Expression of a Polygalacturonase Associated with Tomato Seed Germination[1]

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Radicle protrusion from tomato (*Lycopersicon esculentum* Mill.) seeds to complete germination requires weakening of the endosperm tissue opposite the radicle tip. In common with other cell wall disassembly processes in plants, polygalacturonases (PGs) may be involved. Only calcium-dependent *exo*-PG activity was detected in tomato seed protein extracts. Chromatographic profiles of a partially acid-hydrolyzed fraction of polygalacturonic acid further digested with seed extract were consistent with the presence of only calcium-dependent *exo*-PG activity. In addition, a transcript encoding a previously unknown PG was detected prior to the completion of germination. The mRNA, produced from a gene (*LeXPG1*) estimated by Southern analysis to be represented once in the genome, was also present in flowers (anthers) and in lower amounts in roots and stems. *LeXPG1* mRNA abundance was low during seed development, increased during imbibition, and was even greater in seeds that had completed germination. Expression of *LeXPG1* during germination predominates in the endosperm cap and radicle tip, and in the radicle appears as a distinct band possibly associated with vascular tissue differentiation. We suggest that PG is involved in cell wall loosening of the endosperm necessary for radicle protrusion from tomato seeds and in subsequent embryo and seedling growth.

The tomato (*Lycopersicon esculentum* Mill.) embryo is completely enclosed by the endosperm and testa. Weakening of the micropylar endosperm tissue opposite the radicle tip (the endosperm cap) is the major factor determining whether and when radicle emergence occurs (Groot and Karssen, 1987, 1992; Dahal and Bradford, 1990; Ni and Bradford, 1993). Endosperm cap cell walls are rich in mannans (Groot et al., 1988; Dahal et al., 1997), and weakening of the cap tissue is accompanied by an increase in the activity of endo-β-1,4-mannanase (Bewley, 1997). How-

ever, recent results indicate that, at least in tomato, the presence of mannanase activity alone is not sufficient for germination (Toorop et al., 1996; Dahal et al., 1997; Still and Bradford, 1997). It is probable that other hemicellulases, pectinases, and/or cellulases play a role in weakening the endosperm cap permitting radicle protrusion. Sánchez et al. (1986) showed that cellulase activity correlated well with the completion of germination in *Datura ferox* (like tomato, a member of the Solanaceae), but Leviatov et al. (1995) found that cellulase activity was not closely associated with germination rates of tomato seeds at low temperature.

Based upon microscopic studies of germinating seeds, it has been proposed that the radicle pushes between the cells of the endosperm, which separate rather than break or tear (Karssen et al., 1989). Numerous examples of time- and tissue-dependent loss of cell-to-cell cohesion occur in plant development, including pollen tube growth through transmitting styles (Clarke and Gleeson, 1981; Mu et al., 1994), outgrowth of lateral roots (Peretto et al., 1992), organ abscission (Taylor et al., 1990; Bonghi et al., 1992), dehiscence of seed pods (Jenkins et al., 1996), and intrusive growth of nonarticulated laticifers (Wilson et al., 1976) and fungal hyphae (Hahn et al., 1989). All of these processes are accompanied by partial breakdown in cell wall pectin and are correlated with the presence and activity of polygalacturonase (PG). Furthermore, pollen germination is associated with pectin degradation (Pressey and Reger, 1989; Pressey, 1991). Radicle protrusion from endospermic seeds has much in common with these processes involving loss of cell cohesion or penetration of existing tissues. We therefore tested the hypothesis that a PG may be present in the endosperm cap of tomato seeds. Here we describe the cloning, expression, and activity of a PG during tomato seed germination.

MATERIALS AND METHODS

Plant Material

Tomato (*Lycopersicon esculentum* Mill. cv Moneymaker) seeds were obtained from immature green, mature green, breaker, and red ripe (mature) fruit. The seeds were cleaned (0.1 M HCl for 1 h) and washed in tap water. Dried seeds (5% moisture content fresh weight basis) were stored at 4°C.

Germination conditions were as described previously (Dahal and Bradford, 1990). One gram of seeds was placed on 15 mL of distilled, deionized water on two, 8.5-cm diameter blotting paper discs (Stults Scientific Eng.,

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Springfield, IL) in a Petri dish. Dishes were placed inside of plastic containers lined with water-saturated paper towels and incubated at 25°C in the dark. Whole tomato seeds were harvested every 12 h for 4 d and separated, after 48 h, into those that had or had not completed germination before being extracted for either RNA or protein (see below). Other seeds were treated as above and dissected into the endosperm cap, radicle tip, and the rest of the seed (includes lateral endosperm, hypocotyl, and cotyledons) and RNA obtained from these seed components.

Endo- and *Exo*-PG Assays

Enzyme assays for endo-PG activity were conducted using a gel diffusion assay (Buescher and Burgin, 1992), and a viscometric assay (Christensen, 1954; Hadfield et al., 1998). The latter assay is the most sensitive one available for monitoring endo-PG activity (Tagawa and Kaji, 1988). Various commercially prepared PGA and pectic substrates of different degrees of esterification and from a variety of plants were tested.

Exo-PG activity was determined using a reducing sugar assay (Gross, 1982) with PGA from citrus fruit as the substrate (Sigma, St. Louis). One hundred seeds were pulverized in liquid N_2 and ground in 1 mL of extraction buffer (1 M NaCl, 2.5 mM PMSF, 10 μM leupeptin, and 50 mM NaOAc buffer, pH 5.0). The homogenate was made 80% (v/v) with respect to ammonium sulfate stepwise while stirring on ice. Subsequently, the suspension was placed in centrifuge tubes and left for 30 min on ice. The suspension was centrifuged (20 min, 10,000g) and the pellet resuspended in 50 mM NaOAc buffer, pH 5.0. The extract was dialyzed overnight in 12- to 14-kD exclusion tubing against 50 mM NaOAc buffer, pH 5.0. After dialysis, some aliquots were made 5 mM with respect to $CaCl_2$, some were boiled for negative controls, substrate was eliminated from other control reactions, and three reactions were spiked with serial dilutions of dialyzed commercial PG (*Aspergillus niger*; Megazyme, Sidney, Australia). Some experiments included 50 mM EDTA while in others $CaCl_2$ was not added to the extracts. Each time point and replication was assayed at two different dilutions and proportionality determined. All samples were quantified relative to a standard curve of GalUA. Each experiment was performed three times.

