Suppressors of Systemin Signaling Identify Genes in the Tomato Wound Response Pathway

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Manuscript received March 31, 1999
Accepted for publication July 6, 1999

ABSTRACT

In tomato plants, systemic induction of defense genes in response to herbivory or mechanical wounding is regulated by an 18-amino-acid peptide signal called systemin. Transgenic plants that overexpress prosystemin, the systemin precursor, from a 35S::prosystemin (35S::prosys) transgene exhibit constitutive expression of wound-inducible defense proteins including proteinase inhibitors and polyphenol oxidase. To study further the role of (pro)systemin in the wound response pathway, we isolated and characterized mutations that suppress 35S::prosys-mediated phenotypes. Ten recessive, extragenic suppressors were identified. Two of these define new alleles of def-1, a previously identified mutation that blocks both wound- and systemin-induced gene expression and renders plants susceptible to herbivory. The remaining mutants defined four loci designated Spr-1, Spr-2, Spr-3, and Spr-4 (for Suppressed in 35S::prosystemin-mediated responses). spr-3 and spr-4 mutants were not significantly affected in their response to either systemin or mechanical wounding. In contrast, spr-1 and spr-2 plants lacked systemic wound responses and were insensitive to systemin. These results confirm the function of (pro)systemin in the transduction of systemic wound signals and further establish that wounding, systemin, and 35S::prosys induce defensive gene expression through a common signaling pathway defined by at least three genes (Def-1, Spr-1, and Spr-2).

PLANT defense against herbivores often relies on the rapid synthesis of phytochemicals that adversely affect the growth and development of the attacking pest. One of the best characterized examples of this is the wound-inducible expression of proteinase inhibitors, low-molecular-weight proteins that inhibit the activity of digestive enzymes in the gut of the herbivore (Ryan 1990). Proteinase inhibitor genes, in coordination with many other defense-related genes, are induced in response to wounding both at the site of damage and in distal unwounded leaves of the plant (Bergey et al. 1996). These observations imply a mechanism to transduce signals over long distances (Green and Ryan 1972). Wound-inducible proteinase inhibitors in tomato have provided a model system useful for identifying the signals involved and the mechanisms by which they are communicated between cells.

A search for compounds in leaf extracts that induce proteinase inhibitor expression in tomato led to the identification of an 18-amino-acid peptide called systemin (Pearce et al. 1991). Synthetic systemin can be transported through the plant following its application to wound sites, suggesting that the peptide is a mobile signal for wound responses. Systemin is derived from a 200-amino-acid precursor called prosystemin. A requirement for prosystemin in wound-inducible gene expression has been demonstrated with transgenic plants that express antisense prosystemin mRNA (McGurl et al. 1992). Recent studies indicate that prosystemin expression in tomato leaves is restricted to the vascular bundles, which is consistent with a role for this polypeptide in long-distance signaling (Jacinto et al. 1997). Wounding enhances prosystemin expression in vascular tissue but does not affect its spatial expression pattern. Many gaps remain in our understanding of systemin action, including the mechanism by which systemin is released from prosystemin, how this process is regulated during the wound response, and how cells perceive systemin.

Activation of defense gene expression by systemin involves a signal transduction pathway whose molecular components are only partially understood. Several lines of evidence indicate that the octadecanoid pathway for conversion of linolenic acid to jasmonic acid (JA) is critical for systemin-induced defense gene expression (Ryan and Pearce 1998). It has been proposed that wounding and systemin regulate JA synthesis through the action of lipases that mobilize octadecanoid precursors from membrane lipids (Farmer and Ryan 1992; Conconi et al. 1996; Lee et al. 1997). Allene oxide synthase, which catalyzes the committed step in JA biosynthesis, may also play an important role in the regulation of octadecanoid signaling (Harms et al. 1995; Lauder and Weiler 1998). Wound- and systemin-inducible gene expression also requires ethylene and abscisic acid...
of a 48-kD protein kinase, suggesting the involvement of induced resistance to herbivory. Moyen et al. (1996) suggested the involvement of a phosphorylation cascade early in the signaling pathway. The discovery that systemin evokes rapid cellular responses has provided insight into the early events of the signal transduction pathway. Low concentrations of systemin induce rapid ion fluxes across the plasma membrane, increases in intracellular Ca\(^{2+}\) levels, and transient depolarization of mesophyll cell membranes (Felix and Boller 1995; Moyen and Johannes 1996; Moyen et al. 1998). Systemin also causes rapid activation of a 48-kD protein kinase, suggesting the involvement of a phosphorylation cascade early in the signaling pathway (Stratmann and Ryan 1997; Meindl et al. 1998). It is presently unclear if these responses are components of the signal transduction pathway leading to activation of defense gene expression or if they represent other, unknown signaling pathways that are mediated by wounding and systemin. Another approach to understanding the initial events associated with systemin perception has come from the identification of systemin binding proteins. A plasma membrane protein called SBP50 was shown to bind to the N-terminal part of systemin. Binding was associated with cleavage of the systemin, suggesting that SBP50 functions in systemin degradation rather than as a receptor (Schaller and Ryan 1994). Meindl et al. (1998) have recently identified an additional binding site in cultured tomato cells that shows predicted characteristics of a functional systemin receptor.

