The Wound Response Mutant suppressor of prosystemin-mediated responses6 (spr6) is a Weak Allele of the Tomato Homolog of CORONATINE-INSENSITIVE1 (COI1)

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The systemic defense response of tomato plant in response to insect attack and wounding is regulated by the 18 amino acid peptide systemin and the phytohormone jasmonic acid (JA). Recent genetic analyses based mainly on spr (suppressors of prosystemin-mediated responses) mutant screens have led to the hypothesis that systemin acts at, or near, the site of wounding to amplify the production of JA, which in turn functions as a mobile signal to promote the systemic defense response. In order to identify more components involved in the systemin/JA-signaled defense response, we carried out a larger scale screen for new spr mutants in tomato. Here we describe the characterization of spr6, a mutant impaired in wound- and systemin-induced defense gene expression. Using a candidate gene approach based on genetic linkage, we demonstrate that spr6 is allelic to jai1-1, which is a loss-of-function allele of the tomato homolog of CORONATINE-INSENSITIVE1 (COI1), an F-box protein that is required for JA-signaled processes in Arabidopsis. We show several aspects of the spr6 mutant phenotype distinct from that of jai1-1. First, the responsiveness of spr6 plants to exogenous JA shows a dosage dependency, i.e. it is more sensitive to JA than jai1-1 while less sensitive to JA than the wild-type. Secondly, unlike the sterile jai1-1, the spr6 plant displays normal fertility and seed set and thus can be maintained as a pure line and does not require selection. Therefore, spr6 provides a valuable tool, which can complement the limitations of jai1-1, to study JA signaling in tomato. The gene identification process of Spr6 we described herein represents an example showing the convenience of a candidate gene approach, based on genetic linkage, to identify gene functions of genetic loci defined by tomato wound response mutants.

Keywords: Jasmonic acid — Systemic defense response — Systemin — Tomato (Lycopersicon esculentum L.) — Wound response.

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Genetic analysis provides a powerful approach to dissect the systemin/JA signaling pathway and to elucidate the roles of systemin and JA in this pathway. Transgenic tomato plants that express a 35S::prosystemin transgene (35S::PS) and, as a consequence, constitutively accumulate high levels of PIs and polyphenol oxidase (PPO) activity in the absence of wounding (McGurl et al. 1994), have been used to identify mutations in the systemin signaling pathway (Howe and Ryan 1999, Li et al. 2001). Among the identified ‘spr’ (suppressors of 35S::prosystemin-mediated responses) mutants that were defective in wound-induced systemic PI expression were new alleles of def1 as well as spr1 and spr2, which defined two novel loci that are essential in the systemin/JA-signaled defense responses (Howe and Ryan 1999). The recent identification of Spr2 as a chloroplast co-3 fatty acid desaturase required for wound-induced JA production provided direct evidence that systemin and JA worked in the same signaling pathway and that activation of defense responses by systemin is strictly dependent on the biosynthesis and action of JA (Li et al. 2003). These results not only confirm but also extend the original signaling model for wound response proposed by Ryan and co-workers (Farmer and Ryan 1992, Ryan 2000). Grafting experiments conducted with the JA biosynthesis mutant spr2 and a JA signaling mutant jai1-1, which encodes the tomato homolog of CORONATINE-INSENSITIVE1 (COI1), indicated that systemic signaling requires both the biosynthesis of JA at the site of wounding and the ability to perceive a jasmonate signal in remote tissues. Results from grafting experiments support the hypothesis that JA regulates the production of, or act as, the systemic wound signal (L. Li et al. 2002). These results challenge the previous paradigm that systemin is the long-distance mobile signal for wound-induced defense gene expression (Ryan and Moura 2002, Stratmann 2003, Howe 2005). Recent characterization of the spr1 mutation, which is specifically defective in systemin action, shed new light on the role of the peptide signal (pro)systemin in the systemic defense response. A remarkable feature of the spr1 mutant is that the wound response is severely impaired in systemic leaves, whereas the local response is only slightly reduced. This phenotype, which is distinct from other characterized wound response mutants that lack both local and systemic responses, suggests that systemin is preferentially involved in the systemic response. Grafting experiments with spr1 plants support the idea that systemin acts at or near the wound site to amplify JA production to a level (threshold) that is required for the systemic response (Lee and Howe 2003). Taken together, recent genetic analyses, grafting experiments as well as other data have provided new findings on the roles of systemin and JA in regulating systemic defense responses; these findings support the hypothesis that JA is the mobile wound signal and that systemin promotes long-distance defense responses by amplifying JA production at the wounding site (L. Li et al. 2002, Ryan and Moura 2002, Stratmann 2003, Howe 2005).