The nature of the PG-hydrolyzing activity present in tomato seeds was assayed using HPLC with pulsed amperometric detection (PAD; Townsend et al., 1988). A 1% (w/v) solution of PGA (G12 fraction, Campbell and Labavitch, 1991) was incubated with 1/10th volume of tomato seed extract for 12 h at 37°C. A 25-μL aliquot of each hydrolysate was quantified for monogalacturonic acid and compared with known concentrations from a commercial source (Sigma) using HPLC-PAD as described by Melotto et al. (1994).

PCR Amplification and cDNA Cloning

Degenerate PCR primers were constructed to two flanking conserved sites in known PGs and used according to Hadfield et al. (1998) to obtain putative PG amplicons from diverse plant parts. Amplicons were cloned and used as templates to synthesize DNA probes to screen for expression of their corresponding mRNAs in tomato seeds. Only one of these putative PGs was expressed in seeds prior to radicle protrusion.

Since the PG mRNA expressed in seeds was also present in anthers, 500,000 recombinants in a λgt10 phage anther cDNA library (Twell et al., 1989) were screened and seven hybridizing plaques identified. These were purified, recovered from the vector, and a 1,521-bp cDNA subcloned into pBSIIKS. This cDNA was subsequently digested with *Sca*I, which eliminated the poly(A) tail along with 17 bp of the 3'-UTR. Sequencing revealed that the cDNA did not contain the entire coding region. To obtain the 5' portion of the gene, RACE was performed using a 5'RACE kit according to the manufacturer (Life Technologies, Gaithersburg, MD). The nucleotide sequence of the cDNA and expression of this gene in seeds was verified against the exon sequence of a genomic clone (B. Downie and K.J. Bradford, unpublished data) and by cloning and sequencing the PG using RT-PCR with gene-specific primers on seed RNA, respectively.

Sequencing

Sequencing was performed at the Advanced Plant Genetics Facility (University of California, Davis). An ABI Prism 377 DNA Sequencer (ABI; Perkin-Elmer, Foster City, CA) utilizing dye termination chemistry with AmpliTaq DNA polymerase, FS (*Taq*; FS; Perkin-Elmer/Applied Biosystems Division [PE/ABI], Foster City, CA) was used to read cycle-sequencing reactions using a combination of universal and gene-specific primers (Genset, La Jolla, CA; Operon Technologies, Alameda, CA).

Genomic DNA Isolation and Analysis

Genomic DNA was isolated from lyophilized, expanding tomato leaves using a DNeasy kit (Qiagen, Valencia, CA). Genomic DNA (5 μg per lane) was exhaustively digested with restriction endonucleases, electrophoresed through a 0.8% (w/v) agarose gel in 1× TBE (Sambrook, 1989), and transferred to nylon membrane (Hybond N$^+$, Amersham Life Science, Arlington Heights, IL). The digested DNA was cross-linked to the membrane and hybridized with the 1,504-bp radiolabeled cDNA. Hybridization was performed at 42°C for 12 h in 50% (v/v) formamide, 6× SSC, 5× Denhardt's solution (Denhardt, 1966), 0.5% (w/v) SDS, and 100 μg mL^{-1} boiled, sheared salmon sperm DNA. Blots were first washed at low stringency (5× SSC and 0.1% [w/v] SDS at 65°C) and exposed to film, prior to being re-hybridized with the PG probe, washed at high stringency (0.2× SSC and 0.1% [w/v] SDS, 65°C) and re-exposed to film.

RNA Isolation and Analysis

Tomato seeds were pulverized in liquid N_2 and the RNA extracted and purified by a modification of the method of Ausubel et al. (1987). Extraction buffer consisted of 10 mM

Tris-HCl, pH 8.2, 100 mM LiCl, 1 mM EDTA, 1% (w/v) SDS, and 25 mM DTT. Prior to use, aliquots of the RNA were incubated with DNase I for 1 h at 37°C in digestion buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl$_2$, 20 units of RNasin, and 10 mM NaCl) followed by extraction with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1). The RNA was precipitated in ethanol and dissolved in the original aliquot volume of 2 mM EDTA. Poly(A) selection was conducted using Oligotex resin (Qiagen) as directed by the manufacturer. Poly(A) RNA (5 μg) from whole seeds or 5 μg of total RNA from seed pieces were separated on formaldehyde-containing agarose gels and transferred onto positively charged nylon membranes (Amersham Life Science) in 10× SSC overnight and UV cross-linked. After rinsing the membranes for 5 min in 2× SSC, they were placed in pre-hybridization solution (50% [v/v] formamide, 5× Denhardt's solution, 100 μg mL^{-1} boiled, sheared salmon sperm DNA, 0.2% [w/v] SDS, and 6× SSC, pH 7.0 [Sambrook et al., 1989]) for 4 to 6 h at 42°C (DNA probes) or 62°C (RNA probes). DNA probes to the nearly full-length sequence of the PG (minus the poly[A] tail; see above) were synthesized by random priming (Feinburg and Vogelstein, 1983).