Genetic analysis offers a potentially powerful approach for dissecting the wound response pathway and the role of systemin in it. To facilitate the identification of mutations in the systemin signaling pathway, we employed tomato plants that express a 35S::prosystemin transgene (35S::prosys) and, as a consequence, accumulate high levels of wound response proteins in the absence of wounding (McGurl et al. 1994). Several lines of evidence indicate that the phenotype of 35S::prosys plants results from constitutive activation of the endogenous wound response pathway. For example, genes whose expression is wound inducible in wild-type plants are constitutively expressed in 35S::prosys plants (Bergey et al. 1996). Second, a mutation (def-1) that inhibits wound-inducible gene expression in wild-type plants also blocks 35S::prosys activation of these genes (Howe et al. 1996). Finally, 35S::prosys plants produce a proteinase inhibitor-inducing signal that is transmissible across graft junctions (McGurl et al. 1994). This indicates that 35S::prosys functions in a cell-nonautonomous manner, consistent with the systemic nature of the wound response.

Here we report the identification and characterization of mutations that suppress 35S::prosys-mediated responses. Genetic analysis indicated that we isolated mutations at the Def-1 locus, which is known to be required for responsiveness to systemin and wounding. Eight additional mutants defined four new loci designated Spr (for Suppressor of 35S::prosys-mediated responses). At least two of these genes (Spr-1 and Spr-2) are required for responsiveness to both systemin and mechanical wounding. These results confirm the role of (pro)-systemin in the transduction of systemic wound signals and further establish that wounding, systemin, and 35S::prosys induce defensive gene expression through a common signaling pathway. Analysis of these mutants should help to define further the systemin response pathway and assist in understanding the molecular basis of induced resistance to herbivory.

**MATERIALS AND METHODS**

**Plant material:** Tomato seedlings were grown under 17-hr days (28° with light intensity of 300 \(\mu\)E m\(^{-2}\) sec\(^{-1}\)) and 7-hr nights (17° in total darkness). Agrobacterium-mediated transformation of tomato (Lycopersicon esculentum, var. Better Boy) with 35S::prosys was previously described (McGurl et al. 1994). A transgenic line (called 1-2J) containing a single T-DNA insertion was identified by Southern blot analysis. The phenotype of this line (constitutive accumulation of proteinase inhibitor I, proteinase inhibitor II, and polyphenol oxidase) is referred to here as Inh\(^{-}\), PPO-1. Spontaneous reversion of the Inh\(^{-}\) phenotype was not observed among 241 T\(_1\) plants derived from 1-2J. The following genetic analysis also showed that 35S::prosys behaved as a dominant single gene. A 1-2J plant (Inh\(^{-}\), PPO-1; T\(_1\) generation) was outcrossed to a wild-type cultivar (Castlemart). The resulting F\(_1\) progeny were exclusively Inh\(^{-}\), indicating that the 35S::prosys mutation is dominant. In an F\(_2\) population derived from this cross, a 3:1 segregation ratio of Inh\(^{+}\)PPO\(^{+}\) plants to Inh\(^{-}\)PPO\(^{-}\) plants was observed (\(X^2 = 0.085\) for the 3:1 hypothesis). Furthermore, the Inh\(^{+}\)PPO\(^{+}\) phenotype consistently cosegregated with the ka-namycin-resistant phenotype conferred by the integrated T-DNA. Construction of the def-1(J) 35S::prosys line was previously described (Howe et al. 1996).

**Mutagenesis:** Approximately 1300 T\(_1\) seed collected from 1-2J were mutagenized with ethyl methanesulfonate (EMS). Seed were soaked in water for 2 hr, and then transferred to an unbuffered solution of 70 mm EMS. Seed were soaked in the EMS solution in the dark at 20° for 22 hr, washed extensively under running water, and then sown directly into soil. The germination success (88%) of EMS-treated seed was not significantly different from that of untreated seed. A total of 1126 M\(_2\) seedlings (3 wk old) were transplanted to a field plot in Woodland, California. At maturity, seed were collected from each plant to ensure that mutants identified arose independently. The effectiveness of the EMS treatment was apparent from the frequency of visible mutations segregating in the M\(_2\) generation. These phenotypes, and the percentage of M\(_3\) families segregating the phenotype, included: chlorotic (12%), albino (2.7%), reduced anthocyanin in hypocotyl (1.6%), altered leaf morphology (0.5%), autonecrotic (0.4%), dwarf (0.3%), and high anthocyanin pigmentation (0.2%).