The success of the initial spr mutant screen, together with the fact that this screen had been conducted on only about 25,000 M2 plants from 1,087 M1 families (Howe and Ryan 1999), which is far from saturation of the tomato genome (Van der Hoeven et al. 2002), prompted us to carry out additional genetic screens in order to identify more components involved in the systemin/jasmonate signaling. Here we describe the characterization of spr6, which is one of the newly identified mutants that can block the constitutive accumulation of high levels of PPO in the 35S::prosystemin plants. A remarkable phenotype of spr6 is that its sensitivity to exogenous JA is between that of jai1-1 and the wild-type. Quick mapping of systemin/JA-related genes on specific chromosome regions provides a candidate gene approach to identify gene functions of genetic loci defined by characterized tomato wound response mutants. Co-mapping of the Spr6 locus with the tomato homolog of CORONATINE-INSENSITIVE1 (LeCOI1, also known as Lejai1) on chromosome 5–3 significantly facilitated the identification of spr6 as a weak allele of LeCOI1.

**Results**

The spr6 mutant defines a novel gene in the tomato wound response pathway

spr6 is one of the newly identified mutants that can block the constitutively high activity of PPO in the 35S::prosystemin plants (see below). A standard wound response assay demonstrated that the original spr6 mutant also lost PI-II protein accumulation in response to mechanical wounding (data not shown), suggesting that the spr6 mutation might define an essential component in the tomato wound response pathway. Further characterization of the mutant was carried out using a spr6/spr6 homozygous line in which the 35S::prosystemin transgene was removed by out-crossing, followed by two successive backcrosses to the wild-type cv. Castlemart (see Materials and Methods).

The wound response of the spr6 homozygotes was compared with that of wild-type plants using 16-day-old seedlings containing two fully expanded leaves. Wounding the lower leaf of wild-type plants caused the well-known accumulation of PI-II in the wounded leaf (local response) and upper unwounded leaf (systemic response; Fig. 1A). In contrast, spr6 plants accumulated no detectable PI-II protein in the wounded leaf and the upper unwounded leaf (Fig. 1A). Compared with wild-type...
plants, the wound-induced accumulation of PI-II transcripts is also severely abrogated in spr6 mutants (Fig. 1B). Analysis of PI-II protein levels in an F$_2$ population segregating for spr6 showed that the ratio of wound-responsive to non-responsive plants was 89 : 34. This finding indicates that the deficiency in wound-induced PI-II expression in spr6 is caused by a single recessive mutation (i.e. $\chi^2 = 0.46$ for the 3 : 1 hypothesis) (Table 1). These results suggest that spr6 defines a gene essential for the wound-mediated signaling pathway.

