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Leucine Aminopeptidase RNAs, Proteins, and Activities Increase in Response to Water Deficit, Salinity, and the Wound Signals Systemin, Methyl Jasmonate, and Abscisic Acid[1]

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LapA **RNAs, proteins, and activities increased in response to systemin, methyl jasmonate, abscisic acid (ABA), ethylene, water deficit, and salinity in tomato (*Lycopersicon esculentum*). Salicylic acid inhibited wound-induced increases of *LapA* RNAs. Experiments using the ABA-deficient *flacca* mutant indicated that ABA was essential for wound and systemin induction of *LapA,* and ABA and systemin acted synergistically to induce *LapA* gene expression. In contrast, *pin2* (proteinase inhibitor 2) was not dependent on exogenous ABA. Whereas both *LapA* and *le4* (*L. esculentum* dehydrin) were up-regulated by increases in ABA, salinity, and water deficit, only *LapA* was regulated by octadecanoid pathway signals. Comparison of *LapA* expression with that of the *PR-1* (pathogenesis-related 1) and *GluB* (basic β-1,3-glucanase) genes indicated that these *PR* protein genes were modulated by a systemin-independent jasmonic acid-signaling pathway. These studies showed that at least four signaling pathways were utilized during tomato wound and defense responses. Analysis of the expression of a *LapA1:GUS* gene in transgenic plants indicated that the *LapA1* promoter was active during floral and fruit development and was used during vegetative growth only in response to wounding, *Pseudomonas syringae* pv *tomato* infection, or wound signals. This comprehensive understanding of the regulation of *LapA* genes indicated that this regulatory program is distinct from the wound-induced *pin2*, ABA-responsive *le4*, and *PR* protein genes.**

Plants respond quickly to pathogen and herbivore attacks by activating wound- and defense-response genes (Bowles, 1990; Dixon et al., 1994; Yang et al., 1996). Tomato (*Lycopersicon esculentum*) wound/defense-response genes are often expressed both locally and systemically (Enkerli et al., 1993; Pautot et al., 1993; Bergey et al., 1996). The signals that mediate systemic responses must be transmitted rapidly throughout the plant and may involve cell-to-cell signaling. Putative systemic signals include ethylene (Ecker and Davis, 1987; O'Donnell et al., 1996), SA (Malamy et al., 1990), ABA (Peña-Cortés et al., 1989), JA (Farmer and Ryan, 1990), and systemin (Pearce et al., 1991), as well as electrical and hydraulic signals (Wildon et al., 1992; Malone et al., 1994; Herde et al., 1996; Stankovic and Davies, 1998).

Multiple signal transduction pathways interact to activate or suppress wound- and defense-response genes in the Solanaceae. Wound-response genes, such as the *pin* (proteinase inhibitor) genes are activated by systemin and octadecanoid pathway products such as JA (Farmer and Ryan, 1990, 1992; Pearce et al., 1991; Peña-Cortés et al., 1995). Systemin acts locally and systemically to induce synthesis of JA, which induces the expression of wound-response genes (Narváez-Vásquez et al., 1995). ABA has also been reported as a local and systemic signal for the induction of *pin* genes in potato and tomato (Peña-Cortés et al., 1989, 1991, 1995). However, the role of ABA in the induction of wound-response genes in tomato has remained controversial (Schaller and Ryan, 1995; Birkenmeier and Ryan, 1998).

Several tomato wound-response genes are negatively regulated by SA, which acts at multiple steps in the octadecanoid signaling pathway (Doherty et al., 1988; Li et al., 1992; Peña-Cortés et al., 1993; Doares et al., 1995; O'Donnell et al., 1996). This contrasts to SA induction of the *PR* (pathogenesis-related) protein genes (van Kan et al., 1995). The SA and octadecanoid signaling pathways are reciprocally regulated by a wound mitogen-activated protein kinase (Seo et al., 1995) and a small GTP-binding protein (Sano et al., 1994). This cross-talk may aid in separating early responses to wounding that accompany pathogen or pest attack from long-term responses, such as *PR* gene expression and the development of SAR. The interactions of ethylene with the wound- and SA-signaling pathways are not completely understood. Ethylene is important for the development of necrotic symptoms that accompany

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Abbreviations: JA, jasmonic acid; MeJA, methyl jasmonate; SA, salicylic acid: SAR, systemic acquired resistance.

pathogen invasion, but is not essential for the development of SAR (Bent et al., 1992; Lawton et al., 1994; Lund et al., 1998). Several *PR* transcripts accumulate in response to ethylene or ethephon treatments (Ecker and Davis, 1987; Raz and Fluhr, 1993; van Kan et al., 1995), while ethylene treatments do not induce some wound-response genes (Ryan, 1974; Kernan and Thornberg, 1989). Recent data have suggested that ethylene and JA interact to induce wound-response genes (Xu et al., 1994; O'Donnell et al., 1996).

In addition to responding to wound and defense signals, the expression of each wound- and defense-response gene is modulated during development. While some defense-response genes are silent throughout all of vegetative development and are solely induced in response to stress, other defense- and wound-response genes are expressed in specific vegetative or reproductive organs (Peña-Cortés et al., 1991; Titarenko et al., 1997). Many wound- and defense-response genes are expressed only in floral buds (Peña-Cortés et al., 1991) or in a subset of the floral organs (Lotan et al., 1989; Cote et al., 1991; Uknes et al., 1993; Constabel and Brisson, 1995), while other genes are expressed in all mature floral organs (Peña-Cortés et al., 1991). Finally, the developmental programming may be distinct in different species. For example, the *pin2* genes of potato are only expressed in tubers and developing floral buds, while the *pin2* genes of tomato are expressed in all mature floral organs (Peña-Cortés et al., 1991).

In tomato, *LapA* (Leu aminopeptidase) transcripts, proteins, and activities increase locally and systemically in response to wounding (Pautot et al., 1993; Gu et al., 1996b). Two tomato genes, *LapA1* and *LapA2*, encode the 55-kD subunits of this exopeptidase (Gu et al., 1996a). The *Lap* genes of potato and Arabidopsis are regulated differently than the tomato *LapA* genes, since the Arabidopsis *Lap* gene is constitutively expressed (Bartling and Nosek, 1994) and potato *Lap* RNAs do not accumulate systemically after wounding (Hildmann et al., 1992), nor are they detected in response to pathogens (Herbers et al., 1994).

Given the fact that *LapA* transcripts and proteins are abundant after wounding, pathogen invasion, and insect infestation, LAP-A may play an important role in the tomato defense response (Pautot et al., 1993). For this reason, it was important to develop a comprehensive understanding of *LapA* expression (at the RNA, protein, and activity level) in response to wound/defense-response signals (including ethylene, SA, JA, ABA, and systemin) and during abiotic stress. Comparisons of *LapA* gene expression patterns relative to patterns of expression for the wound-response gene *pin2*, the ABA-response gene *le4*, and three *PR* protein genes (*PR-1*, *PR-4*, and *GluB* [basic β-1,3-glucanase]) in ABA-deficient or ABA-producing lines demonstrated that each gene responded to wound and defense signals in a distinct manner. These data indicated that at least four signaling pathways are required to modulate wound/defense gene expression in tomato plants. Finally, to understand the organ specificity of *LapA* responses to wound signals, transgenic tomato and tobacco (*Nicotiana tabacum*) plants expressing a chimeric *LapA1:GUS* gene were analyzed.

MATERIALS AND METHODS

Plant Growth, Tissue Harvest, and Storage

Tomato (*Lycopersicon esculentum* cv Peto 238R, cv Ailsa Craig *flacca*, and cv Ailsa Craig) plants were grown in soil (University of California mix III) in growth chambers with 16-h (30°C)/8-h (20°C) light/dark cycles. Plants were watered daily and supplemented with 14:14:14 fertilizer (Osmocote, Scotts-Sierra, Maysville, OH). Immediately after treatments, leaves were excised, placed directly into liquid nitrogen, and stored at −80°C until use.

Treatments with Wound- and Defense-Response Molecules

Shoots from 3- to 4-week-old Peto 238R tomato plants were excised 5 cm above the soil for the ABA, MeJA, and ethylene treatments. Shoots from 3-week-old Peto 238R plants were excised below the third leaf from the plant shoot apex for the systemin and SA treatments. The 24-h 10 μM MeJA treatment and control have been described previously (Gu et al., 1996a). For ABA treatments, shoots were placed in flasks filled with 100 μM ABA (pH 6.0) or water (control) for 24 h. For ethylene treatments, excised shoots were placed in flasks filled with water and incubated in airtight glass desiccators containing a ripe banana and apple or without fruit (control) for 24 h. Ethylene levels typically rose to 29 ppm (D.P. Puthoff and L.L. Walling, unpublished results).