**Mutant isolation:** Twenty-five plants per M\(_2\) family were screened for polyphenol oxidase (PPO) activity as follows. The terminal leaflet on the oldest leaf of the seedlings (16–18 days old) was removed and folded in half several times. Using pressure applied from a finger (gloved), the leaf extract was
adsorbed onto a sheet of Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). A 18- x 24-cm sheet of membrane accommodated ~300 such “squeashes.” Wild-type (Better Boy) and 1-2 parental plants grown in the same flat were used as negative and positive controls, respectively, for PPO staining. Membranes were stained for PPO activity using a modification of a procedure used to stain acrylamide gels for PPO activity (Wissemann and Montgomery 1985). Briefly, the membrane was placed (extract side up) onto Whatman 3MM paper saturated with a solution containing 0.2 m sodium phosphate, 0.1 m citric acid, 15 mm catechol, 0.05% p-phenylenediamine (w/v), pH 5.0. Development of a dark purple precipitate in samples containing PPO activity was complete within 10 min (Figure 1). Stained membranes were washed for several minutes under running water and then dried at ambient temperature. PPO activity stains on the dried membranes were stable for at least 3 years with storage in the dark.

RNA gel blot analysis: RNA isolation from leaf tissue and gel blot analysis was performed as previously described (Howe et al. 1996). Samples (5 µg total RNA/lane) were electrophoresed on agarose/ formaldehyde gels in duplicate, with one set stained after electrophoresis with ethidium bromide to ensure equal loading of samples and the intactness of the RNA. Tomato cDNAs used as probes and the methods for 32P labeling were as described in Howe et al. (1996). Hybridization signals were measured with a PhosphorImager (Molecular Dynamics) and normalized to a signal obtained from a constitutively expressed transcript eIF4A. An Arabidopsis cDNA encoding eIF4A was provided by Dr. P. Green (Michigan State University). Hybridization and subsequent washing of eIF4A-probed blots was performed at 60° in 2× SSPE.

Miscellaneous procedures: Radial immunodiffusion assays for Inh-I and Inh-II in response to wounding and elicitors (systemin, JA, and MeJA) were as previously described by Ryan (1997). Spectrophotometric measurements of PPO were performed using 3,4-dihydroxyphenylalanine (DOPA) as a substrate, as described by Constabel and Ryan (1998). The protein content of the extracts was determined by the Bradford assay.

RESULTS

Isolation of 35S::prosys suppressors: Our strategy for obtaining mutants in the wound response pathway employed a 35S::prosys transgenic line that accumulates proteinase inhibitors I and II (Inh-I and Inh-II) and PPO in the absence of wounding (McGurl et al. 1994; Constabel et al. 1995; Bergey et al. 1996). We reasoned that mutations that suppress the constitutive phenotype (designated here as Inh 35S::prosys plants would define genes in the wound/systemin response pathway. A control experiment was performed to determine the extent to which a mutation (def-1) known to block systemin- and wound-mediated signaling would suppress the Inh 35S::prosys genetic background (Table 1). The results showed that plants homozygous for both def-1 and 35S::prosys (designated def-1/35S::prosys) contain levels of Inh and PPO that are comparable to those found in unwounded wild-type plants. Thus, def-1 is a strong suppressor of 35S::prosys-mediated signaling.

Seed from a line (1-2) that is homozygous for a single insertion of 35S::prosys was mutagenized with EMS. Twenty-five M2 seedlings from each of 1087 M1 seed lots were tested for PPO accumulation using a rapid assay in which leaf protein immunobilized to nitrocellulose membrane was stained for PPO activity. This procedure provided a reliable means to distinguish the high leaf PPO content of 35S::prosys plants from low PPO levels found in unwounded wild-type plants or def-1/35S::prosys plants (Figure 1A). Using this screen, 17 M2 families that segregated at least one PPO-deficient (PPO –)
TABLE 1
Proteinase inhibitor and polyphenol oxidase levels in different tomato genotypes

<table>
<thead>
<tr>
<th>Plant genotype</th>
<th>Inh-I (μg/ml leaf juice)</th>
<th>Inh-II (μg/ml leaf juice)</th>
<th>PPO activity (ΔA490/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10 ± 5</td>
<td>11 ± 6</td>
<td>0.21 ± 0.02</td>
</tr>
<tr>
<td>35S::prosys (1-2J line)</td>
<td>147 ± 10</td>
<td>211 ± 23</td>
<td>14.50 ± 0.98</td>
</tr>
<tr>
<td>df-1/35S::prosysa</td>
<td>12 ± 1</td>
<td>15 ± 1</td>
<td>0.15 ± 0.04</td>
</tr>
</tbody>
</table>

Levels of Inh-I, Inh-II, and PPO were determined in leaf extracts prepared from 17-day-old unwounded seedlings. Data represent the mean and standard deviation (n = 18 plants).