In order to define better the position of the wound response pathway affected by the spr6 mutation, we examined the responsiveness of the mutant to exogenous systemin and JA, two major signals playing essential roles in wound-induced defense responses in tomato. Similar to the phenotypes reported for def1 (Howe et al. 1996), spr1 (Lee and Howe 2003) and spr2 (Li et al. 2003), spr6 failed to accumulate PI-II protein in response to exogenous systemin but was fully responsive to exogenous JA (Fig. 1C). It is worth noting that in our standard feeding assay, JA (at the dose of 15 nmol per plant) can readily restore PI-II accumulation in the well-characterized JA biosynthetic mutant spr2 (Li et al. 2003) and acx1/JL1 (Li et al. 2005), but not in the JA signaling mutant jai1-1 (Li et al. 2004) (Fig. 1C). These results led us to classify spr6 into the group of wounding/systemin-insensitive, but JA-sensitive mutants that includes def1, spr1, spr2 and acx1/JL1. As is the case for the molecularly elucidated spr2, which encodes an ω-3 fatty acid desaturase to produce linolenic acid, the metabolic precursor of JA biosynthesis (Li et al. 2003), and acx1, which encodes an acyl-CoA-oxidase that catalyzes the first step in the peroxisomal β-oxidation stage of JA biosynthesis (Li et al. 2005), this class of mutants appears to be defective in JA production, rather than JA signaling. Given the JA-sensitive phenotype of spr6, we crossed this mutant with the known mutants deficient in JA production and found spr6 to be non-allelic to def1, spr2 and acx1/JL1 (Table 1). Together, these results are consistent with the hypothesis that spr6 defines a novel gene that acts upstream of JA action, possibly involved in JA production or production regulation.

To determine whether spr6 plants are defective in JA synthesis, JA levels were quantified from control (unwounded) and wounded leaves of spr6 and wild-type plants using gas chromatography–mass spectrometry (GC–MS). The results showed that the JA level in undamaged spr6 leaves is 85 ± 21 ng (g FW)$^{-1}$, which was statistically similar ($P > 0.05$) to that in unwounded wild-type leaves [95 ± 34 ng (g FW)$^{-1}$]. In wounded wild-type plants, JA levels increased to 460 ± 36 ng (g FW)$^{-1}$, 1 h after wounding. The amount of JA in wounded spr6 leaves at 1 h was 360 ± 22 ng (g FW)$^{-1}$, which was estimated to be approximately 78% of that in wild-type leaves. These results indicate that even though the spr6 mutation does not affect the maintenance of basal JA levels in leaf tissues, it slightly damaged the wound-induced JA accumulation.

Fig. 1 spr6 is deficient in defense gene expression. (A) PI-II accumulation in tomato leaves in response to mechanical wounding. Sixteen-day-old wild-type and mutant seedlings were wounded using a hemostat as described in Materials and Methods. At 24 h after wounding, PI-II levels were measured in the wounded leaf (filled bar) and the upper unwounded leaf (open bar). Values represent the mean ± SD of 12 plants per genotype. (B) Time course of wound-induced gene expression in wild-type (WT) and spr6 plants. WT and mutant plants (16 days old) containing two fully expanded leaves were mechanically wounded with a hemostat on both leaves. At various times (hours) after challenge, damaged leaves were harvested for RNA extraction. RNA blot hybridization was performed using $^{32}$P-labeled cDNAs for proteinase inhibitor II (PI-II). Blots were also hybridized to a probe for eIF4A as a loading control. (C) PI-II accumulation in wild-type (WT) and spr6 plants in response to exogenous systemin and jasmonic acid. WT, spr6, spr2 and jai1-1 seedlings (16 days old) were excised at the base of the stem and supplied with 15 mM sodium phosphate buffer (white bar), or buffer solution with 15 mM systemin (gray bar) and 15 mM jasmonic acid (black bar). PI-II levels were measured 24 h after treatment. Data points represent the mean and SD ($n = 12$).
Genetic mapping of the Spr6 gene