The systemin treatment was a modification of that of Pearce et al. (1993). Excised shoots were placed in a microfuge tubes with 90 μL of 15 mM sodium phosphate buffer (pH 6.5) or 90 μL of 15 mM sodium phosphate buffer with 1 pmol of systemin. After 10 min, shoots were transferred to a flask filled with water for 12 h. Systemin was kindly provided by Dr. C.A. Ryan (Washington State University, Pullman). For SA treatments, shoots were placed in flasks filled with distilled water (control) or 0.1 to 0.5 mM SA (Sigma) for 24 h. The maximum concentration of SA tolerated by tomato shoots without inducing physical damage was 0.5 mM.

Five- to six-week-old *flacca* plants were excised below the third leaf from the shoot apex. Four-week-old Ailsa Craig plants were at a similar stage in development. Shoots were treated with systemin (above) or wounded (Pautot et al., 1991), and were subsequently placed in water with or without 100 μM ABA and incubated in closed desiccators. Systemin-treated and wounded leaves were collected 12 and 24 h later, respectively.

Water Deficit and Salinity Treatments

After 3 weeks of growth (described above), water was withheld from Peto 238R plants and leaves were harvested at 4.5 d (at the initial signs of wilting), 5 d, and 5.5 d later. Control plants were watered once per day and leaves were harvested at the same time as the plants experiencing 5.5 d of water deficit. For salinity treatments, Peto 238R plants were watered with 300 mL of 300 mM NaCl, 400 mM NaCl, or water (control) for 3 d, and leaves were harvested.

Construction of a *LapA1:GUS* Fusion Gene

A 970-bp *Hin*dIII/*Dde*I fragment from the λLapA1 genomic subclone pLapA1-EH (W.S. Chao, V. Pautot, F.M. Holzer, and L.L. Walling, unpublished data), was end-filled using Klenow enzyme and cloned into the filled-in *Bam*HI site of pBI101 (CLONTECH). Site-directed mutagenesis was used to remove residual vector sequences and restore the integrity of the *LapA1* 5′-UTR (Chao, 1996). pLapA1:GUS was transformed into *Agrobacterium tumefaciens* (LBA4404 or EHA105), and transformants were confirmed by minilysates (Gelvin and Schilperoort, 1988; Birnboim and Doly, 1979).

Tobacco and Tomato Transformation

The tomato lines UC82b (Sunseeds Genetics, Hollister, CA) and VF36 (provided by Dr. S. McCormick, U.S. Department of Agriculture/Agricultural Research Service, Albany, CA) plants and tobacco (*Nicotiana tabacum* cv Xanthi) plants were used in the transformation experiments. *LapA1:GUS* transgenic plants were regenerated from tomato cotyledons and tobacco leaf discs using a modification of protocols described by Fillatti et al. (1987) and McCormick (1991). Details were described in Chao (1996). Fifteen independent tomato lines and 12 independent tobacco lines were characterized. DNA blots with *Hin*dIII/*Eco*RI-digested genomic DNAs (10 μg/lane) from T_0 plants and reconstruction lanes with pLapA1:GUS were used to determine transgene copy number (Walling et al., 1988). The expression of the *LapA1:GUS* gene in T_1 and T_0 plants was confirmed by wounding of cotyledon segments and GUS histochemical staining. Transgenic tomato and tobacco plants expressing 35S:GUS (pBI121, CLONTECH) were also made.

GUS Activity Assays

The expression of the chimeric *LapA1:GUS* gene was monitored using histochemical and fluorometric assays for GUS activity (Jefferson, 1987). To reduce endogenous GUS activity, 20% methanol (v/v) was added to the assay buffers (Kosugi et al., 1990). Fluorescence was measured using a mini fluorometer (TKO 100, Hoefer Scientific Instruments, San Francisco). Protein concentrations were determined using a bicinchoninic acid protein assay reagent (Pierce). To reduce interference caused by β-mercaptoethanol, samples were preincubated with an equal volume of 0.1 M iodoacetamide in 0.1 mM Tris-HCl (pH 8) at 37°C for 20 min (Hill and Straka, 1988).

Wounding, MeJA Treatment, and Infection of *LapA1:GUS* Plants

T_1 (*LapA1:GUS*) and UC82b tomato plants with six to eight leaves and T_1 (*LapA1:GUS*) and Xanthi tobacco plants with five to seven leaves were used. Leaves of four to six individual plants per transgenic line were wounded (Pautot et al., 1991) or served as controls. Leaves were harvested into liquid nitrogen 24 h later. Intact 7- to 10-d-old seedlings were treated with MeJA by submerging roots in 10 μM MeJA/0.002% ethanol or 0.002% ethanol (control).

T_1 *LapA1:GUS* and UC82b plants with six to eight leaves were used for the infection studies. Three to four upper leaves were used. Half of the leaflets on a leaf served as the mock-infected control and were gently swabbed with water using cotton-tipped applicators. The remaining leaflets were inoculated with a *Pseudomonas syringae* pv *tomato* suspension (3×10^8 cfu/mL) using cotton swabs (Pautot et al., 1991). Leaflets were harvested 24 h later.

RNA Blot Analyses

RNA blots and washes were performed as described previously (Pautot et al., 1991). Blots were exposed to film (Hyper-MP, Amersham) at −80°C with an intensifying screen (DuPont) for 24 h unless indicated otherwise. Autoradiographic signals were quantitated using a phosphor imager (Molecular Dynamics). Probes were labeled using [α-^{32}P]dCTP by nick translation. Transcript sizes were determined by running an RNA ladder (GIBCO-BRL) in parallel lanes. The pLe4 cDNA was described previously (Cohen et al., 1991; Kahn et al., 1993). The *GluB*, *PR-1*, and *PR-4* cDNA clones from tomato have been described previously (van Kan et al., 1992, 1995), and were kindly provided by Dr. P.J.G.M. de Wit (Wageningen Agricultural University, Wageningen, Netherlands). The tomato *pin2* clone pT2-47 (Graham et al., 1985) and the *LapA* cDNA clone pDR57 (Pautot et al., 1993) have also been described previously.

Total Protein Extraction, Fractionation, and Immunoblot Analyses

Total leaf proteins were extracted and fractionated by two-dimensional PAGE as described by Wang et al. (1992). Electro-transfer and immunoblot procedures were described in Gu et al. (1996b). A 1:500 dilution of the LAP-A polyclonal antiserum and the preimmune serum were used (Gu et al., 1996b).

Aminopeptidase Activity Assay

Native proteins were extracted from leaves of treated and control plants (Gu et al., 1996b). Protein concentrations were determined by a modified Bradford method (Ramagli and Rodriguez, 1985). The assays were performed in triplicate in 96-well microplates with 2 μg of protein and 250 μL of assay solution (1 mM L-Leu-*p*-nitroanilide [Sigma], 50 mM Tris-HCl, pH 8.0, and 0.5 mM $MnCl_2$). After 30 min, the amount of *p*-nitroaniline generated was measured spectrophotometrically at A_{405} using a microplate reader (E-Max, Molecular Devices, Menlo Park, CA).

In Situ Hybridizations

Floral buds (10-mm) were harvested, fixed, and imbedded in methacrylate as described by Kronenberger et al. (1993). Five-millimeter transverse sections of tomato buds were made. Sections were hybridized to a digoxigenin-labeled antisense or sense *LapA1* RNAs. Digoxigenin-

labeled RNAs were synthesized using T3 or T7 RNA polymerase (GIBCO-BRL) and pBS-LapA1 according to the manufacturer's instructions (Boehringer Mannheim). pBS-LapA1 has a 1.6-kb *Eco*RI/*Xba*I fragment from pDR57 inserted into the *Eco*RI/*Xba*I sites of pBS-KS+ (Pautot et al., 1993).

