54 kD (DellaPenna et al., 1986). The difference in size between the mature catalytic PG polypeptide and the PG in vitro translation product is due to a extra 71-amino acid domain at the N-terminus of the in vitro PG translation product (DellaPenna and Bennett, 1988). This domain is composed of a 24 amino acid hydrophobic signal sequence and highly charged 47 amino acid pro-sequence. The nucleotide sequence of a polygalacturonase cDNA (pTOM 6) was reported by Grierson et al. (1986) and encodes a polypeptide of molecular weight of 50,051. It contains an open
reading frame of 1,371 bases (457 amino acids) including the aforementioned pro-sequence of 213 bases (71 amino acids) and four possible glycosylation sites (Asn-X-Ser/Thr) located in the mature PG protein domain (Grierson et al., 1986; Sheehy et al., 1987; DellaPenna and Benett, 1988). The C-terminus of purified PG2A occurred 13 amino acids before the stop codon in the cDNA sequence, indicating the carboxyl terminus is cleaved post-translationally (Sheehy et al., 1987).

The association of the β-subunit with the PG2 isoenzymes to make PG1 alters many biochemical characteristics of the catalytic PG2 protein. PG1 has a lower isoelectric point than the PG2 isoenzymes (8.6 versus 9.4 for PG1 and PG2, respectively; Ali and Brady 1982). The native molecular weight of PG1 is ambiguous but much larger than PG2 (46 kD), reportedly ranging from 84 kD to 199 kD, depending on the method of analysis. This may be caused by differences in experimental methods or differences in the precise assembly (ratio of the two subunits) of PG1, which has yet to be determined experimentally. PG1 is also dramatically more heat stable (50% inactivation at 79°C) than the PG2 isoenzymes (50% inactivation at 57°C) (Tucker et al., 1980). PG1 and PG2 have different ionic strengths for optimum activity and vary in optimum pH (pH 3.6 for PG1 and pH 4.4 for PG2). PG1 activity can be stimulated by low concentrations of divalent cations (such as Ca²⁺), while EDTA is inhibitory to its activity. PG2 isoenzymes show an opposite response to these reagents. Moreover, Pressey and Avants (1973) reported that PG1 and PG2 isoenzymes differed subtly in their mechanisms of pectic polymer cleavage. It was suggested that the PG2 isoenzymes are specific for low molecular weight substrates
and cleave the substrate randomly, while PG1 releases smaller products than PG2 and
does not exhibit substrate-size specificity. The percentages of both isoenzymes as total
activity differ during tomato fruit ripening. At the onset of ripening, only PG1 is found.
At the fully-ripe stage, the relative percentages are dependent upon cultivar. PG1
accounts for 7% of the total activity in ripe Ailsa Craig, 21% in ripe Potentate (Tucker et
al., 1980) and 54% in ripe Tropic (Pressey, 1986).

β-subunit

It is obvious that the distinctly different properties of PG1 and PG2 isoenzymes
result from the association of the β-subunit polypeptide. The β-subunit is a heat-stable
glycoprotein (Tucker et al., 1980; Tucker et al., 1981; Moshrefi and Luh, 1983) and can
survive heating to 100°C for 10 minutes. It can be extracted from both green and ripening
tomato fruit cell walls by high salt buffers (Tucker et al., 1981; Pressey, 1984) and several
investigators have shown that the β-subunit can associate in vitro with and convert PG2
into an isoenzyme that closely resembles PG1 (Tucker et al., 1981; Pressey, 1984; Knecht
et al., 1988).

The presence of PG1 in vivo and the function of the β-subunit remain disputed.
Numerous studies have suggested that PG1 is the active complex in vivo and responsible
for polyuronide degradation (Knecht et al., 1988; Giovannoni et al., 1989; DellaPenna et
al., 1990). Evidence supporting this hypothesis include: (1) PG1 appears first and the
PG2 isoenzymes later during wild-type fruit ripening (Tucker et al., 1980; Brady et al., 1982), (2) only PG1 can be extracted when maximal levels of pectin degradation and solubilization occurs in wild-type and PG transgenic rin tomato fruits (DellaPenna et al., 1990), (3) lack of pectin degradation in transgenic tobacco plants expressing tomato PG and producing only PG2 isoenzymes (Osteryoung et al., 1990), and (4) the in vivo biphase loss of PG activity in heat-treated tissue that mimics that of mixtures of PG1 and PG2 in vitro (Pogson and Brady, 1993b).

However, contrary conclusions were reached by Pressey (1986, 1988) who suggested that PG1 is not an endogenous constituent but rather an artifact formed during extraction as PG2 and the β-subunit are co-solubilized. This conclusion was based on differential extraction at extremes of pH (i.e. pH 1.6 and 10) in which all PG activity is recovered as PG2 and β-subunit is subsequently recovered free of PG2 by further extraction at high ionic strength. Knegt et al. (1991) offered another hypothesis based on their suggestion that the β-subunit protein actually possess two affinity sites. One binding site provides for association with one PG2 molecule and another is for anchoring in the cell wall in vivo. They proposed that the physiologically active form of polygalacturonase in vivo is an enzyme composed of one molecule of β-subunit and one molecule of catalytic PG2 polypeptide, their so-called "PGx isoform". When the other binding site of the β-subunit is freed from the cell wall during extraction, it can bind one additional PG2 to form an extraction artifact, the PG1 isoform by their nomenclature. They reported that these forms (PGx and PG1) have different molecular weights, optimum pH's and
temperatures for 50% heat inactivation. PGx is 71 kD in molecular weight, pH optimum at 4.4 and a 50% inactivation temperature of 83°C (versus 57°C for PG2 isoenzymes). Their artifact form (PG1) is 106 kD in molecular weight, pH optimum at 3.6 and a 50% inactivation temperature of 79°C. It is important to stress that unlike Pressey, Knecht et al. still argued an important role for the β-subunit in regulation PG2 activity in vivo, via the PGx isoform.

These differing opinions on the native and function of the PG1 isoenzyme results from a lack of insight about the protein at the molecular level on the protein and its role in PG isoenzyme production. In order to understand the physiological and biochemical relationships between the β-subunit, PG1, PG2A and PG2B, we have cloned and studied cDNAs encoding the β-subunit. This analysis has provided additional insight into the expression and structure of the β-subunit of PG1. We have investigated its expression pattern in wild-type fruit and non-ripening mutant to determine the factors regulating its expression during fruit development and ripening. Finally, several β-subunit antisense transgenic plant lines have been successfully obtained using T-DNA transformation. We hope the eventual outcome of analysis of these transgenic plants will help to clarify and extend our understanding of the assembly, activity and physiological function of the individual PG isoforms during tomato fruit ripening.
The Dissertation Format

The format of this dissertation follows the "Dissertation Format Option for Including of Published Papers" described by the Graduate College of the University of Arizona. Two papers are included in this dissertation. The first paper was published in The Plant Cell, vol. 4, pp.1147-1156, September 1992. This paper reported isolation and characterization of a full length β-subunit cDNA clone from a tomato mature-green (MG) fruit cDNA library. Three researchers made contributions to the paper, Dr. Dean DellaPenna, Dr. Rick Heupel and myself. Dr. Heupel sequenced the full length cDNA clone. Dr. DellaPenna isolated and purified the β-subunit protein of PG1, designed oligonucleotide probes and did proteolytic experiments and FPLC. The experiments I contributed to the paper are listed below:

1. Isolation and purification of several poly (A)* RNA samples from tomato fruits at different stages.
2. Oligonucleotide northern blot analysis.
3. Synthesis and construction of the MG fruit cDNA library.
4. cDNA library screening.
5. Northern blot analysis.
6. PG1 protein isolation and purification.
7. Genomic DNA isolation and southern blot analysis.
8. PCR experiments and MOPAC-generated probes.
9. Partial cDNA sequencing.

The second paper was submitted to Plant Physiology in early 1994. In this paper, we reported the further characterization of the expression patterns of the β-subunit and catalytic PG2 polypeptide during fruit development and ripening, and in response to treatment with the phytohormone ethylene in wild-type and the ripening mutant rin. All of the experiments described in the paper were performed by myself.

I will also be a co-author on a third paper reporting the characterization of β-subunit antisense plants. My major contributions to the β-subunit antisense work include (1) construction of a chimeric β-subunit antisense gene and sense vector, (2) transformation and regeneration of wild-type tomato plants containing the chimeric genes, (3) designing a procedure for rapid isolation and analysis of β-subunit proteins from tomato fruit, leaf, root and flower cell walls, and (4) developing an immunobloting method for detecting β-subunit proteins in tomato cell wall extracts. In addition, I have analyzed several of the transgenic plants by southern blot analysis. I describe this work in Appendix C.
CHAPTER TWO

PRESENT STUDY: β-SUBUNIT CLONING AND CHARACTERIZATION

The methods, results, and conclusions of this study are presented in the papers appended to this dissertation. The following is a summary of the most important findings in these papers.

Specific Cloning Approach

In the first paper, we reported the results from molecular cloning and characterization of the β-subunit of tomato fruit polygalacturonase 1 isoenzyme. It is the first report of a full length β-subunit cDNA sequence, describes its novel protein structure and the transcript accumulation pattern during fruit development and ripening. The full length cDNA clone, pBsub2.2, was isolated from a tomato mature-green fruit cDNA library I constructed. During the course of cloning, we resolved two fundamental problems which hindered our progress. First, due to its unique protein structure, we obtained very little useful protein sequence data for the design of oligonucleotides. Second, there was no β-subunit expression data available when I began the project, and
we did not know at which stages of fruit development the β-subunit was expressed. In order to overcome these problems, two specific experiments were carried out, oligo-northern analysis and MOPAC reactions. Oligo-northern analysis was performed with several different degenerate antisense oligonucleotides and the results of these experiments indicated that the β-subunit was expressed in early stages during tomato fruit development and absent from ripening fruit. These results were crucial for selecting tomato fruit of a suitable developmental stage for cDNA library construction. The oligo-northern analysis also helped us select the best of several oligonucleotides for MOPAC analysis and library screening. Second, I generated a highly specific DNA-fragment probe directly from poly (A)⁺ RNA by the PCR-based MOPAC procedure (polymerase chain reaction (PCR)-based Mixed Oligonucleotide Primer Amplification of cDNAs). The MOPAC probe dramatically increased the specificity and efficiency of cDNA library screening.