The deficiency in wound-induced PI-II protein accumulation of the spr6 mutant provided a facile assay to identify the Spr6 gene using a map-based cloning approach, by which Spr2 (Li et al. 2003) and LeAcx1 (Li et al. 2005) had been successfully identified in our previous work. The general strategy for mapping Spr6 was essentially similar to that used to map DefI (C. Li et al. 2002), Spr2 (Li et al. 2003) and LeAcx1 (Li et al. 2005). A BC1 mapping population segregating for the spr6 phenotype was constructed from a cross between spr6/spr6 (Lycopersicon esculentum) and Lycopersicon pennellii (LA0716), followed by backcrossing of a resulting F1 plant as the male parent to spr6/spr6. The wound response phenotype of individual BC1 plants was scored by measuring PI-II protein levels in response to mechanical wounding, as described above. Bulked segregant analysis (BSA; Michelmore et al. 1991) was used to identify molecular markers linked to the Spr6 locus. Genomic DNA from 10 wound-responsive (W+) and 10 wound-non-responsive (W−) BC1 plants was pooled to construct a W+ bulk (B+) and W− bulk (B−), respectively. PCR-based SSR (simple sequence repeat) markers that have been mapped on the tomato high-resolution genetic map (www.sgn.cornell.edu) were used to screen the bulks for polymorphism. One such marker named Tom152–153 generated a clear polymorphism between the bulks as well as the parents (Fig. 2A). Linkage of Tom152–153 to Spr6 was further verified using the 20 BC1 individuals that comprised the two bulks. This experiment showed that all W+ plants were homozygous for the L. esculentum pattern, whereas all W− plants were heterozygous (Fig. 2A). The absence of recombinants in this population of 20 BC1 plants demonstrates that Tom152–153 is linked to the Spr6 locus. Given the fact that Tom152–153 had been previously mapped to IL5-3 (Fig. 2B, and Suliman-Pollatschek et al. 2002) using the tomato introgression lines (ILs) harboring defined L. pennellii chromosome segments in the L. esculentum background (Eshed and Zamir 1994), these mapping results indicated that Spr6 located on the chromosome region defined by IL5-3. This conclusion was further confirmed by linkage analysis with restriction fragment length polymorphism (RFLP) markers located on IL5-3 (Tanksley et al. 1992, Eshed and Zamir 1994) using 101 plants (55 W+ and 46 W−) from the above-mentioned BC1 mapping population (data not shown).

Spr6 co-mapped with LeCOI1 on IL5-3

A preliminary mapping effort to position the Spr6 gene on the chromosome region of IL5-3 provides the possibility to identify the mutated gene using a candidate gene approach. Since it is well established that, in the tomato system, systemin and JA interact in the same signaling pathway to regulate wound-induced defense gene expression, we reasoned that if a gene with known function in systemin and (or) JA biosynthesis or signaling maps to the same location with a genetic locus defined by a characterized wound response mutant, this gene could represent a strong candidate for the particular mutant locus. If this happens, in at least some instances it will circumvent the need for intensive chromosome walking effort for gene isolation.

The precious tomato genetic resource of a set of 50 ILs carrying marker-defined segmental substitutions of L. pennellii chromosomes in an otherwise L. esculentum background (Eshed and Zamir 1994) provides us with an efficient approach to quickly map those genes (or cDNA sequences) known or hypothesized to be related to tomato wound response signaling. A cDNA fragment corresponding to a particular gene was converted to an RFLP marker that can distinguish the genomic DNA of L.esculentum cv. M82 from that of L. pennellii (LA0716). This RFLP marker was then used to screen the above-mentioned 50 ILs, and detection of the L. pennellii allele in one of the ILs defines the chromosomal location of the particular probe (gene). As an example, we show here the mapping result of the tomato homolog of LeCOI1, also known as LeJai1, which encodes an F-box protein that plays an essential role in JA-signaled processes (Xie et al. 1998, Xu et al. 2002, Li et al. 2004). Full-length cDNA of LeCOI1 was used as a
the prosystemin gene (designated gene
found that the study, were also integrated into the map. Interestingly, we mapped, including response mutants, mutant loci that have been accurately facilitated the identification of genes defined by tomato wound biosynthesis or signaling related (including JA induced). In order to explore the possibility that this mapping effort could facilitate the identification of genes defined by tomato wound response mutants, mutant loci that have been accurately mapped, including Def1, Spr2, LeAcx1 and Spr6 in the current study, were also integrated into the map. Interestingly, we found that the Def1 locus co-mapped with the JA biosynthetic gene LoxD on IL3-5 and the Spr6 locus co-mapped with both the prosystemin gene (designated LePrs) and the LeCOI1 gene on IL5-3. Our previous mapping results excluded the possibility that LoxD corresponds to Def1 (C. Li et al. 2002). We further tested if LePrs or LeCOI1 corresponds to Spr6.