RESULTS

LapA Was Induced by Wound Signals: Systemin, ABA, MeJA, and Ethylene

To understand the impact of wound signals on *LapA* RNA levels, tomato plants were treated with MeJA, systemin, ABA, or ethylene. RNA blots were hybridized with probes for *LapA1* and genes that respond to one or more of these signals (*le4*, *PR-1*, *PR-4*, and *GluB*). Le4 encodes a dehydrin-like protein and is induced by exogenous ABA and water deficit (Cohen et al., 1991; Kahn et al., 1993). *PR-1*, *PR-4*, and *GluB* RNAs and proteins accumulate in response to SA or ethephon (an ethylene-releasing compound) (Christ and Mösinger, 1989; van Kan et al., 1995; Tonero et al., 1997). *PR-1* and *PR-4* encode extracellular proteins. PR-1 has antifungal activity but its mechanism of action is not known (Niderman et al., 1995). PR-4 is similar to Win and hevein proteins; the role of PR-4 in defense has yet to be elucidated (Linthorst et al., 1991). *GluB* encodes an intracellular, basic β-1,3-glucanase whose activity can hydrolyze pathogen cell walls (van Kan et al., 1992, 1995).

LapA was strongly induced by MeJA and systemin (Fig. 1A). A 57-fold increase in *LapA* transcripts occurred in leaves after 24 h of exposure to 10 μM MeJA. *LapA* RNAs were 2.5-fold more abundant in systemin-treated plants than in MeJA-treated plants. Treatment of shoots with 100 μM ABA increased *LapA* RNA levels 2-fold, which is similar to the increase measured for the well-characterized ABA- and water-deficit-response gene *le4* (Cohen et al., 1991). This may be a minimal estimate of *LapA* induction in response to ABA, since larger increases in *le4* transcripts were observed in an excised leaf assay (Cohen et al., 1991). In contrast to *LapA*, *le4* RNA levels did not increase in response to systemin, MeJA, or ethylene treatments.

Relative to the control, there was a small increase in *LapA* RNAs (2-fold) in plants exposed to ethylene (Fig. 1A). After ethylene treatment, *PR-1*, *PR-4*, and *GluB* transcripts increased 14-, 16-, and 3-fold, respectively. The response of the tomato *PR-1*, *PR-4*, and *GluB* genes to pathogens, ethylene, and SA is well established (Christ and Mösinger, 1989; van Kan et al., 1995; Tonero et al., 1997), but less is known about their responses to wound signals. *PR-1*, *PR-4*, and *GluB* RNAs were unchanged after ABA or systemin treatments (Fig. 1A). While *PR-4* transcripts did not accumulate in response to MeJA, MeJA caused both *GluB* and *PR-1* RNAs to accumulate relative to the control plants. *GluB* and *PR-1* RNA levels were elevated in the MeJA and ethylene controls relative to controls from other treatments (i.e. ABA, systemin, or SA). This may be due to the fact that the MeJA and ethylene treatments were done in a closed environment and a volatile signal may have accumulated to induce *GluB* and *PR-1*. It is clear that *LapA*, *le4*, and *PR-4* transcript levels were not modulated by this additional signal(s).

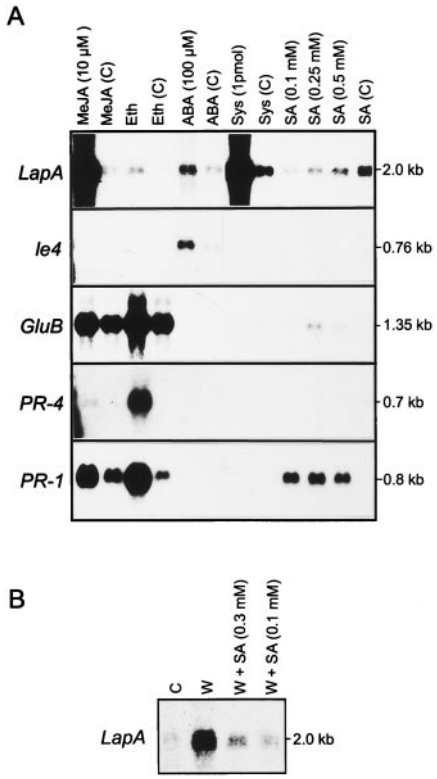

Figure 1. RNA blot analyses of plants treated with wound- and defense-response signal molecules. A, Tomato plants treated with 10 μM MeJA, 29 ppm ethylene (Eth), 100 μM ABA, 1.0 pmol of systemin (Sys), or 0.1, 0.25, or 0.5 mM SA. For each treatment, the corresponding control is shown as a C in parentheses. B, Tomato plants were wounded and incubated in the absence (lane W) or presence of 0.1 mM or 0.3 mM SA (lanes W + SA). Total RNAs were extracted from treated and healthy control (lane C) leaves. The RNA blots were hybridized with ^{32}P-labeled *LapA*, *le4*, *GluB*, *PR-4*, or *PR-1* probes. The RNA sizes are indicated in kb. Data in A and B are from representative experiments. Photographs presented are optimized for visualization of weak autoradiographic signals. Hybridization signals were quantitated using a phosphor imager.

During the course of these studies, we noted that the position of the incision and the age of the seedling used in the excised shoot assay was important (see "Materials and Methods"). When 3-week-old plants were used in this assay (systemin and SA treatments), *LapA* RNAs were detected in controls. This is in contrast to the extremely low to undetectable levels of *LapA* RNAs in leaves from excised 4-week-old shoots (MeJA, ethylene, and ABA treatments) or from intact plants (Pautot et al., 1993). It is clear that the developmental state must influence the tomato response to shoot excision. Several other studies have indicated that plant age may influence wound signaling (Wolfson and Murdock, 1990; Alarcon and Malone, 1995).

LapA RNA Levels Decreased in Response to Exogenous SA

Using the excised shoot assay, *PR-4* transcripts were not detected after any of the SA treatments (Fig. 1A). In con-

trast, *PR-1* and *GluB* transcripts increased after 0.1 and 0.25 mM SA treatments, respectively. The steady-state levels of *PR-1* and *GluB* RNAs were distinct, suggesting differences in either transcriptional or posttranscriptional regulation. SA inhibited the accumulation of *LapA* transcripts, since control leaves had higher levels of *LapA* transcripts than SA-treated leaves (Fig. 1A). When wounding was followed by 0.1 or 0.3 mM SA treatments, *LapA* transcript levels were significantly reduced relative to wounded plants (Fig. 1B).

ABA Was Required for Wound-Induced Activation of *LapA*

To determine if endogenous ABA was required for wound and systemin induction of *LapA*, the expression of *LapA* was examined in the ABA-deficient *flacca* mutant and the ABA-proficient Ailsa Craig lines. Shoots were treated with systemin or were wounded, and subsequently incubated in water or 100 μM ABA (Fig. 2A). High levels of *LapA* and *pin2* transcripts and low levels of *le4* RNAs were detected after wounding in cv Ailsa Craig. In *flacca* plants, the *le4* and *LapA* transcripts were undetectable in healthy or wounded leaves. Low levels of *pin2* transcripts were consistently detected in healthy *flacca* leaves and, after wounding, the levels of *pin2* RNAs increased 2-fold (Fig. 2A).

Treatment of *flacca* shoots with 100 μM ABA caused *LapA* RNAs to rise 5-fold (Fig. 2A). When *flacca* was wounded and ABA treated, *LapA* RNAs increased 7-fold. ABA supplementation of healthy or wounded *flacca* plants caused *pin2* RNAs to increase only 2- to 3-fold. *Le4* RNA levels also increased when healthy *flacca* leaves were treated with ABA; wounding did not further increase *le4* transcript abundance.

To examine if ABA has a role in systemin signal transduction, *flacca* shoots were treated with 15 mM phosphate buffer (control), systemin, or systemin plus 100 μM ABA (Fig. 2A). In buffer-treated *flacca* shoots, *LapA* or *le4* RNAs were undetectable and low levels of *pin2* RNAs were observed. After systemin treatment, *LapA* RNA increased 2-fold in *flacca* leaves. In contrast, a 50-fold induction of *LapA* transcripts was detected when *flacca* shoots were treated with both systemin and ABA. These levels were comparable to *LapA* levels in wounded cv Ailsa Craig leaves, and suggest that ABA and systemin act synergistically. These data indicated that ABA was critical for maximal accumulation of *LapA* transcripts in response to systemin and that *pin2* was regulated in a different manner. *pin2* RNAs increased 9-fold in *flacca* leaves in response to systemin and, when applied simultaneously, systemin and ABA increased *pin2* transcripts 18-fold. *Le4* transcripts did not increase in response to systemin (Figs. 1A and 2A). The level of *le4* RNAs in leaves of *flacca* treated with both systemin and ABA was actually lower than that observed with ABA alone.

