The Induction of Tomato Leucine Aminopeptidase Genes (*LapA*) After *Pseudomonas syringae* pv. *tomato* Infection Is Primarily a Wound Response Triggered by Coronatine

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Tomato plants constitutively express a neutral leucine aminopeptidase (LAP-N) and an acidic LAP (LAP-A) during floral development and in leaves in response to insect infestation, wounding, and *Pseudomonas syringae* pv. *tomato* infection. To assess the physiological roles of LAP-A, a *LapA*-antisense construct (*35S:asLapA1*) was introduced into tomato. The *35S:asLapA1* plants had greatly reduced or showed undetectable levels of LAP-A and LAP-N proteins in healthy and wounded leaves and during floral development. Despite the loss of these aminopeptidases, no global changes in protein profiles were noted. The *35S:asLapA1* plants also exhibited no significant alteration in floral development and did not impact the growth and development of *Manduca sexta* and *P. syringae* pv. *tomato* growth rates during compatible or incompatible infections. To investigate the mechanism underlying the strong induction of *LapA* upon *P. syringae* pv. *tomato* infection, *LapA* expression was monitored after infection with coronatine-producing and -deficient *P. syringae* pv. *tomato* strains. *LapA* RNA and activity were detected only with the coronatine-producing *P. syringae* pv. *tomato* strain. Coronatine treatment of excised shoots caused increases in RNAs for jasmonic acid (JA)-regulated wound-response genes (*LapA* and *pin2*) but did not influence expression of a JA-regulated pathogenesis-related protein gene (*PR-1*). These results indicated that coronatine mimicked the wound response but was insufficient to activate JA-regulated *PR* genes.

Additional keywords: defense response, exopeptidases, *Lycopersicon esculentum*, methyl jasmonate, octadecanoid pathway, phytotoxin.

plant and animal kingdoms, exhibit a high level of amino acid identity, and have similarities in their substrate specificities (Gu and Walling 2000; Gu et al. 1999; Rawlings and Barrett 1993; Taylor et al. 1996; Walling and Gu 1996). In animals, the bovine lens LAP was one of the first aminopeptidases characterized (Smith and Hill 1960). Despite the number of biochemical and biophysical studies that have accumulated on the animal LAP (Kim and Lipscomb 1994; Taylor et al. 1996), the exact role of LAP remains to be established. The animal LAP may be essential for protein turnover because altered LAP activity has been associated with a variety of pathologies, including protein deposition during cataract formation in the lens (Stuart and Doughty 1996; Taylor et al. 1993). In addition, the human LAP is induced by interferon- γ (Harris et al. 1992) and is important for the processing of peptides released by the 26S proteasome for presentation to major histocompatibility complex (Benington et al. 1998).

cDNAs encoding LAP enzymes have been described in *Arabidopsis thaliana*, potato, and tomato (Bartling and Weiler 1992; Hildmann et al. 1992; Pautot et al. 1993; Walling and Gu 1996). There are two classes of 55-kDa LAP proteins in tomato that can be distinguished by their pIs and differential patterns of expression (Gu et al. 1996b). The neutral LAP-N proteins are expressed constitutively and found in all monocots and dicots examined to date (Chao et al. 2000; Gu et al. 1996b). The tomato LAP-N proteins are most similar to the LAP described in *A. thaliana* (Bartling and Nosek 1994) and are likely to be similar to the seed LAPs purified from kidney bean and barley (Mikkonen 1992; Sopanen and Mikola 1975).

The acidic LAP-A proteins accumulate in all developing floral organs and, more specifically, in tomato ovary integument, suggesting a nutritional role of LAP-A for the developing embryo (Chao et al. 1999; Milligan and Gasser 1995; Ruiz-Rivero and Prat 1998; C.-J. Tu and L. Walling *unpublished results*). In addition, LAP-A appears to be part of the plant-defense response because the *LapA* RNAs and proteins accumulate to high levels in tomato leaves after mechanical wounding, insect infestation, and in response to *Pseudomonas syringae* pv. *tomato* infection (Chao et al. 1999; Gu et al. 1996b; Pautot et al. 1993). The potato *Lap* gene described by

Leucine aminopeptidases (LAPs) (E.C.3.4.11.1) catalyze the cleavage of N-terminal amino acids from peptides and proteins. These metalloproteases are widely distributed in the

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Hildmann et al. (1992) appears to be an analog of the tomato *LapA* genes.

Tomato genome has two highly conserved genes, *LapA1* and *LapA2*, encoding LAP-A proteins that are primarily controlled at the transcriptional level (Chao et al. 1999; Chao et al. 2000; Gu et al. 1996a). *LapA* genes are activated by the wound octadecanoid pathway because systemin and methyl jasmonate (MeJA) cause increases in the levels of *LapA* RNA, protein, and activity. In addition, salicylic acid (SA) suppresses wound induction of *LapA* genes (Chao et al. 1999), consistent with SA's role in blocking JA biosynthesis and JA action (Doares et al. 1995; Peña-Cortés et al. 1993).

Tomato pathogenesis-related protein (*PR*) genes and wound-response genes (*LapA*, proteinase inhibitor 1 [*pin1*] and *pin2*) are induced in response to *P. syringae* pv. *tomato* (Jia and Martin 1999; Pautot et al. 1991; Pautot et al. 1993). The gene-for-gene resistance mechanism that is active in *P. syringae* pv. *tomato*-tomato interactions is one of the best characterized mechanisms (Martin 1999). *P. syringae* pv. *tomato* strains expressing the avirulence gene *avrPto* cause a hypersensitive disease resistance response in tomato lines expressing the disease resistance gene *Pto*. Biochemical and genetic studies have shown that during incompatible interactions, AvrPto and Pto interact directly to induce a signaling cascade involving transcription-factor phosphorylation, which leads to the rapid activation of *PR* genes. In compatible interactions, pathogen recognition is slow and *PR* gene expression is significantly delayed.