**spr6 contains a point mutation of the tomato homolog of CORONATINE INSENSITIVE1**

cDNAs corresponding to LePrs and LeCOI1 were converted to RFLP markers to test their genetic relationships with the Spr6 locus using the aforementioned BC1 mapping population. Among the 101 BC1 plants analyzed, we detected four recombination events between LePrs and Spr6 and, interestingly, no recombination event between LeCOI1 and Spr6. These results excluded LePrs as a candidate gene for Spr6.

Co-segregation of LeCOI1 with the spr6 mutant phenotype prompted us to conduct a genetic complementation test between spr6 and JAI1-1, a fast neutron-induced mutation of the LeCOI1 gene (Li et al. 2004). Interestingly, but not surprisingly, F1 plants resulted from a cross using the female-sterile...
Systemin/jasmonate-mediated wound response

jai1-1 as pollen donor showed a similar wound response phenotype to spr6, i.e. deficient in wound-induced PI-II accumulation (Fig. 4). These results showed that spr6 and jai1-1 cannot complement each other, and thus provided direct evidence that spr6 and jai1-1 cannot complement each other, and thus provided direct evidence that spr6 is allelic to jai1-1, which was shown to be a null allele of LeCOI1 (Li et al. 2004). Given that spr6 is an ethyl methane sulfonate (EMS)-induced mutation, it is most likely that the lesion of the LeCOI1 gene in spr6 is relatively weak compared with that in jai1-1. To determine the molecular lesion of the LeCOI1 gene in spr6 plants, we used reverse transcription–PCR (RT–PCR) to obtain full-length cDNAs from both spr6 homozygotes and wild-type plants. DNA sequencing and comparison analyses revealed that spr6 contains a single nucleotide mutation (G to T) at position 1,254 relative to the translation start of the LeCOI1 gene, which resulted in, at the amino acid sequence level, the replacement of Leu418 by a phenylalanine. This polymorphism was further verified by sequencing the corresponding region of genomic DNAs from wild-type and spr6 plants. In addition, the G to T mutation in the spr6 allele disrupts a recognition site of the restriction enzyme Mwo (GCNNNNNNNGC) in genomic DNA of LeCOI1, by which we developed a cleaved amplified polymorphic sequence (CAPS) marker to distinguish spr6 plants from wild-type (Fig. 5).

Dose-dependent responsiveness of spr6 to exogenous JA

Confirmation of Spr6 as LeCOI1 prompted us to compare the responsiveness of the spr6 mutant to exogenous JA with those of jai1-1 and the wild-type. As shown in Table 3, application of various amounts of exogenous JA within the range of 0.0015–15 nmol per plant resulted in PI-II accumulation in wild-type plants. In contrast, JA did not induce PI-II accumulation in jai1-1 plants, even when applied at relatively high concentrations. As expected, the responsiveness of spr6 plants to exogenous applied JA was dose-dependent. JA at concentrations of ≥0.15 nmol per plant induced PI-II protein accumulation in spr6 plants, albeit to levels that were lower than those in wild-type plants. This dose-dependent responsiveness of spr6 to exogenous JA suggests that spr6 is a null allele of LeCOI1, which is involved in the perception and/or signaling of systemin/JA.