Radiolabeled antisense RNA probes for the PG and for G46, a ubiquitously expressed ribosomal protein transcript used as a loading control, were generated by linearizing the appropriate vector and incubating this template at 37°C with T7 DNA-dependent, RNA polymerase (Pharmacia Biotech, Alameda, CA) in a run-off transcription reaction in the presence of [α-^{32}P]UTP (3,000 Ci mmol^{-1}, New England Nuclear Life Science Products, Boston). The DNA probes were added to the prehybridization solution and the membrane hybridized for at least 12 h at 42°C. The RNA probes were hybridized to membranes at 62°C. Membranes were first hybridized with the PG probe and subsequently were re-exposed to the antisense RNA probe for G46.

Regardless of the probe used, the primary wash was done in 2× SSC and 0.1% (w/v) SDS at room temperature for 5 min, then repeated but at 65°C for 30 min. The two final high stringency washes were at 0.2× SSC and 0.1% (w/v) SDS, 65°C for 30 min each. The hybridized probe was detected by autoradiography or on a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

In Situ Hybridization

PG transcripts were localized in situ using nonradioactive RNA probes labeled with digoxygenin (DIG) using colorometric detection. Labeled sense and antisense strands of RNA were obtained by run-off transcription using the T3 and T7 RNA polymerase promoters of pBSII KS (Invitrogen, Carlsbad, CA), respectively. Template was derived from either XbaI- or XhoI-digested PG cDNA in Bluescript. The transcription reaction was conducted in the presence of digoxygenin-labeled UTP. The nearly full-length transcripts were hydrolyzed in base to generate sheared probes with an average M_r of around 300 bp based on agarose gel electrophoresis.

Seeds germinated on water for 0, 36, and 48 h were sliced longitudinally and asymmetrically under ice-cold primary fixative (0.025 M phosphate buffer, pH 7.5, 2% [v/v] acrolein; 3% [v/v] glutaraldehyde, and 2% [w/v] paraformaldehyde) and fixed twice for 15 min under vacuum and subsequently overnight at 4°C. Fixed seeds were dehydrated in an ethanol series and infiltrated with a 4:1 mixture of nitrogen-sparged n-butyl- and methyl-methacrylate (Ted Pella, Redding, CA) to which benzoin ethyl ether, a UV activated catalyst, had been added. The seeds were arranged in molding trays under methacrylate and embedded by polymerizing the resin with UV light at 4°C for at least 24 h. Embedded seeds were fixed to plastic microtome chucks with glue and 2-μm sections obtained along the median, longitudinal plane on an Ultra-microtome with a diamond knife (LKB, Uppsala).

Sections were affixed to poly-Lys coated slides by heating overnight at 40°C. The plastic was removed from the sections by immersion in acetone for 30 min before rehydration through a graded ethanol series. Sections were treated with 500 μg mL^{-1} predigested proteinase K (Boehringer Mannheim) for 45 min, rinsed twice in 2 mg mL^{-1} Gly, and dehydrated through an ethanol series. Prehybridization solution (150 μL per slide; 50% [v/v] formamide, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5% [w/v] dextran sulfate, 1% [w/v] DIG blocking reagent [Boehringer Mannheim], and 150 μg mL^{-1} yeast tRNA) was applied for 3 h at 42°C. The sections were hybridized in 100 μL of prehybridization solution at 42°C containing 1 ng μL^{-1} either sense or antisense sheared probe for 14 to 18 h. The sections were rinsed in 4× SSPE, washed once in 2× SSPE at room temperature, and twice in 0.1× SSPE at 60°C. Sections were washed in 1× PBS and gently agitated in 1:2,500 dilution of anti-DIG-alkaline phosphatase conjugate for 3 h at room temperature.

Sections were washed three times in BSA washing solution (1% [w/v] phosphatase-free BSA, 0.3% [v/v] Triton X-100, 100 mM Tris-HCl, pH 7.5, and 150 mM NaCl) before being washed in TNM-5 buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, and 5 mM MgCl$_2$). Sections were placed in slide mailers containing 32 mL of TNP buffer (100 mM Tris-HCl, pH 9.0, 100 mM NaCl, 10% [w/v] polyvinyl alcohol, and 5 mM MgCl$_2$) with 16 mg mL^{-1} 5-bromo-4-chloro-3-indolylphosphate and 0.33 mg mL^{-1} nitroblue tetrazolium. Color development was terminated with 10 mM Tris, pH 8.0, and 1 mM EDTA and the sections coated with Crystal Mount (Biomedia, Foster City, CA), which was hardened at 42°C overnight.

Phylogenetic Analysis

The deduced amino acid sequence of the tomato PG was aligned with 27 full-length deduced amino acid sequences available in the literature and/or database and three melon PGs reported previously (Hadfield et al., 1998) using Clustal V multiple-sequence alignment software (Higgins et al., 1992) and employing a PG from A. niger (2385) as an outgroup. The amino acid sequences and their protein identification (numbers were: apple fruit (1346704, Atkinson, 1994), Arabidopsis gene ADPG1 (2597824, L.