Superimposed on the activation of *PR* genes are plant responses to the *P. syringae* pv. *tomato* phytotoxin coronatine. This toxin is responsible for the chlorotic halo that surrounds necrotic lesions on tomato plants (Mitchell and Young 1978). Coronatine plays a role in pathogen virulence (Bender et al. 1987) and has been proposed to mimic the action of MeJA/JA. *A. thaliana* mutants that are resistant to coronatine also are insensitive to MeJA (Feys et al. 1994) and MeJA/JA. Coronatine induces similar effects on plants, including inhibition root growth, stimulation of anthocyanin accumulation, and promotion of proteinase inhibitor accumulation (Bender et al. 1999; Feys et al. 1994; Koda et al. 1996; Sembdner and Parthier 1993; Wasternack et al. 1998; Weiler et al. 1994). Coronatine does not strictly mimic MeJA action, however, because this toxin induces other effects, including chlorosis and shrinking of chloroplasts not associated with MeJA treatments (Palmer and Bender 1995).

The induction of *LapA* by wounding and *P. syringae* pv. *tomato* infections seems incongruous given the reciprocal regulation of the wound-induced octadecanoid and the defense-response pathways (Walling 2000). To investigate the role of LAP-A in the defense response and during floral development, transgenic tomato plants expressing an antisense *LapA1* gene construct (*35S:asLapA1*) were evaluated. Levels of *LapA* RNAs, LAP-A and LAP-related proteins, and LAP-A activity were monitored during floral development and in response to mechanical wounding and *P. syringae* pv. *tomato* infection. The growth and development of *Manduca sexta* (tobacco hornworm) larvae on detached leaves from *35S:asLapA1* plants were also determined. In addition, the growth of *P. syringae* pv. *tomato* coronatine-producing (*Cor*⁺) and -deficient (*Cor*⁻) strains in leaves of *35S:asLapA1* lines expressing the bacterial speck resistance gene (*Pto*) or its susceptible allele

(*pto*) were evaluated. The role of coronatine in *LapA* gene expression also was evaluated by measuring changes in *LapA* RNAs, protein levels, and LAP-A activity in response to *Cor*⁺ and *Cor*⁻ *P. syringae* pv. *tomato*. To complement these studies, the impact of exogenous coronatine on the levels of *LapA*, *pin2*, and *PR* gene (*PR-1* and *PR-4*) RNAs were assessed. Collectively, these results suggest that *LapA* genes were induced by wild-type *P. syringae* pv. *tomato* via a wound-response pathway triggered by coronatine. These studies emphasized differences in exogenous coronatine and JA treatments on defense gene expression.

RESULTS

Construction of a *35S:asLapA1* gene and introduction into tomato plants.

A chimeric *LapA1*-antisense gene (*35S:asLapA1*) was constructed by inserting a 1.4-kb fragment of a *LapA1* cDNA (Pautot et al. 1993) between the *Cauliflower mosaic virus* (CaMV) 35S promoter with a double enhancer and the CaMV 35S terminator. This chimeric gene was cloned into the *Agrobacterium tumefaciens* binary transformation vector pBIN19 (Bevan 1984). The recombinant *A. tumefaciens* strain was used to transform UC82b, a *P. syringae* pv. *tomato*-susceptible tomato cultivar. Six independent transgenic plants were selected on the basis of kanamycin resistance. Genomic DNA blot analysis and segregation analyses showed that the transgenic lines contained one to five copies of the *35S:asLapA1* gene at a single locus (data not shown).

Accumulation of *LapA* transcripts in transgenic and control tomato lines.

To determine whether the *35S:asLapA1* altered the levels of *LapA* RNAs that accumulated after wounding, RNAs were isolated from healthy and mechanically wounded leaves from the six T₀ *35S:asLapA1* transgenic lines and UC82b control plants. RNA blot analysis showed that *LapA* RNAs were not detected in healthy plants (Fig. 1A). After mechanical wounding, *LapA* RNAs accumulated to high levels in the

Fig. 1. Levels of *LapA* RNAs in control and transgenic tomatoes expressing the *35S:asLapA1* gene. **A**, Total RNAs were extracted from healthy (H) and 20-h wounded (W) leaves of control (C) and six independent T₀ *35S:asLapA1* lines (1-6). **B**, Total RNAs were isolated from 8-mm green buds (B) and open flowers (F) from control (C) and *35S:asLapA1* plants 3, 4, and 6. Total RNAs (15 µg/lane) were fractionated, blotted, and hybridized to a ³²P-labeled antisense *LapA1* RNA probe. The autoradiographs in **A** and **B** represent 15 h and 1 h 15 min exposures, respectively. The size of the *LapA* RNA is indicated in kb.

leaves of control plants. Three of the *35S:asLapA1* lines (1, 2, and 5) accumulated *LapA* RNAs after wounding. The levels of the *LapA* RNAs, however, were reduced relative to the control plants. A stronger antisense inhibition of *LapA* gene expression was noted in the transgenic *35S:asLapA1* lines 3, 4, and 6. In these plants, the *LapA* RNAs were undetectable or barely detected in healthy and wounded leaves. These data indicated that the *35S:asLapA1* construct effectively down regulated the *LapA1* and *LapA2* genes.

Because *LapA* RNAs and proteins accumulate during floral development in tomato (Chao et al. 2000; Milligan and Gasser 1995), the levels of *LapA* RNAs in developing floral buds and open flowers were examined in the three transgenic *35S:asLapA1* lines (3, 4, and 6) that showed the greatest reduction in leaf *LapA* RNA levels after wounding. In control plants, *LapA* RNAs were abundant in 8-mm green buds and were at low levels in open flowers (Fig. 1B). In contrast, *LapA* RNAs were detected at low-to-undetectable levels in the floral buds from *35S:asLapA1* plants. Transgenic lines 3 and 4 and their homozygous T₂ progeny, which exhibited the most dramatic reduction in *LapA* RNA accumulation after wounding and during floral development, were further characterized.

Accumulation of LAP proteins in the *35S:asLapA1* plants.