Novel Protein Structure

Based on sequencing data of a full length of cDNA clone (pBsub2.2), we found and reported in our first paper that the β-subunit possessed several unique characteristics in its primary protein structure. A 69-kD precursor protein is deduced from the pBsub2.2 cDNA sequence and composed of at least four domains, a signal sequence domain, a
propeptide domain, a mature protein domain and the carboxyl-terminal domain. The signal sequence confirms that the \( \beta \)-subunit is targeted to the endo-membrane system, as would be expected for a cell wall protein. Following the signal sequence, a propeptide domain occurs whose function is unknown. The carboxyl-terminal domain is almost equal in size to the mature protein domain. The function and point of removal of the carboxyl-terminal domain are unknown. We suggest that the carboxyl-terminal domain may serve to neutralize or mask the charge of the mature protein domain (pI 4.9) during processing and secretion, because of its high pI (9.1), or may function in some other fashion to target the \( \beta \)-subunit to its ultimate location in the cell wall. In the mature protein domain, we identified a novel 14-amino-acid motif structure FTNYGxxGNGGxxx, where the \( \text{Phe} \) and \( \text{Tyr} \) residues are invariable and \( x \) is most often a charged or uncharged polar amino. This motif is not found in other domains and accounts for almost the entire mature domain.

A large percentage of the \( \text{Phe} \) residues in this domain are modified. Modified \( \text{Phe} \) residues have not been reported in other plant cell wall proteins, suggesting that this represents a novel post-translational modification in plants. Finally, all six identified \( \text{N-linked glycosylation consensus sequences} \) are located within the mature domain. These unique structural features of the \( \beta \)-subunit protein combined with prior biochemical data (Pressey Avants, 1973; Knegt et al., 1988; 1991) suggest that the \( \beta \)-subunit may represent a class of bifunctional plant proteins that interact both with structural components of the
cell wall and catalytic proteins to localize and regulate metabolic activities within the cell wall.

\[ \text{\textit{p-Subunit Expression}} \]

In the second paper, we investigated the expression patterns of the two subunits of PG1 (PG2 and \textit{p-subunit}), and the factors regulating \textit{p-subunit} expression during fruit development and ripening, by using exogenous ethylene and \textit{rin}, the \textit{ripening inhibitor} mutant. During fruit development, the \textit{p-subunit} was found to have a similar expression pattern in wild-type and \textit{rin} fruit, although PG expression patterns were completely different in the two genotypes. The results strongly suggest that the change in \textit{p-subunit} mRNA levels at the onset of tomato fruit ripening are unrelated to catalytic PG2 expression. They also indicate that \textit{p-subunit} expression is not controlled by ethylene, since wild-type and \textit{rin} fruit differ dramatically in their endogenous ethylene production. Furthermore, this conclusion is supported by results from application of exogenous ethylene to immature-green wild-type and \textit{rin} fruit. During this ethylene treatment, \textit{p-subunit} protein levels of both genotypes were unaffected, while \textit{p-subunit} mRNA levels of both genotypes were only slightly and similarly affected. Thus, we propose that \textit{p-subunit} expression is regulated primary by developmental cues during tomato fruit development and ripening.
Catalytic PG2 Expression

We also found that the catalytic PG2 mRNA could be induced by exogenous ethylene in immature-green *rin* and wild-type fruit. It has been proposed from studies of PG2 expression in ACC-synthase antisense transgenic fruit that PG2 mRNA accumulation is ethylene independent, but that translation accumulation of PG protein requires ethylene (see Literature Review). Our observation indicated that ethylene is able to induce PG2 transcript accumulation in immature-green wild-type and *rin* fruit but, as in ACC synthase antisense fruit, PG2 protein failed to accumulate. These results indicate that regulation of PG2 expression in immature-green fruit, is responsive to ethylene while in mature fruit, development cues predominate. We concluded, in our second paper, that regulation of the tomato catalytic PG2 expression is a complex process involving coordination and interaction of both developmental and hormonal cues.

β-Subunit Expression in Non-Fruit Tissue

We also report in our second paper that the β-subunit protein is present at lower levels in root, leaf and flower cell walls, and that these tissues accumulate very low amounts of the β-subunit mRNA compared with that in fruit tissues. Wild-type and *rin* fruit showed similar β-subunit expression patterns in their non-fruit tissues. Because it has
been reported that other PG enzymatic activities are also found in non-fruit tissues, we hypothesize that β-subunit protein present in non-fruit tissues may possess similar functions as that in fruit, namely to immobilize, regulate or activate other catalytic cell wall proteins.

β-Subunit Antisense Analysis

In order to modify the expression of the β-subunit and investigate its function in vivo, a constitutively expressed chimeric β-subunit antisense gene was constructed and introduced into wild-type tomato plant. A total of twenty-three individual transgenic plants were regenerated from forty-two individually transformed calli selected by kanamycin. After transfer to soil, genomic DNA from several lines were isolated and southern analysis performed to determine whether the transgenic plants contained intact antisense genes. Figure 1 shows the results of southern analysis of six plants probed with the full-length cDNA insert of pBsub2.2. Lane 1, a wild-type control, gives a simple restriction map with BamH I digestion, while transgenic plants showed one extra 2.0-kb band representing the β-subunit antisense gene. β-subunit protein levels in 33-DAP (DAP: day after pollination) fruit from each transgenic line were determined by western analysis. The result from screening by a laser densitometer showed that β-subunit expression in several transgenic antisense fruit was deceased below 5% of the control level.
(Figure 2). However, transgenic fruit did not display any significant change in morphology during fruit development. These transgenic fruit had normal color changes and ethylene evolution patterns during ripening. The results of further molecular and biochemical analysis of the cell walls of these transgenic fruit will enable us to test the hypothesis that the β-subunit plays an important role in regulating polyuronide degradation and that PG1 is the primary responsible for polyuronide solubilization and degradation during ripening.
Figure 1. Southern Analysis of β-subunit Antisense Transgenic Tomato Plants. Five-μg of genomic DNA was digested with BamH I and fractionated in a 0.7% agarose-TAE gel. The probe used was pBsub2.2 cDNA insert. WT: wild-type control.
Figure 2. Western Analysis of β-subunit Antisense Mature-green Fruit. Fruit were harvested at the mature-green stage (33 days after pollination). Total cell wall proteins were isolated and five-µg of each sample were fractionated in a Urea-SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with the β-subunit antibody. WT: wild-type control.
β-subunit (38 kD)

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<th>TA-40</th>
<th>TA-38</th>
<th>TA-18</th>
<th>TA-16</th>
<th>TA-10</th>
<th>TA-9</th>
<th>TA-8</th>
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<td>3</td>
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<td>3</td>
<td>3</td>
<td>2</td>
<td>0</td>
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<tr>
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<td>&lt;1</td>
<td>60</td>
<td>115</td>
<td>4</td>
<td>&lt;2</td>
<td>15</td>
<td>100</td>
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</table>
APPENDIX A

THE β-SUBUNIT OF TOMATO FRUIT POLYGALACTURONASE ISOENZYME 1: ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF UNIQUE STRUCTURAL FEATURES
THE β-SUBUNIT OF TOMATO FRUIT POLYGALACTURONASE ISOENZYME 1: ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF UNIQUE STRUCTURAL FEATURES

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STATEMENT BY AUTHOR

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SIGNED: [Signature]

We have purified and isolated cDNAs encoding the \( \beta \)-subunit of tomato fruit polygalacturonase isoenzyme 1 (PG1), a cell wall protein that associates with, and apparently regulates, the catalytic PG2 polypeptides. Expression of the \( \beta \)-subunit is fruit specific and temporally separated from the expression of PG2 during fruit development. The 37- to 39-kD \( \beta \)-subunit is encoded as a 69-kD precursor protein containing a signal sequence and two propeptide domains. The mature protein is composed almost entirely of the novel 14-amino acid motif FTNYGxxGNGGxxx in which many of the phenylalanine residues are post-translationally modified. The unique structural features of the motif suggest an important role in the function of the protein and hence in the activity of PG1. The \( \beta \)-subunit may represent a class of bifunctional plant proteins that interact both with structural components of the cell wall and catalytic proteins to localize and/or regulate metabolic activities within the cell wall.
INTRODUCTION

The activity of the pectin-hydrolyzing cell wall enzyme polygalacturonase (PG; EC 3.2.1.15) increases dramatically during the ripening of many fruits and has been shown to be the primary enzymic activity responsible for cell wall polyuronide degradation in ripening tomato fruit (Wallner and Walker, 1975; Themmen et al., 1982; Huber, 1983; Brady et al., 1987; Smith et al., 1988; Giovannoni et al., 1989). In tomato, the catalytic PG polypeptide is encoded by a single gene (the PG gene) whose expression and function have been extensively studied in recent years (reviewed in Giovannoni et al., 1990).

The PG gene is transcriptionally activated at the onset of ripening and both PG mRNA and protein accumulate to high levels during the ripening process (Brady et al., 1982; DellaPenna et al., 1987; 1989). Although there is only a single gene for the catalytic PG polypeptide, the total PG activity isolated from ripe tomato fruit is attributable to a mixture of several closely related, post-translationally derived isoenzymes, PG1, PG2A and PG2B (Pressey and Avants, 1973; Ali and Brady, 1982; Moshrefi and Luh, 1983; DellaPenna and Bennett, 1988). The PG2A and PG2B isoenzymes (herein referred to as the PG2 isoenzymes) accumulate late in the ripening process and are each composed of a single catalytic PG polypeptide differing only in degree of glycosylation (Ali and Brady, 1982; DellaPenna and Bennett, 1988). The PG1 isoenzyme accumulates first during ripening and is thought to be composed of one or two catalytic PG2 polypeptides tightly associated with an ancillary glycoprotein, the β-subunit protein
(Moshrefi and Luh, 1983; Knegt et al., 1988). The level of PG1 produced during ripening is apparently determined by the level of β-subunit protein present in the fruit tissue (Giovannoni et al., 1989; DellaPenna et al., 1990; Pogson et al., 1991).

The β-subunit has recently been purified from PG1 and is a heat-stable, acidic, heavily glycosylated protein with an apparent molecular weight of 38 kD (Knegt et al., 1988; Pogson et al., 1991). No enzymatic activity has been identified for the protein. It was reported several years ago that the in vitro association of purified PG2 isoenzymes with a heat-stable, non-dialyzable factor present in both unripe and ripe tomato fruit cell wall extracts (the so-called PG converter) results in the formation of a complex that physically and biochemically resembles the native PG1 isoenzyme isolated from ripening fruit (Tucker et al., 1981; Pressey, 1984). The relationship between the β-subunit and the PG converter protein is still unresolved but a comparison of their physical and biochemical characteristics suggests that they may be the same molecule (Pressey, 1984; Knegt et al., 1991; Pogson et al., 1991).