*This line is homozygous for both df-1 and 35S::prosys.

plant were identified. An example of 1 such family is shown in Figure 1B. PPO − plants from each family were tested for the accumulation of Inh-I and Inh-II, two additional biochemical markers of 35S::prosys signaling. PPO − plants from 4 families accumulated Inh-I and -II to levels comparable to the 35S::prosys parent, suggesting that mutations in these lines affect PPO biosynthesis rather than 35S::prosys signaling. Inh accumulation in PPO − plants from the remaining 13 families was reduced to <20% of that found in the 35S::prosys parent (see below). Subsequent experiments were focused on the characterization of these Inh − PPO − lines.

Inh − PPO − phenotypes could result from mutations that disrupt any physiological process required for 35S::prosys-mediated expression of Inh and PPO. For example, because Inh and PPO accumulation in leaf tissue of 35S::prosys plants increases with plant age (McGurl et al. 1994), plants affected in their rate of vegetative growth constituted a major source of false-positive mutants (appearing to be Inh − PPO −). We were able to identify and eliminate this class of plants by retesting Inh and PPO levels several times during the vegetative growth of the putative mutant. We also considered the possibility that Inh − PPO − mutants might be affected in the biosynthesis or perception of hormones known to play a role in the wound response pathway (O’Donnell et al. 1996; Peña-Cortés et al. 1996; Carrera and Prat 1998). In general, the vegetative growth of all mutants was vigorous, and the Inh − PPO − phenotype was stable throughout vegetative growth. Phenotypes associated with ABA deficiency (e.g., wilting of leaves at low humidity, increased water loss from excised leaves, or reduced seed dormancy) were not observed. Phenotypes associated with ethylene deficiency (e.g., lack of fruit ripening) were also not apparent. Notably, Inh − PPO − mutants from three families failed to produce viable M3 seed. This impeded the further characterization of these mutants, and subsequent studies were focused on 10 fertile Inh − PPO − mutants. Seed set and viability in 8 of these (88C, 532E, 593F, 797A, 823B, 956A, 961E, 1018A) was normal, whereas viable seed yield in two lines (510B and 572A) was reduced to ~10% of that in the other lines.

Quantitative measurements of PPO activity in the 10 mutants were in good agreement with the levels estimated from the PPO activity stain (Figure 2). Lines 88C and 956A, which contained the highest residual levels of PPO activity, also accumulated low but detectable levels of Inh protein. Mutants exhibiting complete loss of PPO activity contained no detectable Inh (e.g., 532E, 593F, 823B, 961E, 1018A). Stable inheritance of the suppressed phenotype of each line has been demonstrated through at least the M5 generation. RNA blot

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**Figure 2.**—Accumulation of polyphenol oxidase and proteinase inhibitors in selected mutants. (A) PPO activity in lysates obtained from leaves of unwounded wild-type plants (WT), 35S::prosys-expressing plants (1-2J), and 10 selected Inh − PPO − lines. Terminal leaflets from the bottom two leaves of 12 18-day-old plants were pooled for protein extraction and spectrophotometric determination of PPO activity. One representative experiment from three independent experiments is shown. (B) Inh-I (solid bars) and Inh-II (open bars) levels were measured in extracts derived from leaves of 18-day-old, unwounded seedlings. Values represent the mean and standard deviation of 6 plants.
analysis was used to examine the constitutive level of several transcripts whose expression is known to be wound inducible in leaves of wild-type plants (Figure 3). In contrast to unwounded wild-type plants, 35S::prosys plants contained high steady-state levels of mRNAs encoding Inh-I, Inh-II, cathepsin D inhibitor (CDI), and leucine amino peptidase (LAP). The abundance of these transcripts in the 10 mutants was reduced to less than 10% of that in the 35S::prosys parental line and comparable to those of unwounded wild-type plants. Mutants 88C and 956A displayed the highest residual expression of 35S::prosys-regulated genes, consistent with the higher expression of Inh and PPO protein in these lines (Figure 2). All mutants expressed high levels of prosystemin mRNA derived from the 35S::prosys transgene. This result excludes the possibility that the suppressed phenotypes are the consequence of reduced expression of 35S::prosys.