Previous studies have revealed that tomato plants have four classes of proteins that are antigenically related to LAP (Gu et al. 1996b). There are two LAP-like proteins with molecular masses of 66 and 77 kDa that are expressed constitutively and two classes of 55-kDa LAPs with distinct pIs (Gu et al. 1996b). A single gene (*LapN*) encodes the 55-kDa LAP-N polypeptides that have neutral pIs (6.6 to 7.0) and are expressed constitutively (Chao et al. 2000). Two genes (*LapA1* and *LapA2*) encode the 55-kDa LAP-A polypeptides that are wound induced and have acidic pIs (5.6 to 5.9) (Gu et al. 1996a). In addition to the epitopes shared between the LAP-A and LAP-N polypeptides (Gu et al. 1996b), the *LapA* genes and the single *LapN* gene-coding regions are 84% identical at the nucleotide level (C.-J. Tu and L. Walling, *unpublished results*). Given this sequence identity, it was important to assess the impact of *35S:asLapA1* gene expression on the accumulation of LAP-A and LAP-N proteins.

To determine the levels of LAP and LAP-like proteins in the control UC82b plants and *35S:asLapA1* lines, proteins were extracted from healthy and wounded leaves and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Gels were subjected to immunoblot analysis with a LAP polyclonal antiserum (Fig. 2A). Similar to the tomato cultivar Peto 238R (Gu et al. 1996b), the UC82b control plants had four immunoreactive proteins (Fig. 2A). The 90-kDa protein was nonspecific and detected by the preimmune serum (Gu et al. 1996b). The LAP antiserum also detected the 66- and 77-kDa LAP-like proteins and low levels of a 55-kDa LAP protein in healthy UC82b leaves. After wounding, the levels of the 66- and 77-kDa polypeptides remained constant and the levels of the 55-kDa LAP proteins rose markedly. When the T₂ progeny from the transgenic *35S:asLapA1* lines 3 and 4 were examined, only the nonspecific 90-kDa and the 66- and 77-kDa LAP-like proteins were detected (Fig. 2A). The 55-kDa LAP proteins were not detected in either healthy or wounded transgenic leaves.

Total proteins from healthy and wounded leaves from control and *35S:asLapA1* plants were fractionated by two dimensional (2D)-PAGE. Immunoblots were incubated with the LAP polyclonal antiserum (Fig. 2B and C). Wounded leaves from the control plants accumulated the 66- and 77-kDa LAP-like proteins as well as the LAP-N and LAP-A polypeptides (Fig. 2B). In contrast, only the 66- and 77-kDa LAP-like proteins were detected in the wounded leaves from the *35S:asLapA1* lines. Neither the constitutive LAP-N nor the wound-induced LAP-A proteins were detected in wounded leaves from the *35S:asLapA1* lines. Some variation in the degree of down regulation of LAP-A and LAP-N imposed by the *35S:asLapA1* gene was noted in repeated experiments. In all cases, however, LAP-A and LAP-N proteins were either undetectable (Fig. 2B) or at low levels (data not shown) in wounded leaves.

Given the role of aminopeptidases in protein turnover (Taylor 1996) and the reduction of the LAP-A and LAP-N polypeptides, it was interesting to determine whether the *35S:asLapA1* plants exhibited differences in their total protein profiles. Total proteins were extracted from healthy and

Fig. 2. Accumulation of LAP and LAP-like proteins in leaves of control and *35S:asLapA1* transgenic plants. Total proteins were isolated from healthy (H) and wounded (W) leaves from control and homozygous T₂ *35S:asLapA1* plants (lines 3 and 4). **A**, Total proteins (25 µg) were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). **B and C**, Total proteins (80 µg) from wounded leaves from control (**B**) and *35S:asLapA1* plants (**C**) were fractionated by two-dimensional (2D)-PAGE. SDS- and 2D-PAGE immunoblots were incubated with a 1:500 dilution of the LAP polyclonal antiserum. Molecular weight markers (M) were run in parallel lanes and are indicated in kD. Data for line 4 plants are shown; similar results were found with line 3 plants.

wounded leaves from control and *35S:asLapA1* plants, fractionated by 2D-PAGE, and stained with silver. Comparison of protein profiles from the control and *35S:asLapA1* plants showed no reproducible differences in healthy and wounded leaves, except for the presence of LAP-A in wounded leaves from the control (Fig. 3).

Impact of LAP down regulation on floral and fruit development.

Given the high levels of LAP proteins and activity in developing flowers from control plants (Fig. 1B) (C.-J. Tu and L. Walling, *unpublished results*), the impact of LAP-A and LAP-N down regulation on floral development and function was investigated. Floral structure was not altered in *35S:asLapA1* plants, and no significant differences in the number and weight of fruit or the number of seeds per fruit were observed in the control and *35S:asLapA1* lines (data not shown).

Influence of LAP down regulation on *P. syringae* pv. *tomato*-compatible and -incompatible interactions.

Leaves from the control and *35S:asLapA1* transgenic plants were inoculated with 2×10^6 CFU/ml of *P. syringae* pv. *to*

mato PT11 (compatible interaction). The levels of LAP-A enzymatic activity and *LapA* RNA were monitored 0, 2, 3, and 4 days after *P. syringae* pv. *tomato* inoculation (Fig. 4). *P. syringae* pv. *tomato* infection caused *LapA* RNA and LAP-A activity levels to rise in the leaves of control UC82b plants after 2 days. While LAP-A activity persisted in leaves 4 days after *P. syringae* pv. *tomato* inoculation, *LapA* RNA levels declined by day 4. In contrast to the control-line response, no LAP-A activity was detected (Fig. 4A) and the accumulation of *LapA* RNA (Fig. 4B) was greatly reduced in infected leaves from the *35S:asLapA1* plants. Similar results were obtained in transgenic line 3 (data not shown).