Biochemical studies of PG1 isolated from tomato fruit and PG1 formed in vitro by reaction of purified PG2 with the β-subunit have shown that this association alters the biochemical and enzymatic properties of the catalytic PG2 polypeptide, especially with regard to pH optimum, heat stability, cell wall binding and activation by Ca\(^{2+}\) (Pressey and Avants, 1973; Knegt et al., 1988, 1991). These studies suggest that the β-subunit plays an important role in regulating PG enzymatic activity in vivo. Evidence supporting this hypothesis comes from studies with wild-type and transgenic tomato fruit that have
demonstrated that maximal levels of PG-dependent polyuronide degradation and solubilization occur at a time when only PG1 can be extracted from the tissue and that subsequent accumulation of extractable PG2 activity during the later stages of ripening is not accompanied by further polyuronide degradation (Giovannoni et al., 1989; DellaPenna et al., 1990). Moreover, transgenic tobacco plants constitutively expressing a chimeric PG gene were found to accumulate only the PG2 isoforms without resulting in increased polyuronide solubilization (Osteryoung et al., 1990). Finally, the β-subunit has been shown to interact strongly with polydextrans and this observation, coupled with the fact that PG1 is bound much more tightly to the cell wall than PG2, has led to the proposal that the β-subunit localizes or restricts PG activity to specific areas of the cell wall (Knegt et al., 1988, 1991). These combined studies suggest that PG1 is the active isoenzyme in vivo with regard to polyuronide degradation and, therefore, implicate the β-subunit protein as an important factor in the localization and/or regulation of pectin solubilization and degradation during the ripening process (Knegt et al., 1988; Giovannoni et al., 1989; DellaPenna et al., 1990; Knegt et al., 1991).

Although the induction of PG activity at the onset of ripening is known to be the major determinant of polyuronide degradation during tomato fruit ripening, the in vivo existence of PG1, the contribution of the various isoenzymes to ripening-associated polyuronide degradation, and the physiological consequences of PG-dependent polyuronide degradation remain unresolved. To extend our understanding of the assembly, activity and physiological function of the individual PG isoforms during tomato
fruit ripening, we have purified the β-subunit from PG1 and isolated cDNA clones which encode the protein. Herein, we report the deduced amino acid sequence of the large β-subunit precursor protein encoded by these cDNAs, the expression pattern of the β-subunit gene during fruit development and several unique structural features of the mature β-subunit protein that are likely to be of functional significance.

RESULTS

Purification and Sequencing of the β-subunit of PG1

When analyzed by SDS-PAGE, purified PG1 was found to contain several polypeptides ranging in size from 37 to 45 kD, as shown in Figure 1A, lane 1. The 44 and 45 kD polypeptides reacted strongly with antiserum raised against purified PG2 (Figure 1B, lanes 1 and 2) and their size and immunoreactivity are consistent with their being the catalytic PG2 polypeptides. Using cation exchange chromatography in urea containing buffers, proteins in the purified PG1 sample could be further separated into two species: the PG2 polypeptides and the β-subunit polypeptide(s) (Figure 1A, lanes 2 and 3, respectively). The purified β-subunit resolves poorly in SDS gels and is heterogeneous in size ranging from 37 to 39 kD with two prominent bands (Figure 1A, lane 3). The β-subunit protein does not react with anti-PG2 antibodies (Figure 1B, lane 3), indicating
Figure 1. SDS-PAGE and Immunoblot Analysis of Purified PG1 and Separated PG2 and β-Subunit Proteins.

(A) Proteins were resolved by SDS-PAGE and visualized by Coomassie Brilliant Blue G250 staining. Lanes 1, 4 μg of purified PG1; Lanes 2, 2μg of purified PG2; Lanes 3, 2 μg of purified β-subunit. β-Sub, β-subunit.

(B) Proteins were resolved by SDS-PAGE and blotted, and PG2 polypeptides were detected by reaction with anti-PG2 antibodies. Lane 1, 2 μg of purified of PG1; Lane 2, 1 μg of purified PG2; Lane 3, 1 μg of purified β-subunit.
that it is immunologically distinct from the catalytic PG2 polypeptides. Similar results have been reported previously by others (Pogson et al., 1991).

Protein sequencing of the purified β-subunit yielded the single amino-terminal sequence shown in Table 1 with high recovery. This result suggests that the observed size heterogeneity of the β-subunit results from differential glycosylation or differential processing at the carboxyl-terminus. Amino-terminal sequencing was repeated three times with identical results, including the blank cycle at residue 9. Additional internal protein sequence data were obtained from amino-terminal sequence analysis of several proteolytically derived β-subunit peptide fragments (Table 1: Glu-C-1, Glu-C-2, Lys-C, and Arg-C peptides). For the Glu-C-1 and Glu-C-2 peptides, the Edman degradation reaction was blank at cycles 8 and 3, respectively, and was also arrested at these positions. The Arg-C peptide yielded a blank amino acid at cycle 8 and the Lys-C peptide yielded a blank amino acid at cycle 9. The Edman degradation reaction continued after the blank cycles for these two peptides.

Identification and Sequence Analysis of β-subunit cDNAs

Purification and characterization of the β-subunit permitted the subsequent isolation of cDNA clones encoding the protein. Numerous β-subunit cDNA clones were isolated from a mature green tomato fruit cDNA library using a mixed oligonucleotide
Table 1. Summary of Protein Sequence Data from the Amino Terminus and Internal Proteolytic Fragments of Purified β-Subunit.

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Peptide Sequence</th>
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<tbody>
<tr>
<td>Amino-terminus</td>
<td>NH$_2$- Glu-Lys-His-Ser-Gly-Asp-Ile-His-[ ?]-Ala-Thr-Tyr</td>
</tr>
<tr>
<td>Lys-C</td>
<td>NH$_2$- Asn-Gly-Asn-Gly-Ala-Asn-Gly-Gln-[ ?]-Val</td>
</tr>
<tr>
<td>Glu-C-1</td>
<td>NH$_2$- Ala-Asn-Ala-Gly-Asp-Gln-Tyr-[ ?]</td>
</tr>
<tr>
<td>Glu-C-2</td>
<td>NH$_2$- Asn-His-[ ?]</td>
</tr>
<tr>
<td>Arg-C</td>
<td>NH$_2$- Gly-Ser-Pro-Arg-Asp-Asn-Lys-[ ?]-Asp-Asn-Tyr-Ala</td>
</tr>
</tbody>
</table>

Underlined sequences indicate amino acid sequence used for the generation of degenerate primers. Bracketed question marks represent blank cycles from the Edman degradation reaction. Additional sequence data were obtained after blank cycles in all cases except the Glu-C-1 and Glu-C-2 peptides.
primer amplified cDNA (MOPAC) probe and degenerate oligonucleotides derived from the protein sequence data shown in Table 1. Figure 2 shows the nucleotide and deduced amino acid sequences of the longest β-subunit cDNA clone, pBsub2.2. Preliminary results from primer extension analysis indicate that the 5' end of pBsub2.2 is within 30 nucleotides of the 5' end of the mature β-subunit mRNA (results not shown). Three sites of poly(A) addition have been observed based on the position of the poly(A) tails in different cDNA clones; however, all cDNAs analyzed contain identical 3' untranslated sequences up to their site of poly(A) addition (results not shown). pBsub2.2 contains a 2227 bp insert, including a 35 bp poly(A) tail, with a single large open reading frame extending from nucleotide 1 to nucleotide 1924 (Figure 2). Assuming that initiation of translation occurs at the first in-frame Met, the open reading frame of pBsub2.2 encodes a 69 kD protein containing 630 amino acids. Included in this open reading frame (Figure 2, single and double underlined protein sequences) are all of the β-subunit protein sequences shown in Table 1, confirming that the cDNA encodes the β-subunit protein.

β-subunit and PG Expression are Temporally Separated During Fruit Development

The expression pattern of β-subunit, PG and D21 mRNAs during tomato fruit development were analyzed by RNA blot analysis (Figure 3). The β-subunit probe, pBsub1.8, begins near the amino-terminus of the mature β-subunit protein domain and is
Figure 2. DNA Sequence and Deduced Amino Acid Sequence of the β-Subunit cDNA Clone pBsub2.2.

The deduced amino acid sequence is shown below the nucleotide sequence in single-letter amino acid code. Wavy underlines identify consensus sequences for N-glycosylation. Solid underlined regions indicate amino acid sequences homologous to the β-subunit protein sequence data in Table 1. The single underlined amino acid sequence is the amino terminus of the mature β-subunit protein, and double underlined amino acid sequences are internal proteolytic fragments. *M* is Met-397, the estimated carboxyl-terminus cleavage site of the mature β-subunit protein (see text for explanation). Sequence data has been submitted to GenBank as accession number M98466.
Figure 3. RNA Gel Blot Analysis of β-Subunit, PG, and D21 Expression during Fruit Development and Ripening.

Total RNA (25 μg) isolated from the indicated tomato tissues was probed with the β-subunit (β-Sub) cDNA clone (pBsub1.8), a cDNA for the catalytic PG polypeptide (pPG1.9; DellaPenna et al., 1987) or a cDNA for the constitutively expressed mRNA D21 (Lincoln et al., 1987). Identical specific activities were used in each hybridization and all blots were exposed for 8 hr.
<table>
<thead>
<tr>
<th></th>
<th>Root</th>
<th>Leaf</th>
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<tbody>
<tr>
<td>β-Sub-</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<tr>
<td>D21</td>
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</tbody>
</table>

β-Sub: (2.3 kb)
PG: (1.9 kb)
D21: (1.0 kb)
approximately 400 bp shorter than the pBsub2.2 sequence shown in Figure 2. This probe recognizes a single mRNA species of approximately 2.3 kb that becomes detectable early in fruit development (10 days after pollination [DAP]) and increases with further development to a maximum level in fruit at 30 DAP (Figure 3, β-subunit panel). Between 30 and 35 DAP (a period coincident with the onset of ripening), the level of β-subunit mRNA decreases below detection, while the PG mRNA level increases dramatically (Figure 3, PG panel). There is no detectable β-subunit mRNA or PG mRNA in leaf, root and flower tissues after an 8-hr exposure (Figure 3); however, after a 6-day exposure faint signals for β-subunit mRNA could be detected in these tissues. The control probe D21 encodes an mRNA of unknown function that is expressed throughout fruit development (Lincoln et al., 1987). The expression pattern of D21 is relatively constant during the early stages of fruit development (10 to 30 DAP) and increases two- to three-fold during ripening (Figure 3, D21 panel). Unlike β-subunit and PG mRNA, D21 expression is not fruit specific.