35S::prosys-mediated hypocotyl phenotypes are suppressed in Inh-PPO- mutants: We observed that hypocotyls of light-grown 35S::prosys seedlings are elongated, and more pigmented, compared to those of wild-type seedlings of the same age (Figure 4, A and B). In crosses between 35S::prosys and wild-type plants, this phenotype was dominant and consistently cosegregated with the Inh-PPO+ phenotype in F2 populations (G. A. Howe, unpublished data). These observations suggested that the hypocotyl phenotype was directly related to 35S::prosys expression or signaling. During the screen for 35S::prosys suppressor mutants it became apparent that plants identified as Inh-PPO- had a wild-type hypocotyl phenotype (e.g., shorter length and less pigmentation), readily distinguishable from Inh-PPO+ siblings in the same M2 family (Figure 4A). In fact, we often identified mutants by their hypocotyl phenotype prior to confirmation of the Inh and PPO deficiency. Measurements of hypocotyl length for each suppressor mutant showed that these differences were significant and heritable in subsequent generations (Figure 4C). These results demonstrate that 35S::prosys conditions increased defense gene expression and changes in hypocotyl morphology and that the mutations identified suppress both of these phenotypes.

Genetic analysis of mutants: A set of crosses was performed to determine the genetic basis of the Inh-PPO- phenotype of each mutant. The induced mutations were determined to be recessive or dominant by crossing each line to the 35S::prosys parent and then testing F1 progeny for PPO and Inh accumulation. F1 plants derived from crosses to all 10 mutants tested positive for PPO activity and accumulated Inh-I and Inh-II to levels similar to or slightly below those in 35S::prosys plants (Table 2). F1 plants from each cross also displayed elongated hypocotyls (data not shown). Inh-PPO+ F1 plants were self-pollinated and the phenotype of F2 progeny were scored. The ratio of Inh-PPO+ to Inh-PPO- plants was ~3:1 in all F2 populations, although slight deviations from this ratio were observed in some cases (e.g., line 956A, Table 2). These results indicate that the suppressor phenotype of each mutant is caused by a single recessive mutation in a nuclear gene.

We also tested the possibility that the mutants identified arose from mutations within the 35S::prosys transgene (intragenic suppressors). Each mutant was outcrossed to a wild-type cultivar (Castlemart) and the resulting F1 progeny were tested for Inh and PPO accumulation. Due to the recessive nature of the suppressor mutations and the dominance of 35S::prosys, the appearance of Inh-PPO+ F1 progeny would indicate that the mutant has a functional 35S::prosys allele. Conversely, Inh-PPO+ F1 progeny would suggest that the mutant harbors a defective 35S::prosys allele. For each of the 10 mutants, hemizygous (35S::prosys/+) F1 plants were Inh-PPO+ and exhibited the elongated hypocotyl phenotype of the 35S::prosys parent (Table 2). These results indicate that all mutations identified are extragenic suppressors of 35S::prosys.

Intermutant crosses were performed to determine the number of complementation groups defined by the mutants (Table 3). F1 progeny obtained from each cross were scored for complementation (Inh-PPO+) or non-complementation (Inh-PPO-). def-1 plants complemented the phenotype of all mutants except 88C and
Consistent with this, 88C did not complement and 1018A. Crosses between the latter four mutants yielded all Inh⁻PPO⁻ progeny. These results indicate that 532E, 593F, 797A, 961E, and 1018A contain mutations in the same gene. Lines 572A, 823B, and 956A complemented the mutant phenotype of all other mutants, indicating the existence of three additional loci. On the basis of these data, at least four complementation groups were obtained in addition to Def-1. These mutants were named spr as follows: spr-1 532E/ 35S::prosys (532E), spr-1 572A/ 35S::prosys (532E), spr-1 823B/ 35S::prosys (532E), spr-1 956A/ 35S::prosys (956A), spr-2 532E/ 35S::prosys (532E), spr-3 572A/ 35S::prosys (572A), spr-4 823B/ 35S::prosys (823B), and spr-1 35S::prosys (956A).

Response of mutants to wounding: Using a representative mutant from each complementation group, we next tested the effect of the suppressor mutations on the wound response pathway. Seedlings were mechanically damaged on their lower leaves and Inh-II protein was measured 24 hr later in the wounded leaves and separately in the upper unwounded leaves. Wild-type and 35S::prosys plants, although differing in their constitutive levels of wound response proteins, accumulated Inh-II in both damaged and undamaged leaf tissue in response to wounding (Figure 5). In contrast, def-1 510B/ 35S::prosys, spr-1 532E/ 35S::prosys, and spr-2 35S::prosys mutants were significantly impaired in wound-inducible Inh-II synthesis. The def-1 510B/ 35S::prosys mutant displayed the most severe deficiency, with no Inh-II accumulation detected in either damaged or undamaged leaves of wounded plants. spr-1 532E/ 35S::prosys and spr-2 35S::prosys mutants accumulated 10–30% of wild-type levels of Inh-II in damaged leaves. However, systemic accumulation of Inh in these mutants was not detected. Other alleles of spr-1 had a similar effect on wound-inducible Inh accumulation, with spr-1 797A being the weakest suppressor of the wound response among this group (data not shown). In contrast to the impaired wound response of def-1,