Despite the down regulation of *LapA* RNAs, the LAP-A and LAP-N proteins, and LAP activities, the *35S:asLapA1* lines and the control plants did not differ in the number and size of the *P. syringae* pv. *tomato*-induced lesions (data not shown). To determine if *P. syringae* pv. *tomato* growth rates were influenced, the impact of the *35S:asLapA1* gene on intercellular bacterial titers during compatible and incompatible interactions was determined. During the compatible interaction with the disease-susceptible UC82b (*pto*) and *35S:asLapA1* line 4

Fig. 3. Total protein profiles in control and *35S:asLapA1* plants. Total proteins (80 μ g) were isolated from healthy (A and B) and wounded (C and D) leaves from control and homozygous T₂ *35S:asLapA1* plants (line 4). Proteins were fractionated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and gels were silver stained. Molecular weight markers were run in parallel lanes and are indicated in kD. The positions of wound-induced LAP-A proteins are indicated by a bracket in C. Brackets also were added in A, B, and D to emphasize the absence of LAP-A proteins in the samples. The left and right ends of the 2D gel represent pH 8 to 4, respectively.

(*pto*), *P. syringae* pv. *tomato* PT11 levels increased during the 4-day period and reached peak levels on day 4 (Fig. 5A). *P. syringae* pv. *tomato* grew at similar rates in the control and *35S:asLapA1* lines. To examine the impact of *LapA* and *LapN* down regulation on *P. syringae* pv. *tomato* PT11 growth during an incompatible interaction, two disease-resistant lines (*Pto* and *35S:asLapA1 Pto*) were infected with *P. syringae* pv. *tomato* (Fig. 5B). The intercellular titers of *P. syringae* pv. *tomato* PT11 during the compatible interactions were over 550-fold higher than with incompatible interactions. Decreased levels of LAP-A and LAP-N did not significantly influence bacterial growth in disease-resistant plants.

Effect of *35S:asLapA1* on insect growth.

Larvae of *M. sexta* were fed a diet consisting of leaves from either control or *35S:asLapA1* transgenic plants to assess the effect of LAP-A and LAP-N on insect growth. Over 21 larvae were monitored on each class of plant. The mass and the developmental stage of the larvae were noted after 9 and 12 days of feeding. No significant difference in larvae growth was observed between the control and *35S:asLapA1* transgenic lines 3 and 4 (data not shown).

Aminopeptidase activity and levels of *LapA* and *pin2* RNAs in response to coronatine-producing and -deficient *P. syringae* pv. *tomato* strains.

Previous studies have shown that *LapA*, *pin1*, and *pin2* RNAs accumulate in tomato leaves during compatible and incompatible interactions with the coronatine-producing *P. syringae* pv. *tomato* strain PT11 (Pautot et al. 1991; Pautot et

al. 1993). To evaluate the induction of *LapA* and *pin2* RNAs by coronatine during compatible interactions, leaves from control (UC82b) plants were inoculated with the *Cor*⁺ or *Cor*⁻ strains of *P. syringae* pv. *tomato*. LAP activity was detected on day 3 after inoculation with the *Cor*⁺ strain PT11, whereas LAP activity was not detected over the 4-day infection period with the *Cor*⁻ mutant PT23.2 (Fig. 6A). Similar findings were found at the RNA level (Fig. 6B). *LapA* and *pin2* RNAs were present at high levels in leaves after infection with the *Cor*⁺ strain of *P. syringae* pv. *tomato* and were not detected after infection by the *Cor*⁻ strain.

To confirm these data, excised seedling shoots were treated with 1 μ mol of coronatine. The levels of transcripts for several tomato wound- and defense-response genes were determined at 0, 2, 4, 8, and 16 h after coronatine treatment (Fig. 7). *LapA* and *pin2* RNAs accumulated in response to exogenous coronatine. For comparison, JA-responsive (*PR-1*) and nonresponsive (*PR-4*) *PR* gene RNA levels were assessed (Chao et al. 1999). *PR-4* and *PR-1* RNAs were not detected or were at very low levels after 16 h of coronatine treatment.

Growth of *Cor*⁻ *P. syringae* pv. *tomato* in control and *35S:asLapA1* plants.

Coronatine is a strong inducer of *LapA* genes of tomato (Fig. 7). To test whether differences in *P. syringae* pv. *tomato* growth rates could be observed with coronatine-deficient *P. syringae* pv. *tomato*, the rate of growth of *P. syringae* pv. *tomato* strain PT23.2 during compatible and incompatible infections of control and *35S:asLapA1* plants was determined. Like the coronatine-producing strain PT11, PT23.2 intercellular titers rose to similar levels in compatible infections of control (UC82b) and *35S:asLapA1* plants (Fig. 5C). The *P. syringae* pv. *tomato* titer on day 4 was substantially lower (40- to 150-fold) than observed for the *Cor*⁺ strain (Fig. 5A and C). This could be attributed partially to the 1.5-fold-lower inoculation concentration of the *Cor*⁻ *P. syringae* pv. *tomato* used in these infections. This difference, however, is more likely attributed to the reduced fitness of coronatine-deficient *P. syringae* pv. *tomato* (Bender et al. 1987). The ability of PT23.2 to grow in control and *35S:asLapA1* plants harboring the *Pto* resistance gene also was assessed (Fig. 5D). Similar to the *Cor*⁺ PT11 during incompatible interactions, intercellular *P. syringae* pv. *tomato* PT23.2 titers were substantially less (6- to 20-fold, reduced) relative to compatible interactions (Fig. 5C and D). No differences in PT23.2 growth rates were observed in the *Pto* or *35S:asLapA1 Pto* lines (Fig. 5D). The reduced fitness that was clearly observed during PT23.2 *Cor*⁻ compatible interactions was not observed as clearly in the incompatible interactions.