DNA gel blot analysis of tomato genomic DNA digested with the indicated enzymes and probed with pBSub1.8 is shown in Figure 4. pBSub1.8 contains a single EcoR V site, two sites each for EcoR I and Hpa I and no sites for Pst I. The relatively simple banding pattern in genomic DNA blot analysis coupled with the observation that pBsub1.8 maps to a single locus on chromosome 5 (Steve Tanksley, personal communication) suggest that the β-subunit is encoded by a single gene or small number of tightly linked genes in the tomato genome.
Figure 4. Genomic DNA Gel Blot Analysis of the β-Subunit of PG1. Ten µg of genomic DNA digested with the indicated restriction enzyme was loaded per lane. The blot was probed with the β-subunit cDNA clone pBsub1.8. At right are the position of molecular weight length markers.
The β-subunit is Encoded as a Large Precursor Protein with Multiple Domains

The predicted primary translation product of the β-subunit contains at least 4 identifiable domains: (1) a hydrophobic amino-terminal signal sequence, (2) an amino-terminal propeptide following the signal sequence (3) the mature protein domain, and (4) a large carboxyl-terminal propeptide, as shown in Figure 5. The predicted cleavage site of the signal sequence was calculated by the method of Von Heijne (1983) to be after Gly-30. The amino acid sequence (Figure 2) and hydropathy plot (Figure 5) of this domain share characteristics common to signal sequences, including a positively charged amino-terminal region, a central hydrophobic core, and a polar carboxyl-terminal region (Von Heijne, 1983). The first amino acid of the mature β-subunit domain (defined by amino-terminal sequence analysis of purified β-subunit protein; see Table 1) is Glu-109, leaving a 78-amino acid propeptide domain between the carboxyl terminus of the proposed signal sequence and the amino terminus of the mature protein.

The carboxyl terminus of the mature β-subunit protein has not yet been experimentally determined but is likely to be near, but after Met-397 (*M* in Figure 2). This prediction is based on both the presence of a single Met residue in amino acid composition analysis of purified β-subunit protein, as shown in Table 2 and Pogson et al. (1991), and the fact that Met-397 is the first Met residue in the mature domain of pBsun2.2 as well as on the observation that digestion of purified β-subunit with cyanogen bromide (which cleaves after Met residues) does not noticeably alter its migration on
Figure 5. Hydropathy Profile and Putative Protein Domains of the Deduced β-subunit Precursor Protein.

Hydropathy was predicted according to the rules outlined in Kyte and Doolittle (1982) using a window of seven amino acids. The abscissa represents the position of the amino acid residues. On the ordinate, positive values indicate hydrophilic and negative values denote hydrophobic characteristics. The black and hatched rectangles at the bottom of the figure denote the signal sequence and amino-terminal propeptide domains, respectively. The estimated mature protein domain and carboxyl-terminal propeptide are labelled. The positions of N-linked core glycosylation consensus sites are depicted by the branched structures on the mature protein domain.
Table 2. Amino Acid Composition of the PG1 β-Subunit protein

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<td>Met</td>
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<td>0.3</td>
</tr>
</tbody>
</table>

* Expressed as residues per 100 residues.

* Results are based on the average of three independent amino acid analyses.

* Based on the deduced amino acid sequence of pBsub2.2 cDNA from residues 109 to 397.
SDS-polyacrylamide gels (results not shown). Cleavage after Met-397 would yield an unglycosylated mature domain (amino acids 109 to 397) of approximately 31.5 kD with a calculated pI of 4.9 and a large carboxyl-terminal domain (amino acids 398 to 630) of 25.6 kD with a calculated pI of 9.1. The size and pI of the predicted mature domain are in good agreement with those previously reported for the purified β-subunit (Pressey, 1984; Pogson et al., 1991). All six identified N-linked glycosylation consensus sequences are located within the predicted mature domain (Fig. 2, wavy underlines; Figure 5, branched structures), consistent with the reported heavy glycosylation of the mature β-subunit protein (Pogson et al., 1991). Table 2 compares the amino acid composition of the mature protein domain deduced from pBsub2.2 and that determined from purified β-subunit protein. The predicted amino acid compositions of the mature β-subunit is notably rich in Gly, Tyr and Phe. The predicted and determined amino acid compositions are generally in good agreement with the exception of an underestimate of Phe residues (1% versus 8% for determined and predicted composition, respectively).

A Novel Repeating Motif is Present in the Mature β-Subunit Protein Domain

Computer-assisted analysis of the deduced amino acid sequence shown in Figure 2 indicated the presence of a repeating motif in Figure 6, within the mature domain with the general structure: FTxYGxxxN(x)₄₋₆, where Phe and Tyr residues are invariable and Thr, Gly and Asn occur with a greater than 50% frequency at positions 2, 5 and 9, respectively.
This structure is not found in other domains of the β-subunit precursor protein. The 14-amino acid version of this motif is repeated 15 of 17 times in the central portion of the mature protein (Figure 6, boxed region). Within the core consensus region, there is a strong bias for specific amino acids or a specific amino acid group at certain "x" positions in the motif, such as Ser, Thr or Asn at position 3, Gly at positions 8, 10 and 11 and charged or uncharged polar amino acids at positions 6, 7, 12, 13 and 14. Inclusion of these residues yields a core consensus of FTNYGxxGNGGxxx where "x" is most often a charged or uncharged polar amino acid. Exhaustive searches of computer databases have failed to identify other proteins with significant overall homology to the mature protein domain or to the 14-amino acid motif and higher order repeats of the motif to 3N. The entire contiguous sequence of the deduced β-subunit mature domain (amino acids 109-397) is shown from top to bottom and left to right. Periods represent gaps introduced into the sequence for alignment purposes. Shadowed residues indicate amino acids that occur with a high frequency at a given position. Bold Phenylalanine (F) residues are those that yielded blank cycles in protein sequence analysis. The boxed area defines the region of the protein from which the core consensus shown below FTNYGxxGNGGxxx was derived. The bold amino acids of the core consensus sequence occur with a 50% or greater frequency in that position.
Figure 6. Optimal Alignment and Consensus Sequence of the Repeating Fourteen-Amino Acid Motif Contained within the Mature β-Subunit Protein Domain and Location of Modified Phenylalanine Residues.
Core Consensus

FTNYG××冈NGG×××
DISCUSSION

The expression and activity of the pectin-hydrolyzing tomato fruit cell wall enzyme PG has been the subject of extensive molecular and biochemical analysis for many years. The presence of multiple PG isoenzymes (PG1, PG2A and PG2B) has been known for some time and the existence of a factor, the β-subunit protein, capable of associating in vitro and apparently also in vivo with the catalytic PG2 polypeptides to generate PG1 has been demonstrated by several groups (reviewed in Giovannoni et al., 1990). Separate lines of evidence from biochemical studies (Tucker et al., 1981; DellaPenna et al., 1990) and transgenic plant studies (Smith et al., 1988; Giovannoni et al., 1989; DellaPenna et al., 1990) in which expression of the catalytic PG polypeptide was modified have suggested that the production of PG1 is an important determinant of polyuronide degradation during ripening. These studies have implicated the β-subunit of PG1 as an important component in the localization and regulation of polyuronide solubilization and depolymerization during ripening.

To extend our understanding of the assembly, activity and function of individual PG isoenzymes in vivo and in particular that of PG1, we have purified and characterized the β-subunit of tomato fruit PG1. The purified β-subunit is heterogeneous in size (37 to 39 kD) and immunologically unrelated to the catalytic PG2 polypeptides. DNA sequence analysis of a full length β-subunit cDNA, pBsub2.2, indicates that the β-subunit is encoded as a large 69 kD precursor protein containing at least four distinct protein domains: an
amino-terminal signal sequence, an amino-terminal propeptide, the mature protein domain and a large carboxyl-terminal propeptide domain. The presence of a signal sequence on the precursor protein is consistent with the β-subunit being targeted to the endomembrane system, as would be expected for a cell wall protein. After insertion into the lumen of the endoplasmic reticulum, the precursor protein clearly undergoes extensive post-translational proteolytic processing during subsequent maturation and targeting to the cell wall. It is interesting to note that both the β-subunit precursor protein and the catalytic PG2 precursor protein (DellaPenna and Bennett, 1988) contain large amino-terminal propeptides; however, no significant homology is observed between the two. The high pI (9.1) and large size of the predicted carboxyl-terminal propeptide domain may serve to neutralize or mask the charge of the mature protein domain during processing and secretion, or may function otherwise in targeting the β-subunit to its ultimate location in the cell wall. Because some of the proposed functions of the β-subunit are to assist in the localization, immobilization or activation of the catalytic PG2 polypeptides at the tomato cell wall, it is also possible that the amino-terminal and/or carboxyl-terminal propeptides play a role in these processes.

The amino acid composition of the mature β-subunit polypeptide domain deduced from pBsub2.2 is in good agreement with that obtained from purified β-subunit protein with the exception of a severe underestimate of the Phe content of the mature protein by ourselves and others (Table 2; Pogson et al., 1991). The cause of this large discrepancy (three Phe residues determined by composition analysis versus 23 predicted from cDNA
sequence analysis) is still unresolved but several observations are relevant. First, the low level of Phe is not compensated for by a corresponding increase in the level of another closely related amino acid such as Tyr (Table 2). Second, in amino acid composition analyses of purified β-subunit protein, two additional peaks were observed in HPLC profiles (results not shown) which did not correspond to any of the 20 common amino acids. Third, and most convincing, comparison of the deduced β-subunit protein sequence (Figure 2) with protein sequence data obtained from the amino-terminus of the protein and four internal proteolytically-derived peptides (Table 1) indicated that whenever a Phe was predicted from the deduced amino acid sequence, a blank cycle was obtained by Edman degradation. In three cases (the amino-terminus and the Lys-C and Arg-C peptide sequences), the Edman degradation continued after this blank cycle and subsequent amino acids were obtained which corresponded to the sequence deduced from pBsub2.2. In two other peptides (Glu-C-1 and Glu-C-2), the anticipated Phe cycle was also blank, but the Edman reaction was arrested.

These results indicated that a large percentage of the Phe residues in the mature β-subunit protein are modified, possibly in 2 ways, neither of which yields identifiable derivatives in protein sequence analysis or amino acid composition analysis. It should be noted that very few modifications have been reported for Phe and that an extensive literature search has not yielded mention of modified Phe residues in other plant cell wall proteins. This raises the possibility that the modified Phe residues in the β-subunit
represent a novel post-translational modification in plant cell wall proteins. The identification and structural determination of these modified Phe residues are underway.

Another striking structural feature of the mature protein domain is the presence of a novel repeating 14-amino acid motif with the general consensus FTNYGxxGNGGxxx, where Phe and Tyr are absolutely conserved. This motif accounts for almost the entire mature β-subunit protein domain (Figure 6) and is not found in other domains of the β-subunit precursor protein. The five modified Phe residues identified by protein sequence analysis are all located in position 1 of this motif (bold Phe residues in the boxed region of Figure 6). It should be noted that the 14-amino acid consensus motif shown is the minimal repeating unit for this motif and that higher order repeats (multiples of the fourteen-amino acid repeat) are also possible with equal or greater conservation. The significance of this protein substructure and the modified Phe residues at position 1 is open to speculation, but the fact that the motif constitutes nearly the entire mature protein domain and is not found in other domains of the precursor implies an important role for it in the structure and/or function of the mature β-subunit protein.