Figure 4.—35S::prosys-mediated hypocotyl phenotypes are suppressed in Inh⁻PPO⁻ mutants. (A) Photograph showing the hypocotyl phenotype associated with 35S::prosys expression. The three seedlings shown are F₂ siblings derived from a cross between the 35S::prosys parental line (1-2J) and mutant 532E. The seedling in the middle (short hypocotyl) is an Inh⁻PPO⁻ segregant from the cross, whereas the seedlings on the left and right are Inh⁻PPO⁺ siblings. Arrows denote the position of the cotyledons. (B) Hypocotyl elongation in developing seedlings. Seeds (35S::prosys-expressing 1-2J line, open circles; wild-type Better Boy cultivar, solid circles) were germinated on moist filter paper in the dark. Synchronously germinated seedlings were transferred to soil and grown under standard growth conditions. Hypocotyl lengths were measured 10, 12, 14, 17, and 20 days after germination. Data represent the mean and standard deviation of 24 plants. (C) Hypocotyl length of Inh⁻PPO⁻ mutants compared to that of wild-type (WT) and 35S::prosys (1-2J) plants. Hypocotyl measurements were made 16 days after germination. Data represent the mean and standard deviation of 12 plants.
Table 2: Genetic analysis of Inh−PPO− mutants

<table>
<thead>
<tr>
<th>Cross†</th>
<th>Generation</th>
<th>Inh−PPO+</th>
<th>Inh−PPO−</th>
<th>χ² d</th>
</tr>
</thead>
<tbody>
<tr>
<td>88C × 1-2J</td>
<td>F₁</td>
<td>6 (80%) c</td>
<td>0</td>
<td>1.85</td>
</tr>
<tr>
<td>88C × wt</td>
<td>F₁</td>
<td>129</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>510B × 1-2J</td>
<td>F₁</td>
<td>13 (107%)</td>
<td>0</td>
<td>5.12</td>
</tr>
<tr>
<td>510B × wt</td>
<td>F₁</td>
<td>210</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>532E × 1-2J</td>
<td>F₁</td>
<td>12 (79%)</td>
<td>0</td>
<td>0.03</td>
</tr>
<tr>
<td>532E × wt</td>
<td>F₁</td>
<td>157</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>572A × 1-2J</td>
<td>F₁</td>
<td>14</td>
<td>0</td>
<td>0.69</td>
</tr>
<tr>
<td>572A × wt</td>
<td>F₁</td>
<td>181</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>593F × 1-2J</td>
<td>F₁</td>
<td>6 (69%)</td>
<td>0</td>
<td>0.21</td>
</tr>
<tr>
<td>593F × wt</td>
<td>F₁</td>
<td>197</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>797A × 1-2J</td>
<td>F₁</td>
<td>11 (72%)</td>
<td>0</td>
<td>0.07</td>
</tr>
<tr>
<td>797A × wt</td>
<td>F₁</td>
<td>227</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>823B × 1-2J</td>
<td>F₁</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>956A × 1-2J</td>
<td>F₁</td>
<td>10 (64%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>956A × wt</td>
<td>F₁</td>
<td>166</td>
<td>42</td>
<td>2.56</td>
</tr>
<tr>
<td>961E × 1-2J</td>
<td>F₁</td>
<td>4</td>
<td>0</td>
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<tr>
<td>961E × wt</td>
<td>F₁</td>
<td>163</td>
<td>39</td>
<td>3.49</td>
</tr>
<tr>
<td>1018A × 1-2J</td>
<td>F₁</td>
<td>6</td>
<td>0</td>
<td>0.08</td>
</tr>
<tr>
<td>1018A × wt</td>
<td>F₁</td>
<td>153</td>
<td>52</td>
<td>0.02</td>
</tr>
</tbody>
</table>

† Mutants were crossed to the 35S::prosys-expressing parental line (1-2J) or to an inbred wild-type cultivar (wt; Castlemart). Reciprocal crosses gave similar results in the F₁ generation.

Phenotypes of segregating F₂ progeny were scored by comparison to the parental lines.

The values in parentheses indicate the total amount of Inh-I and Inh-II in F₁ plants relative to the amount in 1-2J plants of the same age.

Chi-square value was calculated for the expectation that the Inh−PPO− phenotype is conditioned by a recessive mutation in a single nuclear gene (χ² < 3.84 is agreeable at P > 0.05).

spr-1, and spr-2 mutants, spr-3/ 35S::prosys (823B) and spr-4/ 35S::prosys (956A) plants displayed relatively robust responses in both damaged and undamaged leaves.