Fig. 4. Accumulation of LAP-A activity and *LapA1* RNAs in leaves of control and *35S:asLapA1* plants during compatible *Pseudomonas syringae* pv. *tomato* interactions. Native proteins or RNAs were extracted from control and *35S:asLapA1* plant leaves 0, 2, 3, and 4 days after *P. syringae* pv. *tomato* inoculation. **A**, Soluble tomato proteins (40 μ g/lane) and swine LAP (SL; 0.5 μ g/lane) were fractionated by native polyacrylamide gel electrophoresis, and gels were stained for aminopeptidase activity with leucine- β -naphthylamide and Fast Garnet GBC (Gu et al. 1996b). The masses of the tomato LAP-A hexamer (353 kDa) and the swine LAP hexamer (330 kDa) were determined previously (Gu et al. 1996b). **B**, Total RNA blots (15 μ g) were hybridized to a ³²P-labeled antisense *LapA1* RNA probe. The autoradiograph represents a 1-h exposure. The size of the *LapA* RNA is indicated in kb.

DISCUSSION

These studies have investigated the role of LAP-A in the tomato defense response and during floral development. Previous studies showed that *LapA1* and *LapA2* mRNAs encoding LAP-A proteins were induced in response to mechanical wounding, insect infestation, and *P. syringae* pv. *tomato* infection (Chao et al. 1999; Gu et al. 1996b; Pautot et al. 1993). In healthy plants, *LapA* mRNAs accumulated at high levels in closed floral buds. Here an antisense RNA strategy was exploited to gain information about the role of

LAP-A in tomato development and defense responses. Two tomato *35S:asLapA1* lines that displayed greatly reduced levels of *LapA* RNAs and no detectable LAP-A activity in floral buds and after wounding were chosen for further analysis.

Immunoblot analysis with LAP-A polyclonal antibodies indicated that the *35S:asLapA1* plants with genes encoding the acidic (*LapA1* and *LapA2*) and neutral (*LapN*) LAPs were down regulated. These data indicated that the chimeric *35S:asLapA1* gene down regulated the wound-induced *LapA* genes (*LapA1* and *LapA2*) and the constitutively expressed *LapN* gene. Comparisons of the *LapA1* region in the *35S:asLapA1* construct with the corresponding *LapN* region indicated that the *LapA1* and *LapN* genes were 84% identical at the nucleotide level. A few short blocks of 100% nucleotide sequence identity (40 bp) also were located in this region (C.-J. Tu and L. Walling, *unpublished results*). The coordinate down regulation of the *LapA* and *LapN* genes suggest that antisense RNAs can tolerate a moderate percentage of sequence mismatch and still be effective. Similar observations were made with the *Petunia hybrida* chalcone synthase gene family (Van der Krol et al. 1990). In fact, the regions with absolute identity may be critical in determining the success of an antisense strategy because Cannon et al. (1990) showed that a 41-bp region with 100% identity was sufficient to impart an antisense effect.

Fig. 5. Growth of *Pseudomonas syringae* pv. *tomato* in control and *35S:asLapA1* transgenic plants. Leaves from 3.5-week-old control and *35S:asLapA1* lines were inoculated with 3.8×10^6 CFU/ml of *P. syringae* pv. *tomato* PT11 (Rif^R Cor⁺) (A and B) or 2.5×10^6 CFU/ml *P. syringae* pv. *tomato* PT23.2 (Rif^R Cor⁻) (C and D). The number of *P. syringae* pv. *tomato* colony-forming units per cm² was monitored over a period of 4 days after inoculation. Each value is the mean titer from four leaf discs from three different plants of each line. Standard error bars are indicated. **A**, Growth of PT11 during compatible interactions in the UC82b control (open triangles) and *35S:asLapA1* lines (closed squares). **B**, Growth of PT11 during incompatible interactions in the Pto238R (open circles) and *35S:asLapA1* Pto lines (closed triangles). **C**, Growth of PT23.2 during compatible interactions in the UC82b control (open triangles) and *35S:asLapA1* lines (closed squares). **D**, Growth of PT23.2 during incompatible interactions in the Pto238R (open circles) and *35S:asLapA1* Pto lines (closed triangles).

Because *LapA* mRNAs increase in incompatible and compatible *P. syringae* pv. *tomato* interactions (Pautot et al. 1993), the impact of *LapA* down regulation on compatible and incompatible interactions was examined. These experiments indicated that the greatly reduced levels of LAP-A and LAP-N did not significantly impact *P. syringae* pv. *tomato* intercellular growth rates during compatible and incompatible interactions. The severity of the symptoms, number and size of lesions, and growth rate of *P. syringae* pv. *tomato* in leaves of *35S:asLapA1* pto and *35S:asLapA1* Pto relative to their control plants were similar. Additionally, the LAP-A and LAP-N deficiency did not alter the growth of *M. sexta* larvae. Studies in potato showed that down regulation of the potato *Lap* gene did not interfere with *Phytophthora infestans* growth (Herbers et al. 1994).

Like pathogen invasion, the role of LAP-A during floral development remains unclear. The levels of *LapA* RNAs and LAP-A proteins in closed tomato buds and in the ovary integument, in particular, are high (Chao et al. 1999; C.-J. Tu and L. Walling, *unpublished results*). The transgenic *35S:asLapA1* tomato lines that had greatly reduced levels of LAP-A and LAP-N exhibited no obvious phenotypic alterations in floral structure and were fully fertile. Similar results were observed when the potato *Lap* antisense RNA was expressed in transgenic potato plants (Herbers et al. 1994). These data suggested that the tomato LAPs might not play a critical role in floral structure and reproductive development.

Fig. 6. Accumulation of LAP-A activity and *LapA* RNAs in response to wild-type (Cor⁺) and coronatine mutant (Cor⁻) strains of *Pseudomonas syringae* pv. *tomato*. Leaves of 3-week-old UC82b plants were mock-infected (MI), or inoculated with 2×10^7 CFU/ml of *P. syringae* pv. *tomato* PT11 (Cor⁻, minus symbol) or PT23.2 (Cor⁺, plus symbol). Leaves from mock-inoculated and Cor⁺- or Cor⁻-compatible interactions were harvested 0, 2, 3, and 4 days later. **A**, Native proteins were extracted from leaves, and 40 μ g of soluble protein was fractionated by native polyacrylamide gel electrophoresis. The swine LAP (SL; 0.5 μ g/lane) was used as control. LAP-A activity was measured by the in situ gel assay, as described in Gu et al. (1996b). Masses of the tomato LAP-A and swine LAP were determined previously (Gu et al. 1996b) and are shown in kD. **B**, Total RNAs were extracted from leaves. Total RNA blots (15 μ g per lane) were hybridized to ³²P-labeled *LapA1* and *pin2* probes. The sizes of *LapA* and *pin2* RNAs are indicated in kb. Autoradiograms were exposed for 2 h.