Sander, R. Child, P. Uluskov, M. Albrechtsen, B. Joergensen, and B. Borkhardt, unpublished data), gene F5I14.10 (2190556, V.S. Vysotskaia, B.I. Osborne, M. Toriumi, G. Yi, O. Oji, Y.K. Shen, E. Buehler, A.B. Conway, A.R. Conway, K. Dewar, J. Feng, C. Kim, D. Kurtz, Y. Li, P. Shinn, H. Sun, R.W. Davis, J.R. Ecker, N.A. Federspiel, and A. Theologis, unpublished data), gene F6E13.1 (3212875, S.D. Roundsley, S. Kaul, X. Lin, K.A. Ketchum, M.L. Crosby, R.C. Brandon, S.M. Sykes, T.M. Mason, A.R. Kerlavage, M.D. Adams, C.R. Somerville, and J.C. Venter, unpublished data), and PGA2, 3, and 5 (3004442, 3004440, and 2982583, respectively, M. Torki, F. Thomas, R. Mache, P. Mandaron, and D. Falconet, unpublished data), avocado (22631, Dopico et al., 1993; Kutsunai et al., 1993), Japanese cedar (1076242, Komiyama et al., 1994), cotton (3024386, John and Petersen, 1994), kiwifruit (548488, Atkinson and Gardner, 1993), maize (288612 and 288379, Barakate et al., 1993; and 129940, Niogret et al., 1991), melon MPG1, 2, and 3 (3320458, 3320460, and 3320462, respectively, Hadfield et al., 1998), peach (2147957, Lee et al., 1990; 436420, Lester et al., 1994; and 479088, Lee et al., 1990), oilseed rape (304223, Robert et al., 1993; 1212786, Petersen et al., 1996; and 1419408, E.S. Jenkins, W. Paul, S.A. Coupe, S. Bell, L. Davies, and J.A. Roberts, unpublished data), tobacco (22701, Tebbutt et al., 1994), and tomato fruit (129939, Grierson et al., 1986); and tomato abscission zone PGs (TAPG1–5, 2459811, 1575705, 2459809, 2459815, and 2459817, respectively, Hong and Tucker, 1998). The phylogenetic tree was constructed using PAUP software package, version 3.1 (Swofford, 1990). The aligned sequences were analyzed in a heuristic search using simple stepwise addition of taxa. To extend the search
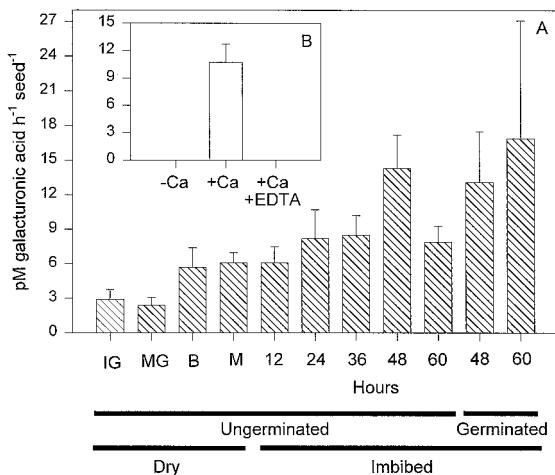

Figure 2. Profiles of a 1% (w/v) solution of partially hydrolyzed polygalacturonic acid exposed to tomato seed protein extract indicate the activity of an *exo*-PG. High-pressure liquid chromatography with pulsed amperometric detection (HPLC-PAD) resulted in the fractionation of a 1% (w/v) solution of partially acid hydrolyzed polygalacturonic acid. The solution is comprised of oligomers with a degree of polymerization from 6 to 19 centered on 10 to 12 (G12 fraction; Campbell and Labavitch, 1991). A, Untreated G12; B, G12 spiked with a commercial source of mono- (1) and di-GalUA (2) (made 2.5 nM with respect to both); C, G12 after exposure to tomato seed extract; D, G12 exposed to boiled tomato seed extract; E, G12 exposed to tomato seed extract containing 50 mM EDTA; F, G12 exposed to a commercial source of endo-PG from *A. niger*. Extracts in chromatograms C to E contained 5 mM CaCl$_2$. Response bars to the left of each chromatogram represent a 100-mV deflection.

Figure 1. PG activity accumulates in conjunction with germination. A, The activity of PG from desiccated developing and mature seeds and mature seeds during germination and subsequent radicle protrusion as assessed using a reducing sugar assay in the presence of 5 mM CaCl$_2$. From 48 h onward seeds were separated into those that had and had not completed germination. IG, Immature green; MG, mature green; B, breaker; M, mature. B, The effect of 5 mM CaCl$_2$ on the activity of PG from whole cv Moneymaker seeds allowed to imbibe for 48 h from which the radicle had protruded. Activity is reported as picomoles of GalUA produced per hour per seed.
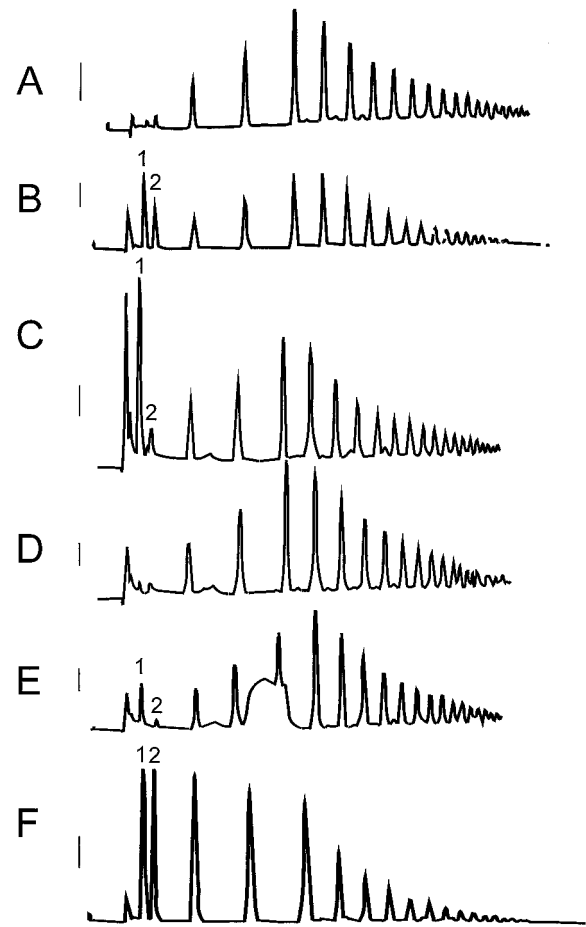
beyond local optima on which stepwise addition is prone to converge (Swofford, 1990), the number of trees held for re-evaluation upon adding the next taxon was set to five throughout tree construction (Swofford, 1990). To further guard against defining a phylogenetic tree of local optima, global (tree bisection and reconnection) branch swapping using 100 bootstrapped replications was performed according to the recommendations of the author (Swofford, 1990) once all 32 taxa had been connected. The tree was rooted to an *A. niger* PG sequence, which was defined as the hypothetical ancestral taxon (outgroup).
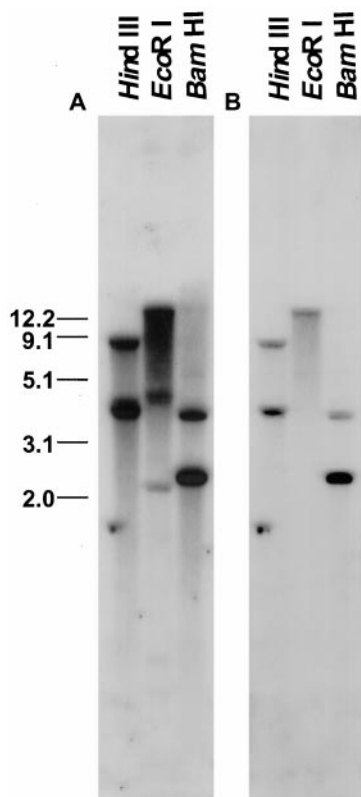
Figure 3. Southern-blot analysis of tomato genomic DNA probed with 1,504 bp of the poly(A)-truncated PG washed at low (5× SSC and 0.1% SDS at 65°C; A) and high (0.2× SSC and 0.1% SDS, 65°C; B) stringency suggest that there is little homology among the PG transcript and other members of this large gene family in tomato. The number of bands visible on the blot corresponds to the number predicted based on the restriction map of the cDNA (accession no. AF138858) and genomic clone (not shown).