The response of mutants to wounding was also examined at the level of Inh-II mRNA accumulation. For these experiments, Inh-II transcript levels were measured both 9 and 24 hr after wounding in both the wounded leaf and in the upper undamaged leaf (Figure 6). The results obtained were in good agreement with the measurements of Inh protein levels. For example, the level of wound-inducible Inh-II mRNA in def-1 355::prosys plants was <5% of that observed in wild-type plants. The spr-1 355::prosys and spr-2/ 35S::prosys mutants accumulated slightly more Inh-II mRNA in the damaged leaves (11 and 6% of wild-type levels, respectively). However, the systemic response of these two mutants was negligible at both time points. The overall level of wound-inducible Inh-II mRNA in spr-3/ 35S::prosys and spr-4/ 35S::prosys plants, determined by taking an average of local and systemic responses at both time points, was ~70 and 160% of that of wild-type plants, respectively.

Response of mutants to systemin and JA: To gain a better understanding of the wound response phenotypes observed, mutants were tested for Inh synthesis in response to exogenous JA and systemin (Figure 7). Both of these compounds have previously been shown to function as potent elicitors of the wound response pathway in tomato plants (Pearce et al. 1991; Farmer and Ryan 1992). In all mutants tested, supplementation of JA through the cut stem induced Inh-I and Inh-II accumulation to levels comparable to those of wild-type plants.
plants. Similar results were obtained after exposure of intact seedlings to methyl jasmonic acid (MeJA), an active volatile form of JA (data not shown). Exogenous systemin induced Inh accumulation to different extents in different mutants. In general, an excellent correlation was observed between the responsiveness of mutants to systemin and to that of wounding. For example, supplementation of \textit{def-1}/35S::prosys, \textit{spr-1}/35S::prosys, and \textit{spr-2}/35S::prosys seedlings with 2.5 pmol of systemin, a concentration known to saturate the wild-type response (Pearce et al. 1991), failed to induce Inh accumulation to levels above that of control plants treated with buffer. Higher concentrations of systemin (25 pmol/plant) were also ineffective in these mutants, as well as in mutants containing other alleles of \textit{spr-1} that were unresponsive to wounding (data not shown). In contrast, the response of \textit{spr-3}/35S::prosys plants to systemin was close to that of wild-type plants. In the case of \textit{spr-4}/35S::prosys, high levels of Inh were induced in plants treated either with systemin or with the buffer control. This indicates that the response of \textit{spr-4}/35S::prosys is triggered by experimental handling during the bioassay.

**DISCUSSION**

We developed a screening strategy to genetically define the wound response pathway in tomato. Our approach was to identify mutations that suppress the Inh\textsuperscript{PPO} phenotype of transgenic plants expressing 35S::prosys. This strategy eliminated many of the potential problems associated with identifying mutants deficient in an inducible phenotype. For example, constitutive signaling by 35S::prosys obviated the need to manually wound plants prior to screening for loss-of-function mutants. Second, the level of accumulation of wound response proteins in 35S::prosys plants is greater, and less variable from plant to plant, than that of mechanically wounded wild-type plants. These features helped to reduce the number of false positive mutants obtained. Finally, the use of simple assays for several independent wound response proteins (Inh-I, Inh-II, Inh-II)}
Figure 6.—Wound-inducible expression of Inh-II mRNA in suppressor mutants. Two-leaf stage wild-type plants, 35S::prosys parental plants (1-2)), and representative mutants from each complementation group (510B, df1; 532E, spr1; 572A, spr2; 823B, spr3; 956A, spr4) were wounded with a hemostat on the lower leaf. Three hours later, the same leaflets were wounded again, proximal to the petiole. RNA was extracted separately from the lower wounded (w) and upper unwounded (uw) leaves either 9 or 24 hr after the initial wound. Leaf tissue from eight plants was pooled for each RNA extraction to obtain an average response. The RNA gel blots shown were hybridized in the same reaction to a32P-labeled Inh-II cDNA probe and subsequently exposed to autoradiographic film for the same length of time (top). As a loading control, a duplicate blot was probed with an Arabidopsis elf4A cDNA that hybridizes to a constitutively expressed homologous transcript in tomato (bottom). Hybridization signals from Inh-II and elf4A were quantitated with a phosphorimager, and Inh-II mRNA abundance was normalized to that of the elf4A transcript.

Figure 7.—Responsiveness of mutants to JA and systemin. Fifteen-day-old wild-type plants (WT) and representative mutants from each complementation group (described in Figure 5) were supplied through the cut stem with a solution containing systemin (2.5 pmol/plant; open bars) or JA (10 nmol/plant; gray bars) for 30 min and then transferred to water. A control set of plants was supplied with an equal amount of buffer solution (solid bars). Plants were assayed for Inh-I (top) and Inh-II (bottom) accumulation 24 hr after treatment. Data represent the mean and standard error of 12 plants.

and PPO) helped to ensure that mutants were specifically affected in 35S::prosys-mediated responses. The PPO activity assay proved to be particularly effective as a primary screen of large numbers of plants.