It is possible that minute quantities of active LAP-A and LAP-N accumulated in the *35S:asLapA1* lines and were sufficient to catalyze their role in development and in curtailment of pathogen invasion or deterring caterpillar growth or feeding. Alternatively, the impact of *LapA* and *LapN* down regulation on plant defense and floral development also could be masked by other exopeptidases that have substrate specificities similar to LAP-A and/or LAP-N. For example, the constitutively expressed tomato-leaf aminopeptidase (AMP1) has a substrate specificity that overlaps with LAP-A. LAP-A and AMP1 hydrolyze substrates with N-terminal leucine (Leu), methionine (Met), and alanine (Ala) residues (Gu and Walling 2000; Gu et al. 1999; N. Ly and L. Walling, *unpublished results*). It is clear that a more comprehensive understanding of the complement of aminopeptidases in healthy and stressed tomato leaves will be necessary to thoroughly evaluate the role of LAP-A and LAP-N in development and in response to stress. Our relative lack of knowledge in this area is indicated by the fact that there have been few comprehensive surveys for developmentally regulated or defense-response aminopeptidases (Walling and Gu 1996). The observation that the aminopeptidase inhibitor, bestatin, activates the tomato-wound response suggests a negative regulatory role for a bestatin-sensitive peptidase in the tomato-defense response (Schaller and Ryan 1996) and emphasizes the need for further studies of peptidases in plant-stress responses. Analysis of transgenic tomato plants that are cosuppressed for *LapA* expression and express a *LapA:asLapA* construct and overexpress *LapA* are being examined for their impact on pathogen and herbivore interactions as well as their roles in protein and peptide turnover.

While *LapA* RNAs accumulated in leaves in response to *P. syringae* pv. *tomato* (Pautot et al. 1993) and the fungal root pathogen *Phytophthora parasitica* (N.-S. Jwa and L. Walling,

unpublished results), the foliar pathogen *Xanthomonas campestris* pv. *vesicatoria* did not induce LAP-A activity (data not shown). In addition, potato *LapA* RNAs did not accumulate in response to *X. campestris* pv. *vesicatoria* or *P. infestans* (Herbers et al. 1994). The fact that i) there is nonuniform induction of *LapA* by pathogens, ii) *LapA* RNAs accumulate with a similar timing in compatible and incompatible interactions with *P. syringae* pv. *tomato* (Pautot et al. 1993), and iii) *LapA* genes are induced by the wound signals MeJA and systemin (Chao et al. 1999) prompted further investigations of the mechanisms underlying the induction of *LapA* by *P. syringae* pv. *tomato*. One feature that distinguishes *P. syringae* pv. *tomato* from the other pathogens mentioned is its ability to produce the phytotoxin coronatine (Bender et al. 1999). Coronatine mimics some of the responses induced by MeJA (Bender et al. 1999; Koda et al. 1996; Wasternack et al. 1998; Weiler et al. 1994), and appears to use similar mechanisms for perception because *A. thaliana* mutants that are resistant to coronatine are also insensitive to MeJA (Feys et al. 1994).

The expression of the tomato *LapA* and *pin2* genes in response to Cor⁺ and Cor⁻ strains of *P. syringae* pv. *tomato* was evaluated. LAP-A activity, *LapA* RNAs, and *pin2* RNAs accumulated to high levels with the Cor⁺ strain but not with the Cor⁻ strain. These data were supported by studies treating excised tomato seedlings with coronatine, indicating that the changes in *LapA* and *pin2* gene expression in response to *P. syringae* pv. *tomato* were mediated primarily by the coronatine. Similar results for *pin2* RNA accumulation in response to coronatine treatments were consistent with coronatine acting as a JA mimic, as reported by Wasternack et al. (1998). Previous studies showed that systemin, a peptide activator of the wound octadecanoid pathway, and MeJA cause *LapA* and *pin2* RNAs to accumulate (Chao et al. 1999; Farmer and Ryan 1990; Pearce et al. 1991; Wasternack et al. 1998).

The responses of two *PR* genes to coronatine also were examined. The tomato *PR-4* transcripts did not accumulate in response to MeJA (Chao et al. 1999) or coronatine. Surprisingly, *PR-1* RNAs accumulated in response to exogenous MeJA (Chao et al. 1999), yet coronatine treatments were insufficient to cause a large increase in *PR-1* transcript levels. These data indicated that, despite similarities in many coronatine and MeJA-induced responses (Koda et al. 1996; Wasternack et al. 1998; Weiler et al. 1994), coronatine and MeJA act in a distinct manner in the excised seedling assay. The in vivo expression of wound- and defense-response genes often is separated temporally as a result of reciprocal regulation of the wound-octadecanoid pathway and defense-response pathways by MAP kinases, small GTP-binding proteins, and SA (Doares et al. 1995; Doherty et al. 1988; Peña-Cortés et al. 1993; Romeis et al. 1999; Sano et al. 1994; Seo et al. 1995). For example, Mittal et al. (1995) showed that Cor⁺ *P. syringae* pv. *tomato* delayed expression of *A. thaliana* defense-response genes relative to Cor⁻ *P. syringae* pv. *tomato* strains. Coronatine and systemin treatments retain the cross talk that coordinates the wound- and defense-response signaling pathways because both treatments induce wound-response genes such as *LapA* and *pin2* but not *PR* genes (Fig. 6 and Table 1) (Chao et al. 1999). In contrast, MeJA treatments do not retain this specificity because exogenous MeJA treatments induce both wound-response genes (*LapA* and *pin2*) as well as JA-regulated *PR* genes (*GluB*, *Chi9*, and *PR-*