LapA Is Induced during Water Deficit and Salinity Stress

ABA is not only an important signal in the wound response of tomato, but it is also an important component in abiotic stresses such as water deficit and salinity (Bray, 1993; Chandler and Robertson, 1994). Therefore, we measured changes in *LapA* RNA levels during water deficit. *LapA* RNA levels increased during water deficit and reached maximal levels in plants stressed for 5 d (Fig. 2B); *Le4* served as positive control (Cohen et al., 1991; Kahn et al., 1993). *Le4* transcripts were present at higher levels than *LapA* RNAs and accumulated throughout the entire stress period. By d 5, *le4* RNA levels had increased 53-fold.

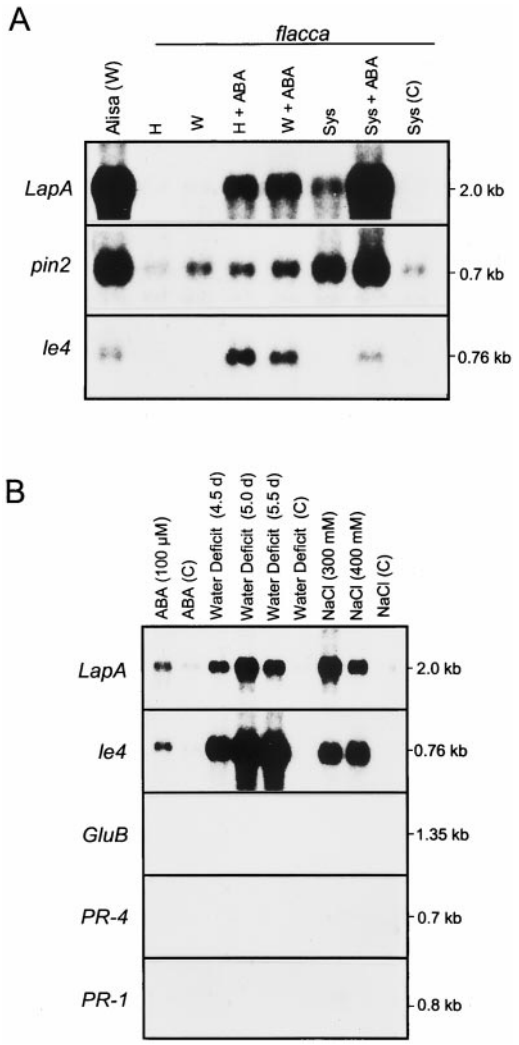

Figure 2. RNA blot analysis of *LapA* gene expression in ABA-deficient (*flacca*) and control (Ailsa Craig) plants and in response to salinity and water deficit. A, Plants were mechanically wounded (W) or treated with 1 pmol of systemin (Sys), and excised shoots were subsequently incubated in water with or without 100 μM ABA. Ailsa Craig leaves were mechanically wounded (Ailsa, W). Shoots of healthy *flacca* plants were incubated in water (H) or ABA (H + ABA). Shoots of wounded *flacca* plants were incubated in water (W) or ABA (W + ABA). *Flacca* shoots were treated with systemin (Sys), systemin + ABA (Sys +ABA), or incubated with 15 mM phosphate buffer (Sys [C]). The blots were exposed to film for 48 h. B, Tomato plants were treated with 100 μM ABA, 300 mM NaCl, or 400 mM NaCl, or were not watered (water deficit) for 4.5, 5.0, or 5.5 d. Total RNA was extracted from treated and control (C) leaves. The RNA blots were hybridized with ^{32}P-labeled *LapA*, *le4*, *GluB*, *PR-4*, or *PR-1* probes. The autoradiographic signals of each band were quantitated using a phosphor imager.

To determine if *LapA* RNAs accumulated in response to salinity, tomato plants were watered with 300 or 400 mM NaCl for 3 d. *LapA* RNAs increased 4- to 6-fold in response to salinity treatments (Fig. 2B), whereas a more dramatic increase (22-fold) in *le4* RNA levels was observed. None of the *PR* gene transcripts accumulated in response to water deficit or salinity stress, which is consistent with the observation that exogenous ABA treatments did not induce *PR-1*, *PR-4*, or *GluB* gene expression (Fig. 1A).

LAP-A Proteins and Activities Were Elevated after Stress Treatments

To determine if there was a coordinate induction of *LapA* RNAs and proteins, total proteins were extracted from leaves that were subjected to water deficit or treated with MeJA, systemin, ABA, or NaCl. Immunoblots showed that four classes of LAP-related proteins and one class of non-LAP protein were resolved (Gu et al., 1996b). The 90-kD proteins were not related to LAP, since they were recognized by preimmune serum (Gu et al., 1996b). The 66- and 77-kD LAP-like polypeptides and 55-kD LAP proteins with neutral pIs (LAP-N) were detected in all control and treated tomato leaf samples (Fig. 3). Only the 55-kD LAP-A polypeptides (with acidic pIs) were induced after stress treatments (Fig. 3, B, D, F, H, and J). LAP-A proteins were most abundant in MeJA- and systemin-treated leaves (Fig. 3, B and J), which is consistent with RNA blot analyses (Figs. 1A and 2B). While the levels of *LapA* RNA varied in the control plants (Figs. 1A and 2B), the LAP-A protein levels varied only slightly (Fig. 3, A, C, E, G, and I).

To determine if LAP-A protein levels is correlated with LAP activities, aminopeptidase activity assays were performed. Relative aminopeptidase activities increased in leaves of MeJA-, systemin-, ABA-, water-deficit-, and NaCl-treated plants (Fig. 4). The large increases in aminopeptidase activities noted in the MeJA- and systemin-treated samples paralleled the large increases in *LapA* mRNAs and proteins (Fig. 1A). Since the activity assays were performed on total soluble leaf protein extracts, a direct correlation between the amount of LAP-A proteins and LAP activities could not be made. The observed changes in aminopeptidase activities may have been due to increases in LAP-A and/or changes in the activities or levels of additional tomato leaf aminopeptidases (Gu et al., 1996b; Walling and Gu, 1996).

There was substantial variation in the aminopeptidase levels detected in the five controls for these studies. This may have been due to the fact that the treatment regimes varied. Four-week-old plants in soil were used for the water deficit and salinity studies and the aminopeptidase activities were similar in their controls. Three-week-old (systemin)- or 4-week-old (ABA and MeJA) excised shoots were used for the other treatments. The impact of seedling age on the *LapA* RNA levels detected in controls was noted (Fig. 1A). Finally, although the ages of the seedlings in the ABA and the MeJA treatments were the same, the ABA- and MeJA-treated plants were incubated in open and closed environments, respectively.

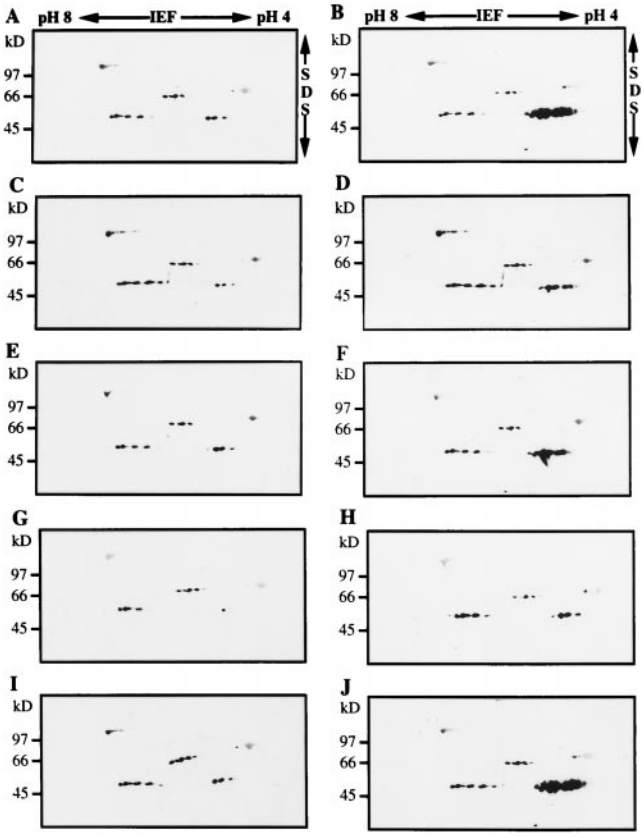

Figure 3. Immunoblots of proteins that accumulated in response to wound signals and abiotic stress. Total proteins (80 μg) were fractionated by two-dimensional PAGE. The gels were electroblotted onto nitrocellulose and the blots were incubated with a 1:500 dilution of the LAP-A polyclonal antiserum. The pH range for IEF and molecular mass markers (in kD) are indicated. The 55-kD LAP-A proteins had a pI range of 5.6 to 5.9 (Gu et al., 1996b). A, Control plants for MeJA treatment. B, Plants treated with 10 μM MeJA for 12 h. C, Control plants for ABA treatment. D, Plants treated with 100 μM ABA for 12 h. E, Control plants for 5-d water deficit treatment. F, Plants 5 d after water was withheld. G, Control plants for 300 mM NaCl treatment. H, Plants 3 d after 300 mM NaCl treatment. I, Control plants for systemin treatment. J, Plants 12 h after treatment with 1 pmol of systemin.