Thirteen independent mutants were identified as being deficient in 35S::prosys-mediated accumulation of Inh-I, Inh-II, and PPO. The high frequency at which these mutants were recovered (1.2% of M1 plants) could attest to the effectiveness of the mutagenesis or a large number of genes in the pathway. The Inh-II/PPO phenotype of the 10 characterized mutants resulted from a coordinate decrease in the expression of Inh- and PPO-encoding genes. In addition to these selected markers, other wound-inducible transcripts whose levels are elevated in 35S::prosys plants were also lacking in the mutants (Figure 3). Thus, we conclude that mutations in these lines affect components of the 35S::prosys signaling pathway leading to gene activation. We also observed that a hypocotyl elongation phenotype of 35S::prosys seedlings was suppressed in Inh-II/PPO mutants. 35S::prosys seedlings exhibit other subtle changes in morphology, including increased pigmentation in the stem and reduced leaf expansion, which were also suppressed in the mutants (Figure 4A; G. A. Howe, unpublished results). At present, the physiological basis of 35S::prosys-mediated changes in seedling morphology is not known. Nevertheless, these observations suggest that it should be possible to exploit the morphology phenotype as a rapid visual screen for additional mutants in the pathway.

Genetic analysis showed that the suppressed phenotype of each mutant is conditioned by a recessive mutation that lies outside the 35S::prosys transgene. This implies that the mutations define genes within the 35S::prosys-mediated signaling pathway. Surprisingly, however, spr3/35S::prosys and spr4/35S::prosys plants were responsive to both wounding and exogenous systemin. The recovery of mutants with this phenotype suggests that there are differences between wound- and systemin-mediated signaling and 35S::prosys-mediated signaling. It is possible, for example, that 35S::prosys signaling involves gene products (e.g., SPR-3 and SPR-4) that are not required for the endogenous wound response pathway. Alternatively, it is possible that the strength of the "wound signal" generated by 35S::prosys is much weaker than that generated by wounding or
exogenous systemin. In this scenario, the spr-3 and spr-4 mutations could reduce signaling capacity below the threshold required for 35S:prosys responses, but not below that required for wound- and systemin-induced responses. Additional experiments are needed to test these hypotheses.

Complementation tests suggested that lines 88C (def-1\textsuperscript{18C}/35S::prosys) and 510B (def-1\textsuperscript{1510B}/35S::prosys) harbor new alleles of \textit{def-1}, a mutation originally identified in a screen for plants that lack proteinase inhibitor synthesis in response to mechanical wounding (Lightner \textit{et al.}, 1993). Additional evidence for this was obtained from analysis of these mutants to wounding and chemical elicitors of the pathway. For example, def-1\textsuperscript{1510B}/35S::prosys plants were responsive to JA but not to systemin and wounding, as is the case for def-1\textsuperscript{111} plants (Howe \textit{et al.}, 1996). The def-1\textsuperscript{18C}/35S::prosys mutant showed incomplete suppression of Inh and PPO accumulation and only partial loss of responsiveness to wounding and systemin (Figures 2 and 3; G. A. Howe, unpublished data). The availability of multiple alleles of def-1 should facilitate future experiments aimed at determining the role of this gene in the wound response pathway.

Our genetic approach revealed two new loci, called Spr-1 and Spr-2, that are required for responsiveness to wounding and systemin. The recovery of this class of mutants, in a screen that did not impose selection for wound response phenotypes, provides strong evidence that prosystemin plays a critical role in the transduction of systemic wound signals. These results also indicate that activation of defense gene expression by mechanical wounding, systemin, and 35S::prosys require common signaling components. The general appearance and growth phenotypes of spr-1 and spr-2 plants suggest that these mutations do not affect the ethylene- or ABA-dependent branches of the wound response pathway. The responsiveness of spr-1/35S::prosys and spr-2/35S::prosys mutants to exogenous JA suggests that these mutations affect a step upstream or outside the JA response pathway. It is possible, for example, that spr-1 or spr-2 affects the perception of systemin, the coupling of systemin perception to the activation of JA biosynthesis, or a step in JA biosynthesis or metabolism. Epistasis analysis between different wound response mutants, together with the study of the physiological defects in these mutants, should provide a better understanding of the wound response pathway and the role of systemin in it.

We acknowledge Ed Green and Teresa Beck-Bunn (Seminis Research Center, Woodland, CA) for generous assistance with production of \textit{M.} tomato seed. We also thank Daniel Kort and Gyu In Lee for excellent technical assistance and Mary Ann Brogan and Greg Wichels for the care of plants. This research was supported in part by National Institutes of Health grants F32GM16888 and R01GM57795 (to G.A.H.), the College of Agriculture and Home Economics, Washington State University Project 1791 (to C.A.R.), and National Science Foundation grant no. IBN 9184542 (to C.A.R.).

**LITERATURE CITED**


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Communicating editor: V. L. Chandler