RESULTS

A Calcium-Dependent *Exo*-PG Is Present in Tomato Seeds

No endo-PG activity was detected in extracts of imbibed tomato seeds regardless of the type of assay used (viscometric, gel diffusion, or HPLC), the substrate employed (PGA or pectin from apple or citrus fruit), or the degree of esterification of citrus fruit pectin (10%, 30%, 60%, or 90%). Crude protein extracts from tomato fruit pericarp and a purified fungal endo-PG consistently exhibited endo-activity in the same assays (data not shown). However, PG activity from tomato seeds was detected using a reducing sugar assay (Fig. 1A), suggesting the presence of *exo*-activity. This activity was low during seed development but increased during imbibition and germination (Fig. 1A). Like *exo*-PGs isolated from pollen (Pressey and Reger, 1989; Pressey, 1991), the PG activity from tomato seeds required Ca^{2+}, and adding EDTA to chelate Ca^{2+} completely inhibited enzyme activity (Fig. 1B).

To further characterize the nature of PG activity in tomato seeds, protein extract was incubated with a 1% (w/v) solution of partially hydrolyzed PGA (G12 fraction from Campbell and Labavitch, 1991) and digestion products

were analyzed by HPLC. The chromatographic profile of the G12 fraction prior to incubation (Fig. 2A) exhibited little mono- or di-GalUA (Fig. 2B). After incubation with tomato seed extract in the presence of 5 mM $CaCl_2$, a large peak of monogalacturonic acid was present, but the remaining profile was essentially unchanged (Fig. 2C). Little or no monogalacturonic acid was present when the G12 fraction was incubated with boiled tomato seed extract (Fig. 2D) or with extract to which EDTA (Fig. 2E) had been added prior to incubation. In addition, the chromatographic profile upon incubation with tomato seed extract in the presence of $CaCl_2$ (Fig. 2C) was considerably different from that obtained when a commercial source of endo-PG digested the G12 fraction (Fig. 2F). Endo-activity shifted the PGA profile to shorter oligomers (Fig. 2F), which did not occur in the presence of the tomato seed extract (Fig. 2C). These results support the contention that the PG activity present in tomato seeds is calcium-dependent and *exo*-acting.

Identification and Cloning of a PG Transcript in Tomato Seeds

Six unique approximately 300-bp amplicons were obtained from a variety of tomato tissues using degenerate
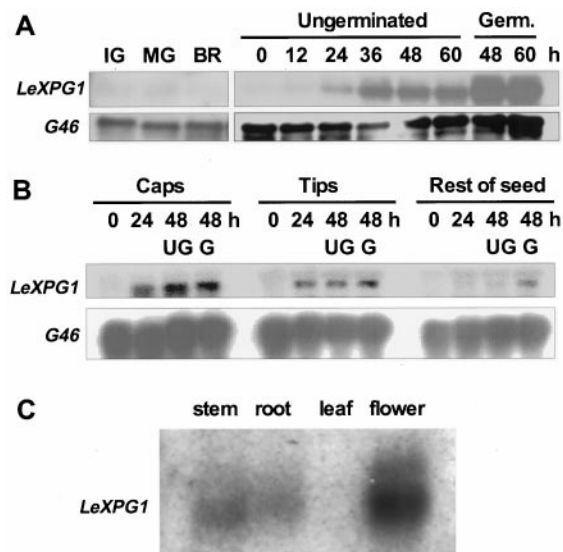