With regard to structural considerations, the β-subunit somewhat resembles plant structural cell wall proteins such as hydroxyproline-rich glycoproteins, glycine-rich proteins and proline-rich proteins that also contain repetitive amino acid motifs and in some cases post-translationally modified amino acids such as hydroxyproline and isodityrosine (Cassab and Varner, 1988). However, the β-subunit differs from the theses classes of proteins in several important respects. First, although the β-subunit does
contain a repeating amino acid motif, the minimum repeating unit is much longer and shows much greater variability than those found in structural cell wall proteins. Second, unlike structural proteins, which are encoded by small multigene families, the β-subunit appears to be encoded by a single gene. Third, β-subunit expression is restricted primarily to a single organ of the plant (the fruit) at a precise developmental time. Other structural proteins, e.g., hydroxyproline-rich glycoproteins, exhibit tissue-specific and/or cell type-specific expression patterns of individual gene family members, but as a group are generally expressed throughout the plant. Finally, and most importantly, unlike structural proteins such as hydroxyproline-rich glycoproteins and proline-rich proteins, the β-subunit binds tightly to and apparently regulates the activity of another protein with enzymatic activity.

In addition to structural studies of the β-subunit, we have also studied the expression patterns of β-subunit and PG mRNA, the two subunits of PG1, during tomato fruit development (Figure 3). Although expression of both β-subunit and PG mRNAs is fruit-specific, they differ dramatically in their temporal regulation during tomato fruit development. β-subunit mRNA is detectable early in tomato fruit development (Figure 3) and increases gradually during fruit development to its highest level at 30 DAP, just prior to the onset of ripening. During the following 5-day period, during which time the ripening process is initiated, the steady-state level of β-subunit mRNA decreases below detectable limits, whereas that for the catalytic PG polypeptide increases from undetectable to its highest level.
The functional significance of this apparent temporal separation in expression of the two components of PG1, the β-subunit and the catalytic PG2 polypeptides, remains to be determined. One possible explanation is that β-subunit expression occurs early in tomato fruit development to allow transport, attachment and localization of the β-subunit protein to specific regions of the cell wall in the absence of the catalytic PG2 polypeptides. Temporal separation would ensure that as the catalytic PG2 polypeptides were expressed and secreted at the onset of ripening they could only associate with the β-subunit at its targeted location in the cell wall rather than in transit. Based on in vitro studies (Pressey and Avants, 1973; Knegt et al., 1988, 1991; Pogson et al., 1991), this association at the cell wall would be predicted to modify the activity or restrict the movement of the associated catalytic PG2 polypeptide(s) in the cell wall. Continued expression of PG2 in excess of the available β-subunit binding sites would then allow the observed accumulation of the PG2 isoenzymes during the later stages of ripening. The precise subcellular localization of the mature β-subunit protein and the presence and location of any β-subunit processing intermediates should provide insight into the assembly, localization and function of the individual PG isoenzymes in vivo.

The results presented in this paper provide further insight into the structure and expression of the β-subunit of PG1 during tomato fruit development and point to the complex regulation of polygalacturonase isoenzyme production and polyuronide degradation during the ripening process. We have shown that the β-subunit protein contains several novel structural features that are likely to be of functional significance.
The β-subunit may be representative of a class of cell wall proteins that interact with structural components of the wall and catalytic proteins to regulate and localize enzymatic activity within the cell wall. To advance our understanding of tomato fruit PG structure and function, future studies will focus on examining in more detail (1) the apparent temporal separation of β-subunit and PG expression during the earlier stages of ripening, (2) the determination of the exact structure of the modified Phe residues in the protein, and (3) the effect of altering β-subunit production during fruit development on the assembly, activity, and function of the individual polygalacturonase isoforms in vivo.

METHODS

Plant Materials

Tomato plants (Lycopersicon esculentum cv. Ailsa Craig) were grown under standard greenhouse conditions. For developmental studies, flowers were pollinated and tagged upon opening and fruit harvested at the indicated days after pollination (DAP). Fruit between 10 and 25 DAP represented different time points of the immature green stage of fruit development. At 20 DAP, fruit were approximately 50% full size. Thirty-, 35- and 40-day-old fruits were full-sized and were classified as mature green, turning stage
and fully ripe, respectively, as judged by external color and locule development (Lincoln et al., 1987).

β-subunit Protein Purification and Preparation of Peptide Fragments

Highly purified polygalacturonase isoenzyme 1 (PG1) was isolated from ripe tomato fruit pericarp tissue following a modification of the purification procedure of Pressey (1988). Ripe pericarp tissue was homogenized in ice cold distilled H₂O at a ratio of 1 kg fruit tissue to 1 liter water, and the resulting slurry was adjusted to pH 3.0. Cell debris was pelleted by centrifugation at 10,000g for 20 min, resuspended in one-half volume of cold dH₂O at pH 3.0 and repelleted. The washed cell debris pellet was resuspended in cold buffer containing 50 mM sodium acetate, 1.25 M NaCl, pH 6.0, and stirred for at least 1-hr at 4°C. The extract was centrifuged at 10,000g for 20 min, and proteins in the supernatant were precipitated by the addition of ammonium sulfate to 70% saturation. After centrifugation, the resulting protein pellet was resuspended in 0.125 M sodium acetate, pH 6.0, and dialyzed extensively against the same buffer. The dialyzed extract was clarified by centrifugation and applied to a CM-Sepharose column equilibrated with 0.125 M sodium acetate, pH 6.0. Bound proteins were eluted by a two step gradient of 0.45 M sodium acetate, pH 6.0, and 1.0 M sodium acetate, pH 6.0. The PG2 isoforms eluted with 0.45 M sodium acetate while PG1 eluted with 1.0 M sodium acetate.
The PG1-containing 1.0 M sodium acetate eluent was concentrated by ultrafiltration, dialyzed against Concanavalin A buffer (500 mM NaCl, 50 mM sodium acetate, 1 mM calcium acetate, 1 mM manganese sulfate, pH 6.0), and further purified by Con-A chromatography as previously described (DellaPenna et al., 1986). PG1-containing fractions were concentrated by ultrafiltration, dialyzed against 50 mM sodium phosphate, 200 mM NaCl, 0.1 mM DTT, pH 6.0, and further purified by Mono S FPLC chromatography (Pogson et al., 1991). The subunits of PG1 were separated and isolated following the method of Pogson et al. (1991). PG1 purification and separation of the PG2 and β-subunit polypeptides were followed by SDS-PAGE. Electrophoretic blotting, and immunological detection methods for the PG2 polypeptides were performed as previously described (DellaPenna et al., 1986). PG1 and PG2 levels during extraction and purification were determined by heat inactivation (Tucker et al., 1981) and activity staining of protein extracts separated by non-denaturing PAGE (DellaPenna et al., 1987).

Amino-terminal sequence analysis of the purified β-subunit was performed with a Beckman 890M gas phase sequenator. Internal β-subunit proteolytic fragments were generated by digestion with Lys-C, Arg-C and Glu-C endoproteases following instructions supplied by the manufacturer (Promega, Madison, WI). The resulting proteolytic fragments (at least 100 pmol) were resolved by SDS-PAGE, blotted to polyvinylidene difluoride membranes and directly sequenced (Matsudaira, 1987). Amino acid analysis was performed with an ABI 420A automated system (Applied Biosystem, Inc., Foster City, CA).
Nucleic Acid Isolation

Root, leaf, flower and fruit pericarp tissues at various stages of development were collected from greenhouse-grown tomato plants and frozen in liquid nitrogen. Total RNA was extracted following the method of Cathala et al. (1983) with modifications. Ten grams of frozen tissue was ground to a powder in liquid nitrogen with a mortar and pestle and homogenized with a polytron in 20 mL of lysis buffer (8 M guanidine hydrochloride, 10 mM EDTA, 300 mM Tris-HCl, pH 7.6, 8% b-mercaptoethanol). Following centrifugation at 3,000 g for 10 min, the supernatant was filtered through miracloth and extracted twice with phenol-chloroform and once with chloroform. RNA was ethanol precipitated and the resulting pellet was washed with 3.0 M sodium acetate, pH 5.5, to remove polysaccharide contaminants. Following a wash with 70% ethanol, the resulting pellet was dissolved in 10 mM Tris, pH 7.6, 1 mM EDTA, 1% SDS and precipitated with 2.5 M LiCl (Sambrook et al., 1989). Total RNA was used for RNA blot analysis or subjected to poly(A)$^+$ selection as previously described (DellaPenna et al., 1986). Genomic DNA was extracted from tomato leaves by the method of DellaPorta (1983).

Generation and Labeling of Probes

Degenerate oligonucleotides were designed from protein sequence data derived from the amino terminus and internal protease fragments of the purified $\beta$-subunit protein
(Table 1). Based on the first seven amino acids of the amino terminus (using two different codon sets for serine at position 4), two degenerate antisense amino-terminal oligonucleotides were synthesized (N-terminal A: 5'-AT[AG]TCNCC[AG]CT[GA]TG[CT]TT[CT]TC and N-terminal B: 5'-AT[AG]TCNCCNGA[GA]TG[CT]TT[CT]TC). Two less degenerate oligonucleotides were designed based on the amino acid sequences from the Glu-C-1 internal peptide fragment (Glu-C oligonucleotide: 5'-AA[TC]GCNGGNGA[TC]CA[AG]AT) and a Lys-C internal peptide fragment (Lys-C oligonucleotide: 5'-GGNAA[TC]GGNGCNAA[TC]GG). These various oligonucleotides were utilized for polymerase chain reaction (PCR)-based mixed oligonucleotide primer amplification of cDNAs (MOPAC) reactions, library screening procedures and positive identification of putative cDNA clones.