Table 2 Summary of mapped systemin/JA-related genes and genetic loci defined by tomato wound response mutants.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession no.</th>
<th>IL mapping</th>
<th>Gene product description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LePrs</td>
<td>M84800</td>
<td>5–3</td>
<td>Prosystemin</td>
<td>McGurl et al. (1992), McGurl et al. (1994), Li and Howe (2001)</td>
</tr>
<tr>
<td>LeSR160</td>
<td>AY112661</td>
<td>2–1</td>
<td>Systemin receptor</td>
<td>Scheer et al. (2002)</td>
</tr>
<tr>
<td>Spr2</td>
<td>AY24874</td>
<td>6–1</td>
<td>ω-3 Fatty acid desaturase</td>
<td>Li et al. (2003)</td>
</tr>
<tr>
<td>LeLoxC</td>
<td>U90926</td>
<td>12–1</td>
<td>Lipoxygenase C (LOXC)</td>
<td>Heitz et al. (1997)</td>
</tr>
<tr>
<td>LeLoxD</td>
<td>U37840</td>
<td>3–5</td>
<td>Lipoxygenase D (LOXD)</td>
<td>Heitz et al. (1997)</td>
</tr>
<tr>
<td>Leaos1</td>
<td>A1895589</td>
<td>4–3,4–4</td>
<td>Allene oxide synthase 1</td>
<td>Sivasankar et al. (2000)</td>
</tr>
<tr>
<td>Leaos2</td>
<td>AF230371</td>
<td>11–4</td>
<td>Allene oxide synthase 2</td>
<td>Howe et al. (2000)</td>
</tr>
<tr>
<td>Leaos3</td>
<td>AF454634</td>
<td>10–1</td>
<td>Allene oxide synthase 3</td>
<td>Itoh et al. (2002)</td>
</tr>
<tr>
<td>LeAc</td>
<td>AW624058</td>
<td>2–5</td>
<td>Allene oxide cyclase</td>
<td>Ziegler et al. (2000)</td>
</tr>
<tr>
<td>LeOpr3</td>
<td>AW034958</td>
<td>7–1, 7–2</td>
<td>12-Oxo-phytodienoate reductase</td>
<td>Strassner et al. (2002)</td>
</tr>
<tr>
<td>LeDes</td>
<td>AF317515</td>
<td>1–4</td>
<td>Divinyl ether synthase (DES)</td>
<td>Itoh and Howe (2001)</td>
</tr>
<tr>
<td>Leact1</td>
<td>AY817109</td>
<td>8–2, 8–3</td>
<td>Acyl-CoA oxidase</td>
<td>Li et al. (2005)</td>
</tr>
<tr>
<td>LeMYC2</td>
<td>AF011557</td>
<td>8–2</td>
<td>Transcriptional activator</td>
<td>Boter et al. (2004)</td>
</tr>
<tr>
<td>LePEX6</td>
<td>BT0127833</td>
<td>10–2, 10–3</td>
<td>An ATPase required for peroxisomal biogenesis</td>
<td>Zolman and Bartel (2004)</td>
</tr>
<tr>
<td>LeCOI1</td>
<td>AY423550</td>
<td>5–3</td>
<td>Tomato homolog of CORONATINE INSENSITIVE 1</td>
<td>Li et al. (2004)</td>
</tr>
<tr>
<td>LebZIP1</td>
<td>AF176641</td>
<td>1–1, 1–2</td>
<td>Wound- and JA-inducible DNA-binding protein</td>
<td>Stankovic et al. (2000)</td>
</tr>
<tr>
<td>Spr6</td>
<td>5–3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Def1</td>
<td>3–5</td>
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All loci listed including those that had been mapped previously were mapped again via introgression lines in this study.
wild-type plants. However, JA at concentrations of <0.15 nmol per plant did not induce PI-II accumulation in spr6 plants, even though this range of JA induced significant amounts of PI-II in wild-type plants (Table 3). These results demonstrate that, in our feeding system, 0.15 nmol per plant appears to be the JA dose threshold to trigger PI-II accumulation in spr6 plants.