Figure 4. Expression of *LeXPG1* mRNA. A, mRNA (5 μg lane⁻¹) from developing (IG, immature green; MG, mature green; BR, breaker) or mature wild-type cv Moneymaker tomato seeds allowed to imbibe from 0 to 60 h was hybridized with the *LeXPG1* cDNA probe. Upon radicle protrusion, mRNA abundance increased as much as 10-fold over that present in mature, desiccated seeds. *G46* is a constitutively expressed cDNA coding for a ribosomal protein that was used as an RNA loading control. Germ., Germinated. B, Northern-blot analysis of 5 μg of total RNA from wild-type cv Moneymaker tomato seed parts revealed that *LeXPG1* mRNA is present in the endosperm cap and radicle tip by 24 h after imbibition. The mRNA accumulated in the rest of the wild-type seed 48 h after imbibition only if the seed had completed radicle emergence. UG, Ungerminated; G, germinated. C, Expression of *LeXPG1* mRNA is not restricted to tomato seeds but also occurs in the stems, roots, and flowers (anthers) of tomato plants. Each lane was loaded with 5 μg of poly(A) RNA.
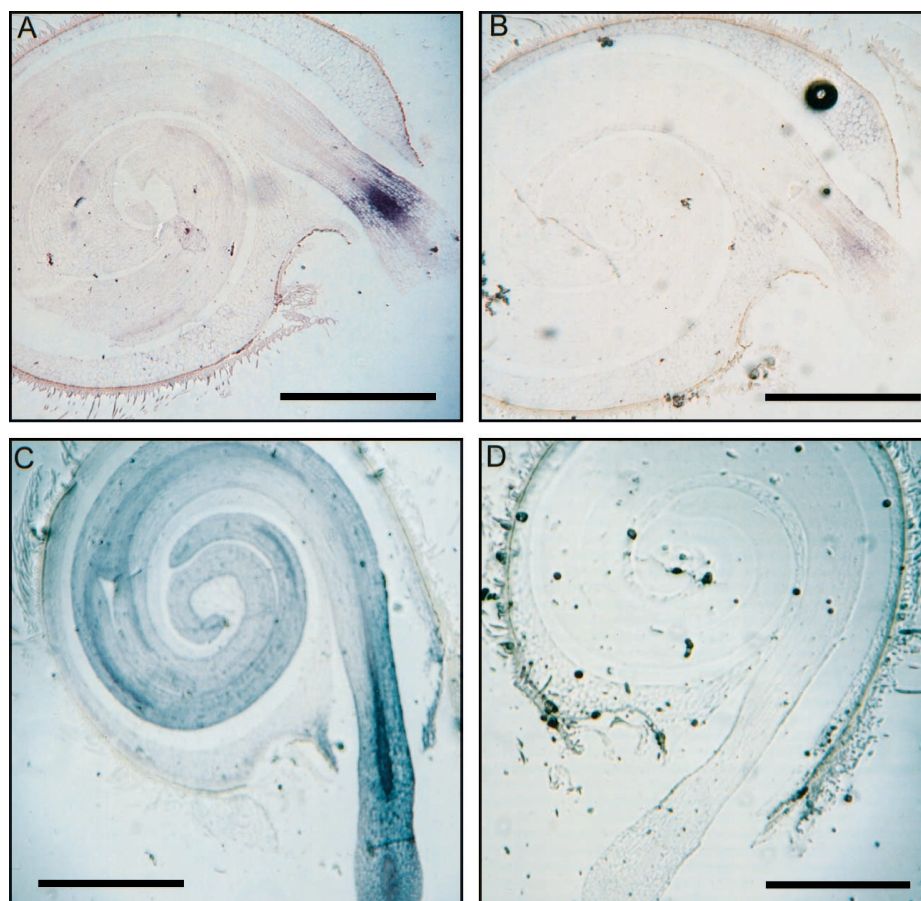
Figure 5. PG mRNA accumulates in the embryo and the endosperm cap. A, In situ analysis detected expression of *LeXPG1* mRNA in tomato embryos in a distinct band behind the radicle tip where it first emerged from the surrounding endosperm. Some expression was also detected in the remaining pieces of the endosperm cap. B, Section of the same seed as in A but probed with the sense strand RNA. C, At later stages of radicle protrusion, expression of *LeXPG1* occurred throughout the embryo, but was most prominent in the vascular trace around the point of egress from the endosperm. Expression remained high in the endosperm cap. D, Section of the same seed as in C but probed with the sense strand RNA. Message was not observed in situ prior to the completion of germination (data not shown). Bars = 1 mm.

PCR primers to conserved regions present in all plant PGs and previously used to clone PGs from melon fruit (Hadfield et al., 1998). Of these six amplicons, only one, obtained from tomato leaf mRNA, hybridized to mRNA from tomato seeds (data not shown). The mRNA was also present in flowers, roots, and stems, which enabled a partial-length cDNA to be recovered from a tomato anther library (Twell et al., 1989). The remaining 5′ sequence was obtained from 5′-RACE reactions using cDNA reverse-transcribed from tomato seed RNA. Analysis of the amino acid sequence derived from the full-length cDNA (accession no. AF138858) revealed that the catalytic domain identifying PGs (block BL00502B; CGPGHGISIGSLG, Bussink et al., 1991; Blocks Database Version 9.3, Henikoff and Henikoff, 1991) was conserved in the tomato seed PG (data not shown). The four regions of universal PG homology described by Kester et al. (1996) were also present. An apparent signal peptide with a cleavage site between amino acids 38 and 39 at the amino terminus of the protein included amino acids comprising a potential transmembrane domain (amino acids 17–48 via RAOARGOS; 18–34

via SOAP; PCGene Software, Intelligenetics, Mountain View, CA, data not shown).

Tomato Seed PG Is Encoded by a Single Gene

A PG cDNA hybridized at high stringency to genomic DNA cleaved with either *Bam*HI or *Hin*dIII identified two bands and a single band was detected in DNA cleaved by *Eco*RI, as predicted from the cDNA sequence (Fig. 3). We conclude that a single gene encodes the PG expressed in tomato seeds. Based on the unique nucleotide sequence and the conservation of catalytic domains characterizing PGs we have termed this gene *LeXPG1*.