The *LapA1* Promoter Was Activated by Wound Signals and *P. syringae* pv *tomato*

LapA genes are primarily controlled at the transcriptional level (W.S. Chao, V. Pautot, F.M. Holzer, and L.L. Walling, unpublished data). Therefore, a *LapA1:GUS* fusion was used to investigate *LapA1* promoter activity in response to pathogens, to wound signals, and during development. The response of the *LapA1* promoter to wounding was characterized using 15 independent *LapA1:GUS* transgenic tomato lines (Table I). No GUS activity was detected in wounded or nonwounded leaves from UC82b control plants. Basal GUS activity levels in nonwounded leaves from the transgenic tomato lines varied (12–1,191 nmol 4-methylumbelliferone min^{-1} mg^{-1} protein). After wounding, GUS activity increased in all *LapA1:GUS* transgenic tomato lines except the U55 line. Increases in GUS

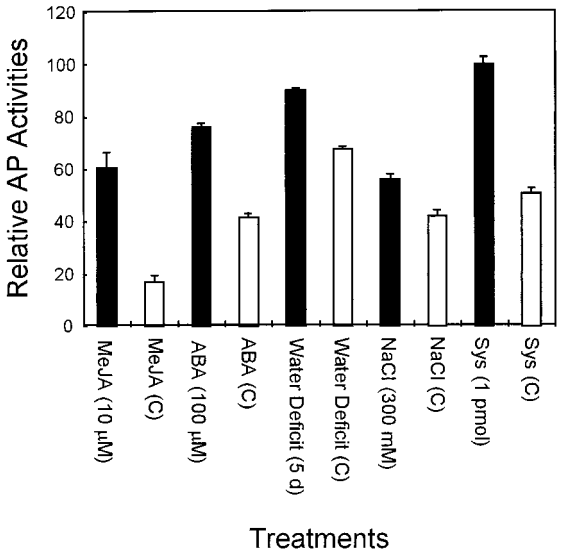

Figure 4. Relative aminopeptidase activities. Native proteins were extracted from control (C) leaves and from leaves treated with 10 μM MeJA, 100 μM ABA, 5 d of water deficit, 300 mM NaCl, or 1 pmol of systemin (Sys). Aminopeptidase activity was measured in triplicate spectrophotometrically at A_{405} by the release of *p*-nitroaniline. Aminopeptidase activity was calculated as the A_{405} per milligram of protein. Relative aminopeptidase activities and SDS are shown; the highest aminopeptidase levels were detected in the systemin-treated leaves (6.2 A_{405}/μg protein); this value was set at 100%. Each analysis was replicated two times.

Table I. *Fluorometric analysis of GUS activity in transgenic tomato lines in response to wounding*

Fifteen independent *LapA1:GUS* transgenic lines were analyzed. Transgenic lines are designated to indicate their parentage: UC82b (U) or VFNT (V). All lines had one to two copies of the *LapA1:GUS* transgene, except U38, which had five copies. GUS activity was measured in leaf extracts. Four to six GUS-positive T_1 plants per line were mechanically wounded or served as healthy controls. Leaves were harvested 24 h later and pooled for each treatment. GUS and protein levels were determined as described in "Materials and Methods."

Transgenic Line	GUS Activity		Ratio (W/H)
	Wounded (W)	Healthy (H)	
	nmol 4-MU min^{-1} mg^{-1} protein		
UC82b	0	0	–
V13	1,074	853	1.3
V14	3,373	84	40.2
V15	2,257	154	14.7
U17	454	43	10.6
U26	634	144	4.4
U33	1,072	88	12.2
U38	1,639	443	3.7
U48	222	22	10.1
U49	2,075	256	4.2
U55	722	1,191	0.6
U63	170	71	2.4
U69	109	12	9.1
U78	3,750	407	9.2
U83	694	404	1.7
U93	2,467	90	27.4

Table II. *Fluorometric analysis of GUS activity in transgenic tomato lines in response to P. syringae pv tomato infection*

GUS activity in leaf extracts from six *LapA1:GUS* transgenic lines was measured 24 h after *P. syringae* pv *tomato* inoculation or mock infection. GUS and protein levels were determined as described in "Materials and Methods."

Transgenic Line	GUS Activity		Ratio (I/M)
	Infected (I)	Mock-infected (M)	
	nmol 4-MU min^{-1} mg^{-1} protein		
UC82b	16	0	–
V14	5,328	806	6.6
V15	1,274	239	5.3
U38	6,703	192	34.9
U49	12,509	72	173.7
U78	10,509	129	82.9
U93	8,196	66	124.2

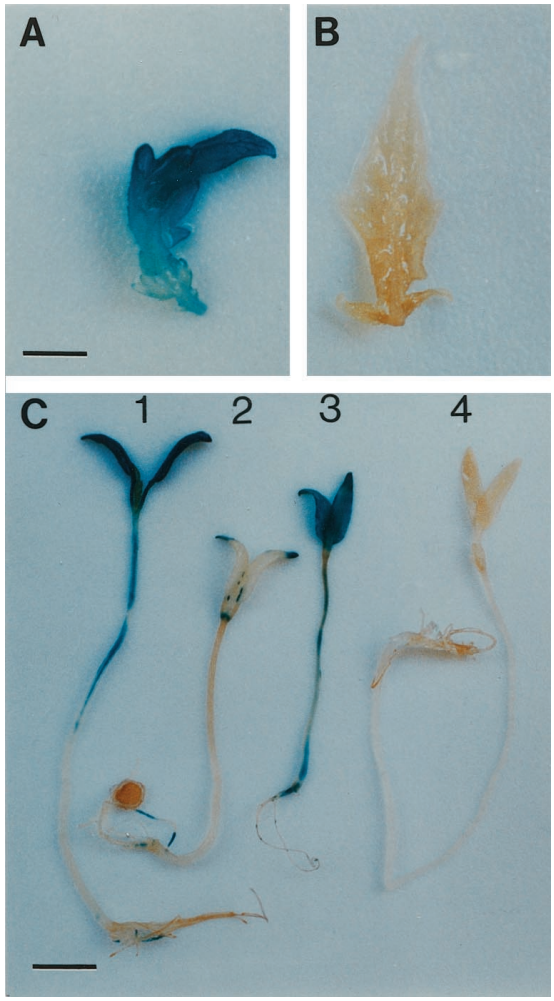

Figure 5. *LapA1* promoter activity in response to MeJA and during seedling development. A and B, Primary leaf from 10-d-old *LapA1:GUS* (line U49) (A) and UC82b control seedlings (B) treated with 10 μM MeJA. Bar = 3.1 mm. C, Seven-day-old *LapA1:GUS* (U49) seedling treated with 10 μM MeJA (1), 7-d-old *LapA1:GUS* seedling (U49) treated with 0.02% EtOH (2), 7-d-old *35S:GUS* seedling (3), and 7-d-old UC82b seedling (4). Bar = 8.6 mm.