Individual MOPAC reactions were performed following the protocol of Lee and Caskey (1990) with 1 μg of poly(A)^+ RNA from immature green (25 DAP), mature green, turning and fully ripe tomato pericarp tissues. A Not I primer adaptor (Promega; 5'-CAATTCGCGGCCGCT) was used as a primer for first strand cDNA synthesis. Subsequent PCR amplification cycles utilized a Not I adaptor oligonucleotide (5'-dCAATTCCGCGCCGCT) as the 3' primer and the degenerate Glu-C oligonucleotide described above as the 5' primer. Aliquots of amplified products were electrophoresed, blotted to nylon membranes and probed with the degenerate Lys-C oligonucleotide. The Lys-C oligonucleotide hybridized strongly to a 1300-bp product generated in MOPAC.
reactions with immature green and mature green fruit poly(A)⁺ RNA (results not shown). This product was recovered and amplified by PCR using the Not I adaptor oligonucleotide (3' end primer) and the degenerate Lys-C oligonucleotide (5' end primer). The resulting 1250-bp Lys-C MOPAC product was used in conjunction with the Lys-C and Glu-C degenerate oligonucleotides for library screening and RNA gel blot analysis. Labelling of the 5' end of oligonucleotides and random primer DNA labelling of double-stranded DNA probes were performed with the appropriate kits following the manufacturer's protocol (Bethesda Research Laboratories, Gaithersburg, MD). Oligonucleotides were end labelled to a specific activity of at least 1 x 10⁶ cpm/µg and used at a concentration of 0.4 pmol/mL hybridization solution.

cDNA Library Construction and Screening

A cDNA library (1.0 x 10⁷ recombinants before amplification) was constructed from mature green tomato fruit poly(A)⁺ RNA in the bacteriophage Zap following the manufacturer's instructions (Stratagene, LaJolla, CA). For primary screening, replicate nitrocellulose filters of six plates (25,000 plaque forming units per 150-mm diameter plate) were probed with the degenerate Lys-C oligonucleotide (Sambrook et al., 1989). Prehybridization was carried out for 4 hr at 37°C in 6 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1 x Denhardt's solution (1 x Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02%SDS), 0.5% SDS, 0.05% sodium pyrophosphate and
100 µg/mL denatured salmon sperm DNA. Hybridization was carried out overnight at 37°C in 50 mL of 6 x SSC; 1 x Denhardt's solution; 0.05% sodium pyrophosphate with 20 µg/mL yeast tRNA and 20 pmol of labelled oligonucleotide probe. Following hybridization, the filters were washed twice for 5 min at room temperature and twice at 37°C for 30 min in 5 x SSC, 0.05% sodium pyrophosphate. A final wash in 5 x SSC, 0.05% sodium pyrophosphate was performed at 40°C for 10 min. The filters were exposed overnight with intensifying screens at -80°C.

Further rounds of screening were performed at a low plaque density using the 1.25 kb PCR-generated MOPAC cDNA fragment, the Glu-C oligonucleotide and the two degenerate amino-terminal oligonucleotides, N-terminal A and N-terminal B. Hybridization and washing conditions for these oligonucleotides were as described above and for the MOPAC-generated cDNA fragment as described by Sambrook et al. (1989) using 5 x 10⁶ cpm/mL. Fifteen primary positive clones were obtained that hybridized to the 1.25 kb MOPAC-generated probe and the Lys-C and Glu-C oligonucleotides. Four of these β-subunit cDNAs also hybridized with the N-terminal A oligonucleotide, consistent with their larger size. The serine codon group of the N-terminal A oligonucleotide was subsequently shown to match that encoded by the β-subunit cDNAs, whereas the N-terminal B oligonucleotide did not. Following plaque purification, plasmids were rescued by in vivo excision as described in the manufacture's protocol (Stratagene). Two of the longest β-subunit cDNAs (pBsub1.8 and pBsub2.2) were sequenced and used to
generate the data in Figure 2. Double-stranded DNA sequencing was performed by the method of Kraft et al. (1988).

RNA and Genomic DNA Blot Analysis

Total RNA (25 μg) was size-fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde and 1 x Mops buffer (20 mM 3-[N-morpholino]propanesulfonic acid, 8 mM sodium acetate, 1 mM EDTA, pH 7.0) and blotted to Nylon membranes as described by Sambrook et al. (1989). The blots were probed with $1 \times 10^7$ cpm of labelled cDNA inserts as described by Sambrook et al. (1989). The cDNA clones pPG1.9 and D21 encode, respectively, the catalytic PG polypeptide and an mRNA of unknown function expressed at near constitutive levels during fruit development. The expression of these two mRNAs during fruit development and ripening has been described in detail elsewhere (DellaPenna et al., 1987, 1989; Lincoln et al., 1987, DellaPenna and Bennett, 1988). Genomic DNA was digested with the indicated restriction enzymes, fractionated on 0.8% agarose gels and transferred to nylon membranes. Prehybridization, hybridization and washing conditions for genomic DNA gel blot analysis were as described by Sambrook et al. (1989).
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REFERENCES


DellaPenna D, Alexander DC, and Bennett AB (1986) Molecular cloning of tomato fruit polygalacturonases: Analysis of polygalacturonase mRNA levels during ripening. Proc Natl Acad Sci USA 83:6420-6424


APPENDIX B

DIFFERENTIAL EXPRESSION OF THE TWO SUBUNITS OF TOMATO PG1 DURING FRUIT DEVELOPMENT AND ETHYLENE TREATMENT IN WILD-TYPE AND RIPENING INHIBITOR MUTANT (rin) TOMATO FRUIT
STATEMENT BY AUTHOR

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DURING FRUIT DEVELOPMENT AND ETHYLENE TREATMENT IN WILD-TYPE
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Abbreviations: PG, polygalacturonase [poly(1,4-a-D-galacturonide) glycanohydrolase, E.C.3.2.1.15]; DAP, days after pollination.
ABSTRACT

The β-subunit of tomato fruit polygalacturonase 1 (PG1) is a cell wall glycoprotein that binds to and apparently regulates the catalytic PG2 polypeptide in vivo. β-subunit and 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 the β-subunit expression is ethylene independent and regulated primarily by developmental cues. This conclusion is supported by results from ethylene-treated immature (20 DAP) wild-type and rin fruit in which no significant differences were observed in β-subunit expression in response to ethylene treatment. Surprisingly, RNA blot analysis indicated that catalytic PG2 mRNA was induced in immature rin fruit after three days of exogenous ethylene treatment. In addition, β-subunit mRNA and protein were also detected at lower levels in root, leaf and flower tissues, suggesting a broader functional role for the protein.
INTRODUCTION

Tomato fruit polygalacturonase or 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 in 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 (Themmen et al., 1982; Ali and Brady, 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 non-catalytic glycoprotein, known as the converter or β-subunit protein (Tucker et al., 1981; Pressey, 1984; Knecht 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 et al., 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.,
Purified β-subunit can also associate with and convert PG2 \textit{in vitro} into an isoenzyme that closely resembles PG1 (Tucker \textit{et al.}, 1981; Pressey, 1984; Knegt \textit{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 \textit{et al.}, 1980, 1981; Knegt \textit{et al.}, 1988).

The presence and function of PG1 and the role of the β-subunit in regulating PG activity \textit{in vivo} remain unresolved. It has been proposed that PG1 is an artifact since PG2 and β-subunit protein can be recovered separately from the cell wall, however, this differential extraction could only be accomplished in buffers of radically different ionic strengths and extremes of pH (Pressey 1986; Knegt \textit{et al.}, 1991). Other lines of evidence from molecular and biochemical studies suggest that PG1 is the physiologically active complex \textit{in vivo} and have implicated the β-subunit as playing an important role in immobilizing and regulating the catalytic PG2 protein \textit{in vivo} (Giovannoni \textit{et al.}, 1989; DellaPenna \textit{et al.}, 1990). Evidence supporting this hypothesis include: (1) the sequential appearance of PG1 and PG2 during fruit ripening (Tucker \textit{et al.}, 1980; Brady \textit{et al.}, 1982), (2) the observation that only PG1 can be extracted when maximal pectin degradation and solubilization are observed in wild-type and ripening inhibitor (rin) plants expressing an inducible PG2 transgene (DellaPenna \textit{et al.}, 1990), (3) lack of pectin degradation in transgenic tobacco plants expressing a tomato PG transgene and accumulating only PG2 isoenzymes (Osteryoung \textit{et al.}, 1990), and (4) the \textit{in vivo} biphasic
loss of PG activity during heat-treatment of intact fruit tissue that mimics 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 (Zheng et al., 1992). It encodes a 69-kD precursor protein that is subject to extensive post-translational 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, FTNYGxxGNGGxxx. Expression of the β-subunit gene was found to be temporally separated from that of the catalytic PG2 gene suggesting that β-subunit and PG2 expression are controlled by different developmental or hormonal cues.

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. To address whether developmental or hormonal (i.e. ethylene) cues regulate the dramatic drop 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. Finally, we report the expression of β-subunit mRNA and protein in non-fruit tissues known to contain other developmentally regulated polygalacturonase activities.
MATERIALS AND METHODS

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill. cv. Ailsa Craig, and ripening inhibitor, *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 8 times to Ailsa Craig). Flowers were hand-pollinated and tagged at anthesis and fruit harvested at the indicated days after pollination (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-hr 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 uL.L⁻¹ of ethylene in water saturated air (continuous
flow at 1.5 L.min⁻¹), while the control group was treated with only water saturated air (1.5 L.min⁻¹). Fruit samples were collected at zero, one, three and five days 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 per gram powdered tissue. The mixture was vortexed vigorously and extracted with an equal volume of phenol/chloroform. Following centrifugation at 12,000g 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 1/10 volume of 3 M sodium acetate, pH 5.4, and 2.5 volumes of ethanol, followed by incubation for 2 hours at -20°C. The precipitated RNA was recovered by centrifugation at 12,000g for 30 min and the pellet 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 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 x Denhardt's reagent (1 x Denhardt's solution is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA), 0.1% SDS (w/v), 5 x SSPE, 100 μg/ml low molecular weight denatured DNA at 42°C overnight. Probes were labeled by random priming (BRL) with 32P-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 polygalacturonase 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

500 mg (fruit or root) or 200 mg (leaf or flower) of frozen powdered tissue 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, centrifuged at maximum speed in an microcentrifuge for 5 min at 4°C and the supernatant discarded. The cell wall pellet was again suspended in 1 ml 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 hours. 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 determined by a BCA assay following the manufacture's protocol (Pierce). The protein concentration of all samples was diluted to 1 μg.μl⁻¹ with 1.5 M NaCl, 10 mM sodium acetate, pH 6.0, and stored at -80°C until use.