Expression of *LeXPG1* Is Associated with Germination

LeXPG1 mRNA was undetectable in developing seeds obtained from fruits at the immature green, mature green, breaker, and ripe stages of development (Fig. 4A). *LeXPG1* mRNA began to accumulate within 24 h of imbibition and increased by 36 h of imbibition, just prior to radicle pro-
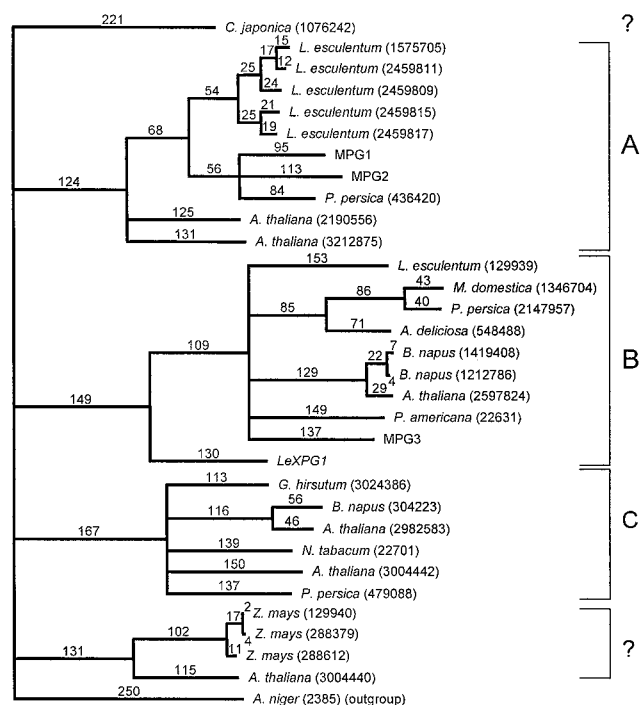
Figure 6. Homology of *LeXPG1* to other reported PGs. Based on alignment of the deduced amino acid sequences from 31 PGs reported in the literature, the tomato seed PG (*LeXPG1*) is most homologous to a PG from avocado, sharing 40% similarity at the amino acid level. *LeXPG1* does not segregate with PGs associated with pollen but is rather distantly related phylogenetically to PGs associated with abscission zones and fruit ripening that are predicted to contain a prosequence (Hadfield et al., 1998). The numbers above the lines on the tree represent theoretical divergence among PGs proportional to the number of amino acid substitutions.

trusion (Fig. 4A). Abundance of the mRNA increased further in germinated seeds compared with ungerminated seeds at 48 and 60 h (Fig. 4A). To determine the tissue location of expression, imbibed seeds were dissected into the endosperm cap, the radicle tip, and the rest of the seed. *LeXPG1* mRNA was present at 24 and 48 h in the endosperm caps and radicle tips, but was present in lesser amounts in the rest of the seed until after radicle emergence had occurred (Fig. 4B). In addition to the germinating seed, *LeXPG1* mRNA was also present in tomato stems, roots, and flowers but not in leaves (Fig. 4C). Because the cDNA was recovered from an anther cDNA library, at least part of the expression detected in the flowers is from the anthers, although expression in other floral organs is not ruled out.

In situ hybridization localized the expression of the *LeXPG1* gene in germinated seeds primarily to the embryo and the endosperm cap (Fig. 5, A and C). The expression in the embryo at 36 h was localized to a distinct band of tissue comprised of vascular, cortical, and epidermal cells and, while expression expanded by 48 h to include the whole of the embryo, it was most prominent in the vascular tissues in the region of radicle egress from the endosperm (Fig. 5C). Much weaker expression was also evident in the vascular tissue in the hypocotyl and cotyledons regardless of

the time after imbibition (Fig. 5, A and C). Relative to the sense-hybridized control (Fig. 5B), there was also some expression in the peripheral cells of the endosperm cap that remained following radicle protrusion at 36-h imbibition (Fig. 5, compare A and B). The expression in the cap was more evident in the 48-h imbibed seed (Fig. 5C) since there was more cap tissue present in the plane of the section.

Sequence Homology with Other Known PGs

Based on alignment of the deduced amino acid sequences from 30 plant PGs, *LeXPG1* is most highly homologous to a PG from avocado fruit (PID 166951), sharing 40% similarity at the amino acid level. *LeXPG1* shares 13 highly conserved Cys residues with almost all of the PGs compared in the alignment. However, *LeXPG1* does not cluster with PGs associated with angiosperm pollen (Pressey and Reger, 1989; Pressey, 1991). Instead, it is rather distantly related phylogenetically to PGs associated with abscission zones and fruit ripening that generally contain a prosequence (clade B in Hadfield et al., 1998). However, a PG isolated from Japanese cedar pollen also fell outside the clade defining PGs from angiosperm pollen (Fig. 6). In addition, *LeXPG1* has been cloned from and detected in tomato anthers and flowers, suggesting that it too might be present in pollen. The assignment of *LeXPG1* to clade B (Hadfield and Bennett, 1998; Hadfield et al., 1998) is in accordance with, but not due to, the presence of a predicted prosequence in *LeXPG1*.

DISCUSSION

This is the first report, to our knowledge, of *exo*-PG enzyme activity in seeds. The assignment of the tomato seed PG as an *exo*-enzyme is based on the absence of endo-activity in viscometric and gel diffusion assays and the exclusive detection of calcium-dependent activity in reducing sugar and HPLC assays. In addition, the products of the action of the tomato seed enzyme on oligomeric PGA substrates resolved by HPLC are consistent with *exo*-activity but not with endo-activity (Fig. 2). Finally, *exo*-PGs are known to require calcium for activity, as did the enzyme from tomato seeds (Pressey and Reger, 1989; Pressey, 1991). Whereas *LeXPG1* was the only PG mRNA detected in tomato seeds, it remains to be confirmed that it codes for the *exo*-enzyme detected by activity assays. There was, however, a correlation between calcium-dependent *exo*-PG activity and the accumulation of *LeXPG1* mRNA (Figs. 1 and 4, A and B).