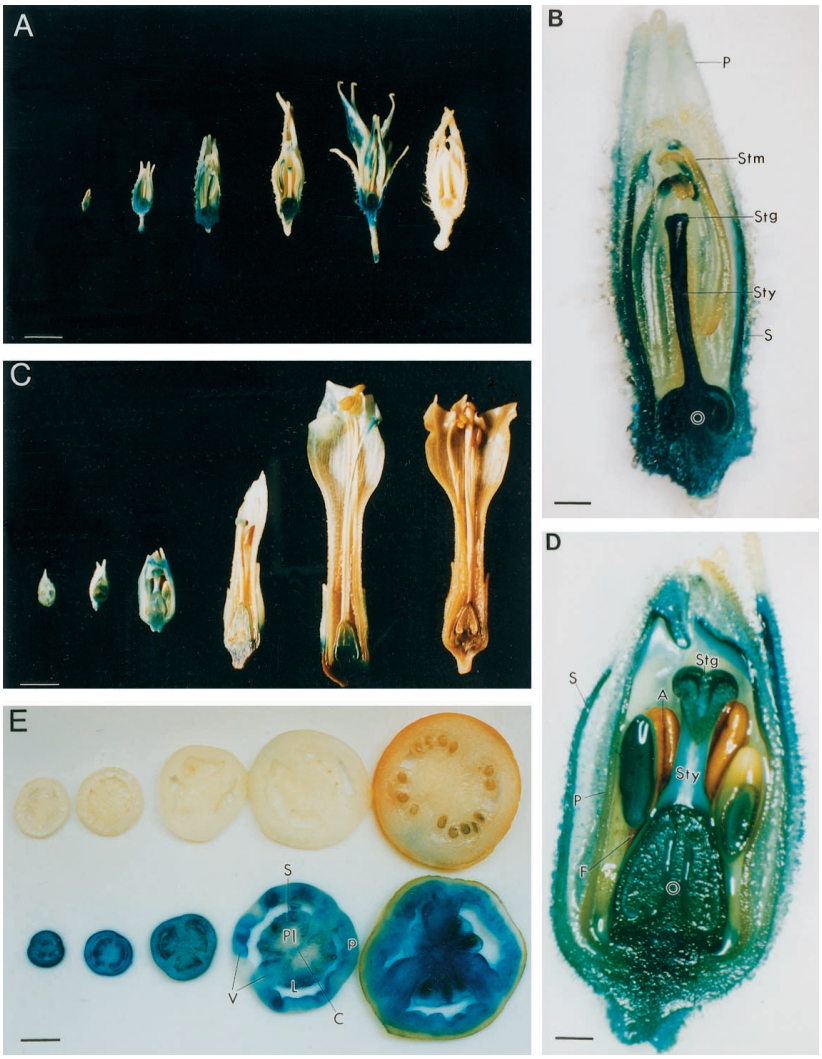

Figure 6. *LapA1* promoter activity during flower and fruit development. A, Flowers of *LapA1:GUS* tomato plants (U49) were excised, cut in half, and infiltrated with GUS histochemical substrate. Flowers displayed are 0.3, 0.8, 1.2, 1.6, and 1.6 cm in length (left to right). A 1.5-cm UC82b flower is presented at the right end of the panel. Variation in GUS activity in tomato and tobacco styles was due to the fact that GUS activity was not readily detected unless the style was bisected. Bar = 4.3 mm. B, Eleven-fold enlargement of the 1.2-cm *LapA1:GUS* tomato flower. Bar = 0.92 mm. S, Sepal; P, petal; O, ovary; Sty, style; Stg, stigma; Stm, stamen. C, Flowers of *LapA1:GUS* tobacco plants (line X2; Chao, 1996) were excised, cut in half, and infiltrated with GUS histochemical substrate. Flowers displayed are 0.5, 0.5, 1.0, 3.0, and 5.0 cm in length (left to right). A 5-cm *N. tabacum* cv Xanthi flower is present at the right end of the panel. Bar = 6.3 mm. D, Fourteen-fold enlargement of the 1.0-cm *LapA1:GUS* tobacco flower (line X2). Bar =0.71 mm. S, Sepal; P, petal; O, ovary; Sty, style; Stg, stigma; A, anther; F, filament. E, Top, Fruit from control UC82b; bottom, fruits of *LapA1:GUS* (U49) ranging in size from 0.5 mm to 7 cm incubated with GUS histochemical substrate. Bar = 1.25 cm. P, Pericarp; L, locular tissue; S, seed; Pl, placental tissue; V, vascular bundle; C, collumella.

activity levels after wounding was variable and ranged from 1.3-fold (line V13) to 40-fold (line V14). Wound induction of the *LapA1* promoter was also noted in the 12 independent *LapA1:GUS* transgenic tobacco lines characterized (Chao, 1996). In general, wound induction was less dramatic, ranging from 2- to 8-fold; however, one transgenic *LapA1:GUS* tobacco line exhibited a 72-fold induction (data not shown).

GUS activity increased significantly in six transgenic tomato lines 24 h after *P. syringae* pv *tomato* inoculation (Table II). Nontransformed UC82b leaves had low to undetectable levels of GUS activity in infected and mock-infected leaves, respectively. The levels of GUS activity in mock-infected *LapA1:GUS* transgenic tomato plants were variable, but were similar to basal levels in untreated leaves (Table I). Line U49 showed the most dramatic increase in GUS activity (174-fold) in response to *P. syringae* pv *tomato* infection. These data were well correlated with the wounding results (Table I).

Histochemical staining for GUS activity showed that, like the control line UC82b, *LapA1:GUS* seedlings did not display significant GUS staining of cotyledons, hypocotyls, roots, or primary leaves (Fig. 5, B and C). Occasionally, GUS staining was detected at random sites on the *LapA1:GUS* seedlings, and this was correlated with sites of inadvertent mechanical wounding. *35S:GUS* seedlings served as a positive control, and uniform GUS staining in the cotyledons, hypocotyls, and roots was detected (Fig. 5C). When treated with MeJA, the *LapA1:GUS* seedlings showed strong GUS staining in the aerial portions of the transgenic tomato plants: primary leaves (Fig. 5A), cotyledons, and hypocotyls (Fig. 5C). Cotyledons showed the highest level of GUS staining and the most apical portion of the hypocotyl in most seedlings also exhibited strong GUS staining. GUS staining was rarely detected in roots of JA-treated or control plants.

The *LAPA1* Promoter Was Active in Flowers and Fruit

Examination of the *LapA1:GUS* transgene expression in both tomato and tobacco indicated that the *LapA1* promoter was active in reproductive organs and in developing tomato fruit (Fig. 6). Strong GUS staining was consistently observed in the stamens, ovaries, stigma, and styles of

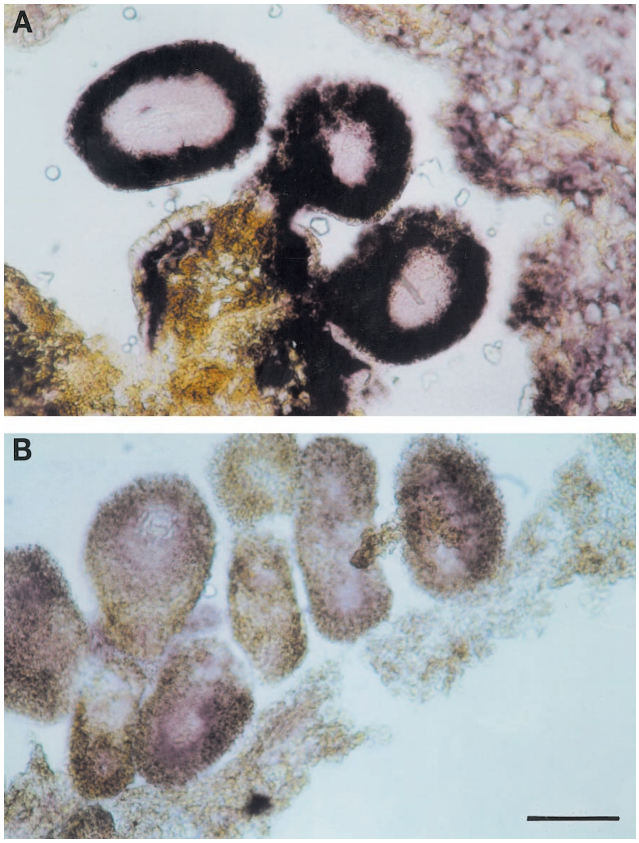

Figure 7. In situ hybridization of an antisense *LapA* RNA with a tomato floral bud. A methacrylate-imbedded transverse section of a 10-mm floral bud of tomato was hybridized with digoxigenin-labeled antisense (A) and sense (B) *LapA* RNAs. Bar = 88 μM.