Discussion

The spr6 phenotype is caused by a point mutation in LeCOI1

We describe here the characterization of the tomato wound response mutant spr6 that was identified based on its ability to suppress the constitutive expression of high levels of PPO and PIs in 35S:prosystemin plants. Using a candidate gene approach that relied on the genetic linkage of Spr6 to LeCOI1, we demonstrate that spr6 affects the function of a tomato homolog of COI1. In Arabidopsis, it has been established that the F-box protein COI1 is part of an SCF E3 ligase complex (designated SCF^{COI1}) that includes ASK1 or ASK2 and CUL1 (Xie et al. 1998, Xu et al. 2002, Devoto et al. 2002). SCF^{COI1} is proposed to selectively recruit yet to be identified regulatory proteins for degradation by the 26S proteosome (Moon et al. 2004). Given the strong JA-insensitive phenotypes of tomato jai1-1 and Arabidopsis coi1 mutants and the high sequence similarity of COI1 proteins in these two species, Li et al. (2004) concluded that an equivalent SCF^{COI1} exists in tomato and performs a similar function in JA signal transduction.

Compared with the recently described jai1-1 mutant, which was shown to be a null mutation in LeCOI1, the spr6 mutant shows a relatively weak phenotype in several aspects. First, spr6 is more sensitive to exogenous JA than jai1-1. In our experimental system, even though spr6 is deficient in wounding- and systemin-induced defense gene expression, it is responsive to applied JA at a concentration >0.15 nmol per plant, whereas jai1-1 is non-responsive to JA at virtually all the concentrations tested (Table 3). This observation suggests that the point mutation in the LeCOI1 gene of spr6 plants might partially impair its protein function, and supports the hypothesis that the JA level induced by mechanical wounding and systemin is lower than the threshold needed to trigger defense gene expression in spr6 plants. The JA dose-dependent responsiveness of spr6 plants supports the hypothesis that JA can regulate, in this case stimulate, the activity or signal output of the SCF^{COI1} complex in a dose-dependent manner. In this scenario, when the activity increase of SCF^{COI1} stimulated by JA cannot complement the activity reduction by the mutation lesion, spr6 shows a JA-insensitive phenotype, otherwise it shows a JA-responsive phenotype. Given that the Leu-to-Phe substitution caused by spr6 is located in the leucine-rich repeat (LRR) domain previously shown to be important for protein–protein interaction (Devoto et al. 2002, Xu et al. 2002), it is possible that this mutation impairs the binding of SCF^{COI1} to its putative substrates, which accounts for the partial loss of JA responsiveness of the spr6 plants. We propose that JA works to promote the binding of SCF^{COI1} to its substrates in a dose-dependent manner. In agreement with this speculation, it has been demonstrated that auxin stimulates binding of SCF^{TIR1} to its substrate proteins AUX/IAAs, and their degradation (Gray et al. 2001, Dharmasiri et al. 2005). Identification of the putative SCF^{COI1} substrates will be essential to confirm this hypothesis. Secondly, in contrast to the sterile jai1-1, spr6 is fertile. jai1-1 shows complete female sterility and reduced pollen viability; this remarkable phenotype established the idea that COI1-dependent JA function is essential for female reproduc-

<table>
<thead>
<tr>
<th>JA concentration (nmol plant^{-1})</th>
<th>PI-II protein (µg ml^{-1} leaf juice)</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>spr6</td>
</tr>
<tr>
<td>15</td>
<td>144 ± 22</td>
</tr>
<tr>
<td>1.5</td>
<td>97 ± 17</td>
</tr>
<tr>
<td>0.15</td>
<td>56 ± 9</td>
</tr>
<tr>
<td>0.015</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>0.0015</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>Buffer</td>
<td>15 ± 5</td>
</tr>
</tbody>
</table>

Various amounts of exogenous JA (dissolved in 15 mM sodium phosphate buffer) were applied to cutting stems of spr6, wild-type and jai1-1 seedlings (16 days old). Control plants were treated with 15 mM phosphate buffer. PI-II levels were determined 24 h after JA or buffer treatment. Values represent the mean PI-II concentrations of eight plants ± SD. This experiment was repeated three times and similar results were obtained.