An *exo*-PG present in liverwort cell cultures increased in parallel with cell growth until the cessation of the exponential growth phase, when the activity declined (Konno et al., 1983), and an *exo*-PG extracted from cultured carrot cells degrades pectic polymers from these cultures (Konno et al., 1984). Based on this developmental correlation, Konno et al. (1983) suggested that *exo*-PGs act coordinately with other cell wall-modifying enzymes to promote remodeling and/or loosening of the plant cell wall, permitting growth. The expression of *LeXPG1* in a conspicuous band of cells in the radicle tip and later throughout the expand-

ing embryo, is consistent with a role for PGs in potentiating the cell elongation necessary for growth. The detection of *LeXPG1* in stems and roots undergoing secondary thickening and its presence in young expanding (initial PCR amplicon) but not mature expanded leaves (Fig. 4C) also correlates well with a role in mediating cell elongation. Pectin is deposited in a highly esterified form abundant in cell walls prior to the onset of elongation (Moore and Staehelin, 1988; Carpita and Gibeaut, 1993; Catesson, 1994). Pectin methylesterase activity is necessary to modify this esterified pectin to permit PG-mediated hydrolysis (Jona, 1989; Konno et al., 1989). PME is abundant from the earliest stage of tomato seed germination in both the endosperm cap and radicle tip (Downie et al., 1998). In protruded radicles, PME activity occurs in the tip of the radicle and in a distinct band of cells (Downie et al., 1998) reminiscent of the PG gene expression observed here. It is possible that this localized region of expression is associated with the transition between root and hypocotyl tissue.

The prominent expression of *LeXPG1* in the vascular tissue of the radicle tip and its less prominent expression in the vascular tissue of the rest of the embryo (Fig. 5, A and C) implies a role for *LeXPG1* in vascular tissue differentiation. Differentiation of the vasculature is one of the few changes discernable microscopically during seed germination prior to radicle protrusion (Sundås et al., 1992). The localization of a PG to the vascular cylinder in maize anther filaments (Dubald et al., 1993) may indicate a role for PGs in vascular tissue differentiation. Esterified pectin has been shown to be rapidly de-esterified in the stele of growing roots, particularly the vessel elements (Dolan and Roberts, 1995), which would potentiate hydrolysis by PGs. Not surprisingly, differentiation of *Zinnia* cell cultures into tracheary elements is accompanied by a marked decrease in pectin (Ingold et al., 1988) potentially brought about by PG activity.

The expression of *LeXPG1* in the endosperm cap is correlated with the well-characterized weakening of this region necessary for radicle protrusion. Our understanding of the biochemical action of PGs on the plant cell wall is limited (Hadfield and Bennett, 1998), so a role for a single PG in both endosperm weakening and embryo growth (and/or vascular tissue differentiation) is plausible.

Alternatively, the PG in the endosperm cap could function as part of a plant defense mechanism that is upregulated prior to radicle protrusion through the endosperm. Some PGs are thought to play a role in trimming large pectic oligomers cleaved by exogenous (pathogen produced) PGs to sizes effective in eliciting plant protective responses and/or in the removal of these signals, enabling the plant to stand down upon the cessation of pathogen attack (Aldington and Fry, 1993; García-Romera and Fry, 1995). PGs are capable of stimulating ethylene evolution (Baldwin and Pressey, 1990) leading to pathogenesis-related protein induction (Boller, 1988). Conceivably, *LeXPG1* transcription and protein accumulation in the endosperm cap may be initiated in response to imminent endosperm rupture by the radicle, which would expose the endosperm to the external biotic environment. Pathogenesis-related proteins, such as a vacuolar targeted, class I β-1,3-

glucanase, have been shown to accumulate exclusively in the cells of the endosperm cap of germinating tobacco seeds (Vögeli-Lange et al., 1994; Leubner-Metzger et al., 1996). In conjunction with endosperm weakening in tomato, both β-1,3-glucanase and chitinase are expressed in the endosperm caps of tomato seeds prior to the completion of germination (C.-T. Wu, G. Leubner-Metzger, F. Meins, Jr., and K.J. Bradford, unpublished results). Whether the PG in tomato seeds is involved in cell wall disassembly or functions in a manner to increase resistance to pathogens remains unresolved.

Phylogenetic analysis of *LeXPG1* puts it into a clade of PGs associated with abscission zones and fruit softening, although the clade distinctions are by no means absolute. Hence, a PG from cedar pollen, like the PG from seeds and anthers, falls outside the clade containing most pollen PGs (Fig. 6). In addition, a Cys residue thought to be conserved in pollen PGs (Kester et al., 1996) is present in neither *LeXPG1* (amino acid 306) nor in the PG from cedar pollen. Conversely, a PG cloned from melon fruit (MPG2) does contain this Cys (Hadfield et al., 1998). The presence of a potential prosequence in *LeXPG1* is also consistent with its placement with clade B, as other PGs identified with clade C do not contain prosequences (Hadfield and Bennett, 1998).

The identification of *LeXPG1* in germinating tomato seeds provides another candidate for enzymes that may be involved in endosperm weakening and/or embryo expansion related to the mechanism of radicle protrusion. Hydrolysis of PG-susceptible, acidic pectin in the middle lamella of the walls of endosperm cap cells would likely reduce cell-to-cell cohesion and facilitate penetration by the radicle. Alternatively, the PG in the cap could be performing a purely protective function. The localization of the message for the enzyme in the vascular tissues corroborates previous evidence of PG expression in the vascular tissue of maize (Dubald et al., 1993) and implicates PG activity in vascular tissue differentiation (Ingold et al., 1988). The enzyme could also potentially be involved in cell wall modification permitting turgor-driven elongation of the radicle culminating in radicle protrusion through the endosperm.

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