**SDS-PAGE and Immunoblot Analysis**

We have found that β-subunit protein resolved well in an SDS-PAGE gel containing 8 M urea, while catalytic PG2 protein resolved 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, while for catalytic PG polypeptide analysis, a standard 10% SDS-PAGE gel was used. Cell wall extracts (1 μg μl⁻¹) were diluted with an equal volume of water, and two volumes of 2 x 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 hours. Separated polypeptides were electroblotted onto a nitrocellulose membrane in 100 mM glycine, 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 x PBS containing 2% (w/v) non-fat dry milk and 0.01% (v/v) Tween-20 for one hour, then incubated with a primary antibody (1:1000 dilution for both PG2 and β-subunit antibodies) in 1 x PBS, 0.2% (w/v) non-fat dry milk and 0.01% (v/v) Tween-20 for 1 hour. 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 DellaPenna et al. (1986). After three ten minute washes with 1 x PBS, 0.1% (v/v) Nonidet P-40, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:3000 dilution, Bio-Rad) in 1 x PBS, 0.2% (w/v) non-fat dry milk and 0.01% (v/v) Tween-20 for 1 hour 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 two to three days at 20C, detached 30-DAP wild-type fruit began to produce endogenous ethylene and ripen with the first external color change visible at 0.5 nL gram\(^{-1}\) hour\(^{-1}\). RNA blot analysis indicated that expression of the two subunits of PG1 overlapped during the initial stages of wild-type fruit ripening (Figure 1A). PG2 mRNA levels increased gradually as ethylene evolution rose, while β-subunit mRNA decreased from maximal to undetectable levels during the same period. β-subunit mRNA levels exhibited a transient and reproducible decrease as ethylene levels increased to 0.5 nL gram\(^{-1}\) hour\(^{-1}\). D2I mRNA levels (D2I is a constituitively expressed mRNA control) remained relatively constant during this period indicating that this decrease was not due to uneven loading of RNA.

Immunoblot analysis of cell wall protein extracts isolated from the same tissue samples showed that β-subunit protein levels were relatively constant during ripening and apparently unaffected by increasing ethylene evolution (Figure 1B). The PG2 polypeptide first became detectable in cell wall protein extracts of fruit producing 1.9 nL gram\(^{-1}\) .hour\(^{-1}\) ethylene and increased rapidly as ripening progressed. PG2 protein lagged behind the appearance of its mRNA which became detectable at a much earlier ripening stage (0.3 nL ethylene gram\(^{-1}\) .hour\(^{-1}\) )
Figure 1. RNA blot and immunoblot analysis of β-subunit and PG2 mRNA and 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 C_2H_4 g^-1 hr^-1, indicated above each lane). (A) RNA blot analysis: 20 μg of total RNA was fractionated in a 1.2% agarose formaldehyde gel, transferred to a nylon membrane, and sequentially hybridized to the indicated {}^{32}\text{P}-labeled cDNA clones. (B) Immunoblot analysis: 5 μg of total cell wall proteins were separated by SDS-PAGE, blotted to nitrocellulose and detected with β-subunit or PG2 antisera as described in "Methods and Materials". PG1 lane, 1 μg of purified PG1 protein.
A. Ethylene Production

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B. Ethylene Production

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β-subunit and PG2 Expression during Wild-type and \textit{rin} Fruit Development

RNA blot analysis (Figure 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 at 30-DAP (Figure 2). During the onset of ripening (30 to 35-DAP), β-subunit mRNA decreased below detection, while PG2 mRNA reached its maximal level at 35-DAP and decreased by 40-DAP. These results agree with previous studies (Zheng et al., 1992). In \textit{rin} fruit (Figure 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 wild-type, β-subunit mRNA remained at a relatively high level in 35-DAP \textit{rin} fruit and was still detectable up to 50-DAP. PG2 mRNA was not detected in \textit{rin} fruit at any developmental stage. D21, a constitutively expressed control, showed very similar expression patterns during wild-type and \textit{rin} fruit development.

Immunoblot analysis of these same tissues showed β-subunit protein was detectable throughout wild-type and mutant fruit development (Figure 3). Densitometric scanning of immunoblots indicated the amount of β-subunit protein increased gradually during wild-type fruit development until 25-DAP and remained relatively constant thereafter (Figure 3). In mutant fruit, β-subunit protein levels increased up to 15-DAP and then remained constant. Catalytic PG2 polypeptides became detectable at 35-DAP in
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 separated in a 1.2% formaldehyde agarose gel, blotted to a nylon membrane and probed sequentially with the indicated $^{32}$P-labeled cDNA clones. Equal specific activities were used in each hybridization and all blots were exposed overnight. (DAP, days after pollination).
Wild-Type Fruit (DAP)

10 15 20 25 30 35 40

Mutant Fruit (DAP)

10 15 20 25 30 35 40 45 50

β-Sub
(2.3 kb)

PG2
(1.9 kb)

D21
(1.0 kb)
Figure 3. Immunoblot analysis of β-subunit and PG2 protein during wild-type and rin fruit development. 5 μg of total cell wall proteins were separated by SDS-PAGE, blotted to nitrocellulose and detected with β-subunit or PG2 antisera as described in "Methods and Materials". (DAP, days after pollination; PG1 lane, 1 μg of purified PG1 protein).
Wild-Type Fruit (DAP)

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Mutant Fruit (DAP)

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the wild-type fruit (Figure 3), but was not detected at any developmental stage in rin fruit (Figure 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 one- and three-day air treatments (Figure 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 zero-day fruit after five days. However, in ethylene-treated wild-type and mutant fruit, β-subunit mRNA levels dropped more sharply after one-day of ethylene treatment compared to air treatment. In ethylene treated wild-type fruit, β-subunit mRNA levels had recovered by 5 days (Figure 4), whereas in ethylene treated rin fruit, β-subunit mRNA levels remained somewhat depressed (Figure 4).

PG2 mRNA was consistently detected by RNA blot analysis in the immature-green wild-type fruit after one-day of ethylene treatment (Figure 4) and increased dramatically during the course of ethylene treatment. Surprisingly, PG2 mRNA was also detected in immature rin fruit after three days of ethylene treatment and continued to increase after
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 fractionated in a 1.2% formaldehyde agarose gel, blotted to a nylon membrane and probed sequentially with the indicated 32P-labeled cDNA clones.
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<th>Wild-Type Fruit</th>
<th>Mutant Fruit</th>
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<tr>
<td></td>
<td>Air (Days)</td>
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- **β-Sub (2.3 kb)**
- **PG2 (1.9 kb)**
- **D21 (1.0 kb)**
five days of treatment (Figure 4). However in comparison with identically treated wild-type fruit, PG mRNA levels in rin fruit after five days of ethylene treatment were much lower. Similar results were obtained in multiple experiments. Immunoblot analysis of the same tissue (Figure 5) showed that the amounts of β-subunit protein isolated from cell walls of both genotypes were not significantly affected by air or ethylene treatments. No immunologically detectable PG2 protein was detected in either wild-type or rin fruit cell wall extracts, despite the induction of PG2 mRNA in both genotypes by ethylene treatment (results not shown).

β-subunit Expression in Other Tissues

After an 8-hr exposure to X-ray films, β-subunit mRNA could not be detected by northern blot analysis in root, leaf and flower tissues of both wild-type and mutant plants, although it was easily detected in fruit tissues in the same exposure time (data not shown). However after exposure for six days, low levels of β-subunit mRNA were detected in root, leaf and flower tissues of both genotypes (Figure 6). The non-fruit mRNA was identical in size to that in fruit. PG2 mRNA was not detected in any non-fruit tissues of both wild-type and rin (data not shown).

Immunoblot analysis revealed that a protein identical in size to the fruit β-subunit protein was also present in cell wall protein extracts of roots, leaves and flowers of both
Figure 5. Immunoblot analysis of β-subunit protein levels in air and ethylene treated immature wild-type and rin fruit. 5 μg of total cell wall proteins were separated by SDS-PAGE, blotted to nitrocellulose and detected with β-subunit or PG2 antisera as described in "Methods and Materials". β lane, 500 ng purified β-subunit protein.
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 proteins from the indicated tissues were separated by SDS-PAGE, blotted to nitrocellulose and detected with β-subunit as described in "Methods and Materials". PG1 lane, 1 μg of purified PG1 protein. RNA analysis: 20 μg of total RNA from the indicated tissues was fractionated in a 1.2% agarose formaldehyde gel, transferred to a nylon membrane, and hybridized with a 32P-labeled β-subunit cDNA insert. RNA blots were exposed for six days.
### Immunoblot Analysis

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**β-Sub (38 kD)**

### RNA Blot Analysis

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**β-Sub (2.3 kb)**
wild-type and *rin* plants (Figure 6). The amount of protein detected was highest in floral tissue, while root tissue contained the lowest amount. In addition, a second larger band was also consistently detected at lower levels in root and leaf tissues. PG2 protein was not detected in non-fruit tissues of both wild-type and *rin* plants (data 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 if this smaller band represents differential processing, a separate gene product or a specific degradation product. The later is unlikely to be a result of extraction as PG2 and D21 mRNAs in the same samples were intact (Figures 1, 2, and 4).

**DISCUSSION**

The ripening of wild-type fruit is due in large part to hormonal and developmental-mediated alterations in gene expression. The regulation of a specific fruit or ripening-associated gene can be determined by studying its expression in wild-type fruit, mutants genetically blocked from ripening and in response to exogenous application or removal of ethylene in both genotypes (Giovannoni *et al.*, 1990; Theologis 1992; Gray *et
We have previously shown that during wild-type fruit ripening, β-subunit mRNA levels drop dramatically, while PG2 mRNA levels rise rapidly (Zheng et al., 1992). These results raise several possibilities: (1) that the β-subunit and PG2 respond coordinately but oppositely to the same developmental signal (ripening), (2) they respond independently of each other, or (3) that β-subunit expression responds negatively to elevated ethylene produced at the onset of ripening. In order to further understand the factors regulating β-subunit expression at the onset of ripening, we have studied β-subunit expression in wild-type and mutant rin fruit during development, ripening and in immature fruit of both genotypes treated with ethylene.

Wild-type and rin fruit grow and develop similarly prior to ripening. However, fruit containing the rin mutation, show a complete inhibition of ripening, produce only background levels of ethylene and trace amount of PG protein (Herner and Sink, 1973; McGalssson et al., 1975; DellaPenna et al., 1987). The genetic ripening deficiency of rin fruit allows one to resolve developmental effects on gene expression from ripening or ethylene related effects. β-subunit expression was similar during wild-type and rin fruit development, although the PG expression patterns of the two genotypes were completely different (Figures 2 and 3). Furthermore, when immature-green fruit of both genotypes were treated with exogenous ethylene, β-subunit protein levels were unaffected (Figure 5), and β-subunit mRNA levels were only slightly affected (Figure 4). 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 non-fruit tissues. Root, leaf and flower tissues of both genotypes produced significant amounts of immunologically detectable β-subunit protein identical in size to that in fruit and in some cases lower levels of a larger immunoreactive protein (Figure 6). These results do not agree with those reported by Pogson et al. (1993a) who could not detect β-subunit protein in stem and leaf tissues. This contradiction may be explained by differences in the antibodies used by the two groups.