2-mm buds to fully opened flowers (1.6-cm) from *LapA1:GUS* transgenic tomatoes (Fig. 6, A and B). GUS staining was more uniform in petals and sepals of younger buds (2 mm) than in older buds (0.8 cm and larger) or petals of fully opened flowers (Fig. 6A). This is well correlated with the accumulation of *LapA* RNAs during tomato flower development (V. Pautot, F.M. Holzer, J. Chaufaux, and L.L. Walling, unpublished data; Milligan and Gasser, 1995). In situ hybridizations with a *LapA* antisense RNA probe showed that *LapA* transcripts were the most abundant in the integument of the ovaries and in the placental region (Fig. 7). *LapA* RNAs were also detected at lower levels throughout other floral organs (V. Pautot, F.M. Holzer, J. Chaufaux, and L.L. Walling, unpublished data), and LAP-A proteins were detected in all floral organs of open flowers (C.J. Tu, F.M. Holzer, and L.L. Walling, data not shown). Similar results were obtained when transgenic *LapA1:GUS* tobacco lines were examined (Fig. 6, C and D). All *LapA1:GUS* flowers (0.5–5 cm) had GUS activity in sepals, petals, stamens, pistils, and sepal trichomes. In tobacco, pistils, ovaries, and stigmas exhibited the strongest GUS staining throughout floral development (Fig. 6, C and D).

The *LapA1* promoter was active in all stages of tomato fruit development in the different *LapA1:GUS* transgenic lines (Fig. 6E). The pericarp generally had the highest levels of GUS staining, but staining was also seen in locular tissue, seeds, placental tissue, vascular bundles, and columella. In most cases, GUS staining was most uniform in the earlier stages of fruit development (data not shown). While all fruit exhibited GUS staining, the degree of GUS staining was variable, and approximately 10% of the fruit had lower levels of GUS activity (data not shown).

DISCUSSION

Multiple Signal Transduction Pathways Regulate Wound- and Defense-Response Genes

The *LapA* RNAs and proteins accumulate in response to wounding and *P. syringae* pv *tomato* infection in tomato (Pautot et al., 1993; Gu et al., 1996b). Therefore, it was important to determine if *LapA* genes were regulated by the octadecanoid- or SA-dependent defense-response pathways or if *LapA* utilized one of the more recently identified JA-independent (Titarenko et al., 1997) or SA-independent signal transduction pathways (Penninckx et al., 1995; Pieterse et al., 1996). To this end, tomato plants were treated with wound/defense signals and assessed for levels of *LapA* and three *PR* gene transcripts. These studies indicated that at least four signaling pathways were important for the expression of wound- and defense-response genes in tomato (Fig. 8). Similar to *pin2*, *LapA* was modulated by MeJA and systemin, signals associated with the octadecanoid-signaling pathway (Fig. 1; Schaller and Ryan, 1995). Consistent with the role of SA in blocking the octadecanoid signaling pathway (Peña-Cortés et al., 1993; Doares et al., 1995; O'Donnell et al., 1996), wound induction of *LapA* was suppressed by SA. *LapA* was not strongly induced by exogenous ethylene (Fig. 1). However, if it were similar to *pin* genes, *LapA* would

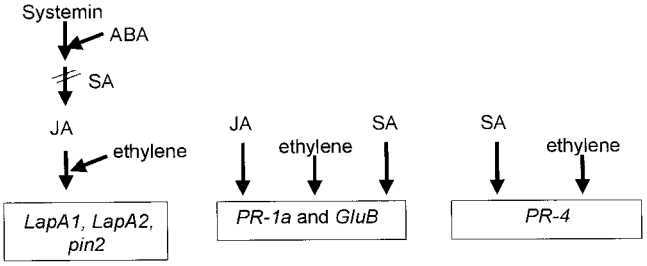

Figure 8. Schematic diagram showing the four independent signal transduction pathways that activate wound and defense genes in tomato. The PR genes (*PR-1*, *PR-4*, and *GluB*) utilize two signaling pathways: a SA-dependent and an ethylene-dependent pathway. *PR-1* and *GluB* utilize a third pathway, a systemin-independent, JA-dependent pathway, which may be analogous to the SA-independent pathway used to induce defensins in Arabidopsis (Penninckx et al., 1995). The fourth signaling pathway is the octadecanoid pathway, which in tomato utilizes systemin, ABA, JA, and ethylene to activate the wound-response genes (*LapA1*, *LapA2*, and *pin2*) and SA to down-regulate the pathway. There is evidence for a fifth signaling pathway in Arabidopsis. A JA-independent mechanism for wound-response gene activation was recently described, but tomato genes activated by this pathway have yet to be identified (Titarenko et al., 1997).

require JA for maximal activation by ethylene (O'Donnell et al., 1996).

PR-4 may be regulated by two signaling pathways that were not utilized by *LapA*: an ethylene-dependent and a SA-dependent pathway. *PR-4* was strongly induced by exogenous ethylene (Fig. 1; van Kan et al., 1995) and therefore utilized an ethylene signaling mechanism distinct from that used by *LapA* or *pin* genes. The excised-shoot assay utilized in these studies did not detect increases in *PR-4* transcripts in response to 0.5 mM SA. However, excised-leaf assays have demonstrated that SA is an important regulator of *PR-4* gene expression (van Kan et al., 1995). The SA signaling pathway has been elegantly elucidated in Arabidopsis (Dangl et al., 1996; Ryals et al., 1996; Yang et al., 1996), and is assumed to function in a similar manner in the Solanaceae.

A more complex circuitry was used to modulate expression of the tomato *PR-1* and *GluB* genes. *PR-1* and *GluB* RNAs accumulated in response to exogenous SA and ethylene, and similar observations were made by other investigators (Christ and Mösinger, 1989; van Kan et al., 1995; Tonero et al., 1997). The ethylene and SA signal transduction pathways utilized by *PR-1*, *GluB*, and *PR-4* were probably the same. However, while SA increased the levels of both *PR-1* and *GluB* RNAs, the steady-state levels of these RNAs were distinct. These data indicate that there are substantial differences in transcriptional and/or posttranscriptional controls that modulate these genes in response to SA.

Finally, *PR-1* and *GluB* genes were also regulated by a signaling pathway that was activated by JA but not by systemin. At present it is not known why the JA generated after systemin treatments was insufficient for *PR-1* and *GluB* transcript accumulation. It is possible that the systemin-induced JA was present in a subset of tomato leaf cells that were not competent for *PR-1* and *GluB* gene expression; similar theories regarding JA compartmentalization have been proposed by Harms et al. (1995). Alternatively, systemin may have induced an inhibitor to interfere with JA induction of *PR-1* and *GluB* expression, which would be consistent with the reciprocal regulation of the oxylipin and SA signal transduction pathways (Seo et al., 1995). At the present time, it is not known how the SA-independent and JA-dependent signaling pathways identified in Arabidopsis or tobacco relate to the pathways being elucidated in tomato (Penninckx et al., 1995; Pieterse et al., 1996; Vidal et al., 1997). However, it is clear from studies in Arabidopsis that some genes (such as defensins) can be induced by exogenous JA and ethylene but not by SA (Penninckx et al., 1995). These data indicate the independence of these signaling mechanisms. The fact that Arabidopsis defensins are not wound induced suggests that they may utilize the JA and ethylene signaling pathways that are similar to those used by the tomato *PR-4*, *PR-1*, and *GluB* and are distinct from JA-dependent or -independent wound responses (Titarenko et al., 1997).

ABA Is Essential for Wound Induction of *LapA*

The role of ABA in the modulation of wound-response gene expression in tomato remains controversial. Peña-Cortés et al. (1989, 1991, 1995) have concluded that ABA is essential for *pin2* gene expression and acts early in the octadecanoid pathway. On the other hand, other studies have concluded that ABA does not have a primary role in oxylipin signal transduction pathway (Schaller and Ryan, 1995; Birkenmeier and Ryan, 1998). These discrepancies suggested to us that the role of ABA in wound-response gene induction needed to be re-evaluated, since significant differences in plant genotypes and treatments were present in the previous studies. The studies reported here with ABA-producing lines indicated that *PR* gene expression was not influenced by exogenous ABA. Our data support the idea that the systemin-independent, JA-responsive mechanism for *PR-1* and *GluB* gene expression is distinct from the JA-signaling mechanisms utilized by *LapA* and *pin2* genes.

Examination of *LapA*, *pin2*, and *le4* transcript levels in the ABA-deficient line *flacca* indicated that there were significant differences in the role of ABA in the regulation of each of these genes. First, *pin*2 RNAs accumulated in nonwounded and wounded *flacca* leaves, whereas *LapA* RNAs and *le4* RNAs were undetectable. Second, the impact of exogenous ABA on *le4* and *LapA* gene expression was accentuated in *flacca* plants relative to ABA-producing plants. Third, ABA was critical for maximal accumulation of *LapA* transcripts in response to systemin, and ABA and systemin appeared to act synergistically to modulate *LapA* RNA levels. In contrast, *pin*2 transcript accumulation was not dependent on exogenous ABA for systemin induction. Finally, while ABA promoted *le4* transcript accumulation, *le4* did not respond to the signals of the octadecanoid pathway.