Fig. 7. Coronatine RNA blots. Shoots were excised from 3-week-old seedlings and 1 μ mol of coronatine in 0.002% ethanol was applied to excised seedlings. Control plants were treated with 0.002% ethanol in a similar manner. Leaves were harvested 0, 2, 4, 8, and 16 h after treatment. Total RNAs were extracted from leaves. Total RNA blots (15 μ g per lane) were hybridized to ³²P-labeled *LapA1*, *pin2*, *PR-1*, and *PR-4* probes. The sizes of RNAs are indicated in kb. The *LapA1*, *pin2*, *PR-1*, and *PR-4* RNA blots were exposed to film for 72 h.

1) (Chao et al. 1999; D. Puthoff, C. LeVesque, W. Chao, T. Perring, and L. Walling, *unpublished results*).

Although the signal transduction pathways that are responsible for mediating coronatine and MeJA inhibition of root growth and wound response overlap (Feys et al. 1994; Rojo et al. 1999), the data presented here shows that there must be elements of the wound- and defense-signaling pathways that respond to exogenous MeJA and coronatine differently. One interpretation is that MeJA treatments did not produce a regulator that suppressed the defense pathway, thereby leading to up regulation of *PR-1*, and that coronatine and systemin treatments may have produced this regulator. Alternatively, coronatine and systemin may have induced an inhibitor of JA-regulated *PR-1* expression or prevented the accumulation of a defense-response factor critical for its expression. It also is possible that the JA that accumulated after systemin treatments and the coronatine that was taken up in the seedling assays may be compartmentalized and therefore not impact cells that can express *PR* genes (Chao et al. 1999; Harms et al. 1995; Schaller and Ryan 1996). Perhaps MeJA and/or coronatine were transported or catabolized at different rates in different populations of cells, allowing coronatine to solely induce wound-response genes and MeJA to activate wound- and defense-response genes. These surprisingly complex interactions stress that intricate networks are utilized to coordinate and prioritize the multiple signal transduction pathways active in response to pathogens and pests (Dempsey et al. 1999; Pieterse and van Loon 1999; Ryan 2000; Walling 2000).

MATERIALS AND METHODS

Construction of transgenic tomatoes expressing 35S:asLapA1.

The 1.4-kb *EcoRI*–*SpeI* fragment containing 81% of the *LapA1* coding region (nucleotides 324 to 1,736 in the *LapA1* cDNA sequence; GenBank accession no. U50151) was used to design a *LapA1* antisense construct. This fragment was shuttled into pBluescript-KS+ to facilitate further manipulations. The *LapA1* fragment was excised from pBluescript-KS+ with *XbaI* and *EcoRI* and subcloned into pUC18. The pUC18 clone was digested with *EcoRI* and *SalI*. The *LapA* fragment was cloned in the antisense orientation between the CaMV 35S promoter with a double enhancer and the transcriptional termination region from the CaMV 35S gene in the pLBR19 plasmid vector (T. Michael, Norwich Research Park, Colney, Norwich, U.K.). pLBR19 was derived pJIT60 (Guerineau et al. 1992) and the *EcoRI* site of pJIT60 was abolished by adding an *XbaI* linker. The chimeric 35S:asLapA1 gene was excised with *XhoI* and *KpnI* and ligated into the binary transformation vector pBin19 hydrolyzed with *KpnI* and *SalI* (Bevan 1984). This construct was introduced into *A. tumefaciens* C58 (An et al. 1988).

Conditions for plant and *A. tumefaciens* growth and procedures for tomato transformation have been described (Hamza and Chupeau 1993). *Lycopersicon esculentum* Mill. cv. UC82b (Harris Moran Seed Co., Salinas, CA, U.S.A.) cotyledons were infected with *A. tumefaciens* (35S:asLapA1). Plants were regenerated in the presence of kanamycin. The presence of the 35S:asLapA1 transgene in T₀ lines 1 to 6 was detected by genomic DNA blot analysis. Homozygous T₂ progeny from lines 3 and 4 were identified. The 35S:asLapA1 plants, similar

to their parental line UC82b, were susceptible to *P. syringae* pv. *tomato* infection (*pto* allele).

Homozygous 35S:asLapA1 *pto* (line 4) plants were used as the female parent in a cross with a homozygous *Pto* line (Peto238R). Peto238R is an inbred tomato line with the *Pto* resistance gene, which confers resistance to *P. syringae* pv. *tomato* race 0 strains. Peto238R was initially provided by J. Williams (Petoseed Co., Saticoy, CA, U.S.A.). F₁ and F₂ progeny expressing the *Pto* gene were identified by screening for resistance 72 h after inoculation of leaves with 5×10^6 CFU/ml *P. syringae* pv. *tomato*, as described previously (Stockinger and Walling 1994). The presence of the *Pto* allele was confirmed with allele-specific primers (*Pto2* primer: 5'-T GCTTTAAGCTCGAGTTATC-3'; *Pto5* primer: 5'-TCCCTTG GAAGAGATTGAACT). The presence of the 35S:asLapA1 transgene was followed with primers to the linked kanamycin resistance gene (Npt-F primer: 5'-CCGCGTACCTGCCCAT C-3'; Npt-R primer: 5'-GCGATAGAA-GGCGATGCG-3'). Annealing temperatures for the *Pto* and *nptII* primers were 52 and 63°C, respectively. Polymerase chain reaction conditions have been described previously (Van de Ven et al. 2000). A homozygous 35S:asLapA1 *Pto* plant was identified and used in *P. syringae* pv. *tomato* infection studies.