Polygalacturonase activity has been most extensively studied in fruit tissues but has also been reported in non-fruit 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 polygalacturonase and we hypothesize that β-subunit protein present in non-fruit tissues may perform similar functions as that in fruit, namely to immobilize or regulate these non-fruit PG polypeptides. Although we did not detect PG2 mRNA or PG2 proteins in non-fruit 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 polygalacturonases expressed in non-fruit tissues may
be sufficiently divergent both immunologically and at the DNA sequence level from the fruit PG to be undetected by the PG2 cDNA and antibodies.

Our 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 (Figure 4) has not been previously reported. PG gene induction in immature fruit therefore contrasts with previous data indicating transcriptional activation of the PG gene is ethylene independent (Oeller et al., 1991; Picton 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 point, 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 non-fruit tissues raises the possibility that differential processing of a single gene product occurs in these tissues or that multiple β-subunit genes exist. Our recent isolation of two closely related β-subunit genomic clones supports this later 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 understand β-subunit function in fruit development and ripening and in other non-fruit tissues.
ACKNOWLEDGMENTS

We wish to thank Fangcheng Gong for his assistance with laser densitometric scanning of western blots.
REFERENCES


Allen RL, Lonsdale DM (1993) Molecular characterization of one of the maize polygalacturonase gene family members which are expressed during late pollen development. Plant Journal 3(2):261-271


Pogson BJ, Brady CJ (1993a) Accumulation of the β-subunit of polygalacturonase 1 in normal and mutant tomato fruit. Planta 191:71-78


Theologis A (1992) One rotten apple spoils the whole bushel: The role of ethylene in fruit ripening. Cell 70:181-184


APPENDIX C

β-SUBUNIT ANTISENSE AND SENSE STUDIES

Antisense and Sense Gene Constructions

1. Construction of Antisense Gene pBant18

The vector pBI121 was used in antisense and sense constructions. The antisense
and sense DNA constructions were derived from the pBsub2.2 cDNA (2.2 kb, Figure 1), a
β-subunit cDNA clone from a MG tomato fruit cDNA library (Zheng et al., 1992).

For antisense construction (Figure 2) pBI121 was digested with Sma I (Blunt end)
and Sst I (5' overhead) to remove the GUS gene from the vector. pBsub2.2 was digested
with Nru I (blunt end, single site at 1790 of the cDNA sequence) and Sst I which is
located in the 5' polylinker of the clone. After digestion, phenol/chloroform extraction
and precipitation, each digested DNA sample was dissolved in 10 μl of water and
fractionated in a 0.8% low-melting point agarose gel. The digested pBI121 vector (Sma
I-Sst I digested) and the cDNA insert (Nru I-Sst I fragment) were recovered for ligation
reaction. Tubes containing the vector or insert, were removed from the freezer and placed
on a 65°C water bath. After the agarose melted, a ligation reaction was set up in a
microcentrifuge tube at room temperature as follows:
11 µl water
2 µl Vector DNA (in Low Melting Agarose)
2 µl Insert (in Low Melting Agarose)
4 µl 5 x ligation buffer (BRL)
1 µl Ligase (BRL) (1 U/µl)

Ligation was carried out overnight at 15°C. In the morning, an additional 1 µl of ligase was added and the reaction incubated at 25°C for 4 hours. After ligation, 20 µg of yeast tDNA was added, the reaction brought up to 100 µl, phenol/chloroform extracted and precipitated. After a 70% ethanol wash, the pellet was dissolved in 10 µl of TE, pH 8.0. The resulting solution was used directly to transform XL1-Blue bacteria. Seventeen white colonies were picked for minipreps. Fifteen were recombinant as confirmed by southern analysis using the full length insert of the pBsub2.2 clone. The antisense construction was named as pBant18 (Figure 2).

2. Construction of Sense Gene pBsen20

In order to obtain appropriate restriction sites for sub-cloning of the β-subunit sense construction into pBI121, the pBsub2.2 cDNA insert was digested and sub-cloned into the Kpn I-Xba I site of pUC19. The resulting plasmid, named pUsen20, was purified
Figure 1. Schematic diagram of pBsub2.2.
Figure 2. Schematic diagram of β-subunit antisense construction.
and digested with Sst I and Xba I. The Xba I-Sst I β-subunit fragment was recovered from a LMA gel and ligated with purified pBI121 previously cut with Xba I and Sst I restriction enzymes to remove the pBI121 GUS fragment. Ligated DNA was used directly to transformed XL1-Blue bacteria. The recombinant colonies were confirmed by southern analysis and named pBsen20 (Figure 3). Bacteria with the antisense and sense recombinant plasmids were used for mating with Agrobacterium.

*Agrobacterium* Tri-Parental Mating

The method of *Agrobacterium* tri-parental mating was as described by Beven (1984) with modifications. One colony of LBA4404 (*Agrobacterium*) grown on a M9 plate containing 250 μg/ml streptomycin was inoculated to a liquid M9 sucrose medium without antibiotics and grown at 28°C. The culture should be 2 days old and the cells grow in a fine suspension in this medium.

E. coli cultures (pRK2013 containing the helper plasmid, XL1-Blue with pBant18 or pBsen20) were streaked onto LB plates containing 50 μg/ml kanamycin and incubated at 37°C. After one day, a well-isolated colony from each plate was inoculated into 3 ml of LB liquid medium without antibiotics, respectively. The culture was incubated at 37°C with shaking overnight. 50 μl of each of three cultures (LBA4404, pRK2013, XL1-Blue with the antisense or sense construction) was combined on LB plates without antibiotics.
Figure 3. Schematic diagram of β-subunit sense construction.
The plates were placed in an incubator at 28°C overnight. The resultant culture was scraped off and placed into 2 ml of LB liquid medium containing 50 µg/ml kanamycin and 250 µg/ml streptomycin. The amount of inoculum should be the size of an inoculation loop. The liquid medium was incubated at 28°C overnight with vigorously shaking.

50 µl from the overnight culture solution was diluted with LB liquid medium. The diluted solution was spread on M9 sucrose plates containing 50 µg/ml kanamycin and 250 µg/ml streptomycin. The plates were kept in an incubator at 28°C overnight. Several colonies were picked and checked by minipreps (the E coli. alkaline lysis method works well if the cells are in early log phase). LBA4404 colonies with the pBant18 or pBsen20 were streaked on M9 sucrose plates with 50 µg/ml kanamycin and 250 µg/ml streptomycin and incubated at 28°C for two days. They were used for transforming tomato cotyledons.

Tomato Transformation

Dry tomato seeds were sterilized in solution containing 20% household bleach, 0.1% Tween-20 for 15 minutes, followed by three or more rinses of sterile water (about 1,000 seeds/4 gm for AC wild type). The sterilized seeds were moved onto 0.5 x MSO medium in large Petri dish plates (about 100 seeds/plate). The plates were placed in a growth chamber at 26°C under 16L/8D light conditions for 10 to 14 days. When there
were no or minimal true leaves present on seedlings, the tops of seedlings (approx. top half of hypocotyl and attached cotyledons) were cut off in batches and floated in liquid MSO for cutting. Cotyledons were cut near the proximal (wide) end and placed upside down on 100 x 15 mm Petri dish plates with MSO containing 1 mg/L zeatin. Before cutting cotyledons, the plates were covered with 3 ml of Nicotiana tabacum feeder cells, then a 3 MM filter paper disc that were previously washed with water extensively and autoclaved. Feeder plates with cotyledons were kept in the growth chamber at 26°C overnight (20 - 34 hours).

The cotyledons were removed from the feeder plates to small Petri dish plates. Fresh Agrobacterium culture (LBA4404 with pBant18 or pBsen20) was poured over the cotyledons. In general, 60 pieces of cotyledons were covered with 5 ml of diluted bacteria (about $2 \times 10^8$ to $1 \times 10^9$ cells, assuming an $\text{OD}_{590}$ of 1 unit = $10^9$ cells). The cotyledons were allowed to soak the Agrobacterium bacteria for 10 minutes, then gently transferred onto a sterile paper and blotted dry. They were moved back onto the feeder plates. The plates were taped shut with two pieces of parafilm film and incubated in a growth chamber at 26°C (16L/8D) for two days. After 48-hours co-cultivation, the cotyledons were transferred to deep plates (100 x 50 mm) with D1 medium containing 25 µg/ml kanamycin + 100 µg/ml cefotaxine. The plates were sealed with tapes and returned to the growth chamber for incubation.

Callus, green bumps, or shoots usually were seen at the cut edges within 10 days, and certainly after three weeks incubation, if the transformation was successful. The
cotyledons were transferred at three weeks to D2 medium (deep plates) containing 25 μg/ml kanamycin + 100 μg/ml cefotaxine for further shoot organogenesis. Before the first transfer to D2 plates, the entire explant should be transferred at subsequent three week transfers to fresh plates.

After another three weeks, shoots with true meristems arose, were cut off cleanly from the callus /cotyledons and transferred to rooting medium (MSO + 25 μg/ml kanamycin + 50 μg/ml cefotaxine, without hormones). Shoots usually rooted within 7-10 days, then were transferred into very wet soil in a 2-inch pot within a magenta box, with the lid tightly closed. Any residual agar was rinsed from the roots before transplanting. These magenta boxes were incubated under low light conditions and the lid of the magenta box slowly tilted off over a period of five days. The transformants were left within the magenta box (without the lid) until they were a reasonable size. They were then removed from the magenta box, eventually potted in size and transferred to the greenhouse.
REFERENCES


Buescher RW, Tigchelaar EC (1975) Pectinesterase, polygalacturonase, Cx-cellulase activities and softening of the rin tomato mutant. HortSci 10:624-625


Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. The Plant Journal 3:1-30


DellaPenna D, Alexander DC, Bennett AB (1986) Molecular cloning of tomato fruit polygalacturonase: Analysis of polygalacturonase mRNA levels during ripening. Proc Natl Acad Sci USA 83:6420-6424


Ecker JR and Davis RW (1986) Inhibition of gene expression in plant cells by expression of antisense RNA. Proc Natl Acad Sci, USA 83:5372-5376


Hobson GE (1967) Effects of alleles at the "never-ripe" locus on ripening of tomato fruit. Phytochemistry 6:1337-1341


Lyons JM, Pratt HK (1964) Effect of stage of maturity and ethylene treatment on respiration and ripening of tomato fruit. Proc Amer Soc Hort Sci 80:491-500


Ng TJ, Tigchelaar EC (1977) Action of the non-ripening (nor) mutant on fruit ripening of tomato. J Amer Hort Sci 102:504-509


Pogson BJ, Brady CJ (1993a) Accumulation of the β-subunit of polygalacturonase 1 in normal and mutant tomato fruit. Planta 191:71-78


Strand LL and Barman RJ (1978) Linkage of the non-ripening (nor) and uniform ripening (u) genes. Rpt Tom Genet Coop 28:20

Theologis A (1992) One rotten apple spoils the whole bushel: The role of ethylene in fruit ripening. Cell 70:181-184