Collectively, these data indicate that although both *LapA* and *pin2* genes utilized the octadecanoid signaling pathway, they responded differentially to ABA. *LapA* responses to ABA were more similar to those of *le4* than to those of *pin2*. Wound induction of *LapA* was either dependent on ABA or required ABA levels that exceeded the residual levels in the *flacca* line. These data may indicate that *pin*2 gene expression was more sensitive to the residual levels of ABA in *flacca* plants (Neill and Horgan, 1985) than were *le4* (Cohen and Bray, 1990) and *LapA*. Alternatively, the basal *pin*2 transcript levels detected in *flacca* plants were reflective of ABA-independent expression. Data from Peña-Cortés and colleagues (1989, 1996) support the idea that *pin2* expression in nonwounded *flacca* leaves was due to residual ABA levels. Using an excised leaf assay, the *sitiens* mutant (which accumulates less ABA than *flacca*) exhibited no increase in *pin2* RNAs, while the ABA-producing control showed a marked increase in *pin2* mRNAs. This interpretation is also supported by Carrera and Prat (1998), who showed that transgenic tomato plants expressing the mutant *abi1* allele from Arabidopsis, which blocks the ABA signal transduction cascade, prevents the accumulation of *pin2* and *LapA* transcripts in response to ABA.

Comparison of the data presented here and those from Peña-Cortés et al. (1989, 1996), Carrera and Prat (1998), and Birkenmeirer and Ryan (1998) showed that the results obtained from excised shoot versus excised leaf assays are different. First, using the excised leaf assay, *pin2* mRNAs

are not detected in healthy, ABA-proficient plants (Peña-Cortés et al., 1989, 1996; Carrera and Prat, 1998). Excised shoot assays routinely detect *pin2* transcripts (Birkenmeirer and Ryan, 1998; Fig. 1). Second, exogenous ABA caused larger increases in *pin2* RNA levels in excised leaves than in excised shoots. This is consistent with the difference in *le4* expression noted in our studies and in previous studies that utilized detached leaf assays (Cohen et al., 1991).

LapA Genes Are Induced during Abiotic Stresses That Are Accompanied by ABA Accumulation

Endogenous ABA levels increase when plants are exposed to a saline environment (Downton and Loveys, 1981; Walker and Dumbroff, 1981), water deficit (Zeevaart and Creelman, 1988), or low-temperature stress (Chen et al., 1983). Like the ABA- and water-deficit-response gene *le4* (Cohen and Bray, 1990; Cohen et al., 1991; Kahn et al., 1993), *LapA* RNAs increased in response to water deficit and increases in salinity. However, differences in the *le4* and *LapA* responses were noted. *le4* RNAs increased more dramatically and persisted for a longer period of time than the *LapA* transcripts. It is also important that while tomato *LapA* RNAs increased during water-deficit stress, water deficit did not change potato *Lap* transcript levels (Hildmann et al., 1992). Several barley *JIP* (jasmonate-induced protein) genes are also water-deficit, ABA, and JA-induced; however, these genes are not induced by salinity stress, suggesting differences in stress signaling pathways in barley and tomato (Reinbothe et al., 1992).

It is not clear if the signal transduction pathways used for expression of *LapA* genes in response to water deficit and salinity stress were the same and corresponded to the octadecanoid pathway, or if they represent different signal transduction pathways. Clearly, *le4*, which is not modulated by systemin or MeJA, must utilize a signal transduction pathway distinct from the octadecanoid pathway. However, it is possible that there is cross-talk between the octadecanoid and abiotic-stress signal transduction pathways to coordinate *LapA* gene responses. Alternatively, *LapA* RNA induction by abiotic stress might solely utilize the wound-response octadecanoid pathway, since increases in JA have been measured for several abiotic stresses (Creelman and Mullet, 1995). It is also possible that the active oxygen species that accumulate during water deficit (Davies and Mansfield, 1983; Inzé and van Montagu, 1995) might activate the octadecanoid signaling pathway by causing lipid peroxidation and lipoxygenase production (Keppler and Novacky, 1989; Ádám et al., 1989). Rises in ABA could further activate the octadecanoid pathway to ultimately increase JA levels and activate *LapA* gene expression.

The *LapA1* Promoter Is Responsive to Wound and Developmental Signals

Analysis of transgenic tomato and tobacco plants expressing a chimeric *LapA1:GUS* transgene demonstrated that the *LapA1* promoter sequences that responded to wound and developmental signals were located within the 1st kb of the *LapA1* 5′-flanking sequences. Similar results have been reported for the expression of the tomato *LapA1* gene in potato (Ruíz-Rivero and Prat, 1998) and the tomato *LapA2* gene in Arabidopsis (A. El Amrani and V. Pautot, unpublished data). The *LapA1* promoter was not active in vegetative organs unless tissues were wounded, *P. syringae* pv *tomato* infected, or treated with a wound signal such as MeJA.

The lack of *LapA1* promoter activity in cotyledons after germination indicated that the LAP-A protein does not have a role in the mobilization of storage protein reserves (Walling and Gu, 1996). Aminopeptidases with properties similar to LAP-A have been characterized from kidney bean and barley seeds (Sopanen and Mikola, 1975; Mikkonen, 1992). The data presented here indicate that the kidney bean and barley seed LAPs are likely to be analogs of the constitutively expressed LAP-N of tomato and Arabidopsis LAP (Bartling and Nosek, 1994; Gu et al., 1996b; C.J. Tu and L.L. Walling, unpublished data).

Compared with other wound- and defense-response genes (Lotan et al., 1989; Cote et al., 1991; Uknes et al., 1993; Constabel and Brisson, 1995), the *LapA1* promoter has a unique developmental specificity. In transgenic tomato, the *LapA1* promoter was active in all floral organs, which is similar to the activity seen for tomato *pin2* RNA accumulation (Peña-Cortés et al., 1991). However, unlike *pin2* genes, *LapA* genes were active throughout all of fruit development. Other wound-response genes (i.e. the wound-induced ACC synthase gene and a *pin1*-like gene) are expressed only during the ripening phase of fruit development (Margossian et al., 1988; Li et al., 1992), when endogenous ethylene levels rise. Recently, Ruíz-Rivero and Prat (1998) reported an analysis of the tomato *LapA1* promoter in transgenic potato, and their results differed from the data reported here. They did not detect *LapA1* promoter activity in stigmas, styles, or ovaries, although expression in other floral organs was reported. At present, it is not known what signals are responsible for the activation of the *LapA1* promoter in tomato flowers and fruit. However, the signaling mechanisms appear to be different in potato and tomato (Peña-Cortés et al., 1991; Ruíz-Rivero and Prat, 1998). The availability of tomato mutants that impact biosynthesis or perception of ABA, JA, and ethylene may aid in resolving their roles in developmental programming of *LapA* gene expression.

Plants utilize intricate systems for the expression of defense genes during floral and fruit development. The overlapping patterns of expression of the vast array of defense- and wound-response genes may ensure production of viable seeds. LAP-A proteins may play a defensive (or protective) role in tomato flowers and fruit by protecting gametes from damage by insect or pathogen attack. While the role of protease inhibitors in the control of insect predation is established and highly publicized (Johnson et al., 1989; Xu et al., 1996), a few studies have shown that proteases are important in plant defense. For example, one study showed that a Cys endoprotease confers resistance to maize against fall armyworm (Jiang et al., 1995). It is possible that exopeptidases such as LAP-A or the tomato wound-induced carboxypeptidases may have important

roles in plant defense (Pautot et al., 1993; Mehta et al., 1996; Walling and Gu, 1996).

In animals, exopeptidases are important in the activation and inactivation of bioactive peptides and regulation of protein half-lives (Taylor, 1996; Varshavsky, 1996; Bradshaw et al., 1998). In a similar manner, LAP-A may serve to modulate levels or activities of regulatory proteins or peptides. Alternatively, LAP-A may facilitate turnover of proteins that are damaged due to reactive oxygen species generated during wounding, or may hydrolyze proteins to supply the pool of amino acids to support the substantial changes in protein synthesis associated with wounding. Current studies using antisense plants and plants overexpressing *LapA* are in progress. These studies will help to resolve the roles of LAP-As during floral and fruit development and during wounding and defense responses.

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