P. syringae pv. *tomato* infections, wounding, and coronatine treatments.

Control and 35S:asLapA1 transgenic tomato plants were grown in a greenhouse under day–night temperatures of 30°C (day), 15°C (night), or in growth chambers with a 16-h day cycle at 27 to 28°C and an 8-h night cycle at 18 to 20°C. Completely open flowers and 8-mm green buds were harvested from greenhouse-grown plants. Tissues used for LAP activity assays were used immediately; tissues used for RNA extractions were frozen in liquid nitrogen and stored at –80°C until use. Methods for *P. syringae* pv. *tomato* inoculation, wounding, tissue harvest, and tissue storage have been described previously (Pautot et al. 1991).

A rifampicin-resistant *P. syringae* pv. *tomato* PT11 was selected by their ability to grow on King's B media plates containing 50 µg of rifampicin per ml and 0.2 % (vol/vol) dimethyl sulfoxide (DMSO). PT11 (Rif^R) produces the phytotoxin coronatine (Cor⁺), whereas PT23.2 (Rif^R) is defective in coronatine production (Cor[–]). For compatible interactions, *P. syringae* pv. *tomato* was inoculated on control (UC82b) and 35S:asLapA1 leaves. For incompatible interactions, *P. syringae* pv. *tomato* was inoculated on control (Peto238R *Pto*) and 35S:asLapA1 *Pto* leaves. Leaves from 3.5-week-old control and 35S:asLapA1 plants were inoculated with 3.8×10^6 CFU/ml of the *P. syringae* pv. *tomato* Cor⁺ and 2.5×10^6 CFU/ml of the Cor[–] strains. At various times after inoculation, two leaves were harvested from each inoculated plant and four disks (0.78 cm²) were excised. Disks from a single plant were

Table 1. Expression of wound- and defense-response genes.

Gene	Treatments		
	Systemin	Coronatine	MeJA
<i>LapA</i>	+	+	+
<i>pin2</i>	+	+	+
<i>PR-1</i>	–	–	+
<i>PR-4</i>	–	–	–

pooled and three independent plants were assessed per time point. Four disks were rinsed three times in 50 ml of 1× phosphate-buffered saline (PBS) (170 mM NaCl; 6.2 mM KCl; 12.6 mM Na₂HPO₄; 2.2 mM KH₂PO₄, pH 7.4). Leaf disks were homogenized in 1× PBS and the homogenate was serially diluted in 1× PBS to determine the number of colony forming units per milliliter. Titers of rifampicin-resistant *P. syringae* pv. *tomato* were determined on King's B medium with 50 µg of rifampicin per ml and 0.2% DMSO.

For coronatine treatments, 3-week-old tomato plants were excised at the base of the shoot. A 90-µl drop of 1 µmol of coronatine in 0.002% ethanol was applied to the shoot. After 3 to 4 min, the drop was taken up by the transpiration stream and shoots were placed in water for 2 to 16 h. Control seedlings were fed 90 µl of 0.002% ethanol.

Insect feeding experiments.

M. sexta eggs were obtained from Plant Genetic Systems (Gent, Belgium). The greatest survival rate for larvae occurred when new emerging larvae were fed on leaves. Raising larvae on an artificial diet prior to transfer to control or 35S:*asLapA1* tomato plants decreased survival significantly. For this reason eggs were placed on detached leaves from 35S:*asLapA1* plants that did not accumulate or had low levels of LAPs and were incubated at 25°C. Seven 2- to 3-day-old first-instar larvae (6 mg each) were selected and placed on each 5-week-old control and 35S:*asLapA1* plant in insect cages in the greenhouse. Leaves from six independent plants of each transgenic line were used in feeding trials. After 9 and 12 days, surviving larvae were removed, their developmental stages determined, and their masses recorded. Larvae survival rate on control and antisense plants was 50%.

RNA isolation and RNA blot hybridizations.

Procedures for the isolation of total RNAs, fractionation of RNAs by formaldehyde agarose gel electrophoresis, RNA transfer to nylon membranes, and RNA blot hybridization have been described (Pautot et al. 1991). All gels were stained with ethidium bromide to assure equivalent loading per lane. All RNA blots were exposed to XAR5 film (Kodak, Rochester, N.Y., U.S.A.) at -80°C with one Quanta III intensifying screen (Kodak). ³²P-labeled *LapA1*-antisense RNAs were synthesized in vitro with T3 RNA polymerase (Gibco BRL, Cergy Pontoise, France) and [α-³²P]-CTP (Amersham, Les Ulis, France) from the pBluescript-KS *LapA1* subclone. This subclone contained the 1.6-kb *EcoRI*-*XbaI* *LapA1* fragment from pDR57 (Pautot et al. 1993). DNA probes were labeled with [α-³²P]-dCTP and the random priming method of Feinberg and Vogelstein (1983) or with a nick-translation kit (Gibco BRL). Under the stringent hybridization conditions used in these studies and described in Pautot et al. (1991), the ³²P-labeled *LapA1* probe detected *LapA1* and *LapA2* RNAs but did not cross hybridize with the constitutive, rare-class *LapN* transcripts (C.-J. Tu and L. Walling, unpublished results). The proteinase inhibitor II (*pin2*; pT2-47), *PR-1* (pP6), and *PR-4* (pPR-P2) cDNA clones have been described (Graham et al. 1985; Linthorst et al. 1991; Van Kan et al. 1992).

Protein extraction, fractionation, immunoblots, and LAP activity assays.

The procedures for the extraction of native, soluble proteins and aminopeptidase gel activity assays have been described

(Gu et al. 1996b). While the activities of the LAP-A and the fast migrating aminopeptidase (AMP1) were visualized in this assay, LAP-N activity was not detected. Swine LAP was purchased from Sigma (St. Louis, MO, U.S.A.) Total protein extraction, SDS-PAGE, 2D-PAGE, silver staining, and immunoblots were performed according to Gu et al. (1996b). A 1:500 dilution of the LAP-A polyclonal antisera was used in the immunoblot analyses. Immunoblots were performed with proteins from homozygous progeny of the 35S:*asLapA1* lines 3 and 4 plants.

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