^a The amount of JA applied to each plant was an approximate estimation based on our standard feeding system.

^b ND, not detectable, i.e. below the detection limit of the immunodiffusion assay for PI-II measurement (approximately 5 µg PI-II ml^{-1} leaf juice).
tive development, but is not absolutely required for male fertility in tomato. Because spr6 shows normal fertility in both male and female reproductive development and a seed set rate comparable with the wild-type (J. Zhao and C. Li, unpublished data), it is possible that the mutation in COOl of the spr6 plant is not severe enough to impair its normal function in male and female development. Given that spr6 is deficient in wound-induced defense gene expression, we speculate that the threshold of SCF<sup>COOl</sup> ‘activity’ required for defense gene expression is higher than that required for reproductive development. As a fertile allele of jai1 (coil), spr6 may provide a valuable tool to study JA signaling, which can complement the limitations of the sterile jai1-1 (coil) in at least two aspects: first, the fertile spr6 can be maintained in a pure line and therefore does not require selection. Secondly, spr6 may aid in the genetic screening for suppressors of jai1 (coil) mutations.

Candidate gene approach based on genetic linkage facilitated identification of gene function defined by tomato wound response mutants

An ever-growing body of biochemical and genetic evidence indicates that systemin and JA worked together in the same signaling pathway to promote local as well as systemic expression of defense-related genes. The systemin/JA signaling pathway for induced plant resistance to herbivores provides a unique opportunity to investigate, in a single experimental system, the mechanism by which peptide and oxylipin signals interact to coordinate systemic expression of defense-related genes. The robust nature of wound-inducible PI expression in tomato offers a facile assay to dissect the systemin/JA-signaled defense response pathway using the powerful forward genetic approach. Several genetic screens led to the current collection of tomato wound response mutants that can be categorized as being defective in JA biosynthesis, JA signaling or systemin function (Howe 2005). Identification of gene identity and function defined by a tomato wound response mutant usually involves mapping of the target locus to a specific chromosome region. We reasoned that if a gene with known function in systemin and (or) JA biosynthesis or signaling maps to the same location with a genetic locus defined by a characterized wound response mutant, this gene could represent a strong candidate for the particular mutant locus. If this happens, in at least some instances it will circumvent the need for chromosome walking effort for gene isolation, which is a laborious and time-consuming process in tomato. Of equal importance is the fact that the positioning of systemin- and JA-related genes in specific chromosomal regions also permits the exclusion of most genes as candidates for a locus defined by a particular wound response mutant which has been accurately mapped to a specific chromosome region. Genetic mapping of gene sequences related to a particular biological phenomenon or responses represents a simple, yet powerful tool for gaining insight into specific gene function (Giovannoni et al. 1999). For example, mapping of tomato loci homologous to the <em>Arabidopsis ETR1</em> gene revealed co-segregation of a related sequence with the genetic locus defined by the tomato never-ripe mutant that is insensitive to ethylene (Lanahan et al. 1994, Yen et al. 1995). This significantly facilitated subsequent verification that the NR gene in fact represents a tomato ethylene receptor (Wilkinson et al. 1995). We reasoned that this approach is particularly useful for genetic dissection of the tomato wound response pathway for several reasons. First, extensive studies in this model system in the past four decades produced a wealth of knowledge relevant to the mechanism of systemic wound signaling. Secondly, rapid progress of genome research in tomato and related solanaceous species provides an ever-growing amount of gene sequence information including those related to systemic defense responses (http://www.sgn.cornell.edu/). Thirdly, extensive genetic resources, especially the tomato ILs population (Eshed and Zamir 1994), facilitate quick localization of gene sequences to specific chromosomal regions using the method described herein. Finally, as mentioned above, several well-characterized defensive proteins such as PIs and PPO provide a convenient and robust assay to identify wound response mutants.

As more genes and mutant loci are mapped, the probability of identifying genes corresponding to functionally characterized loci will increase. The gene identification process of Spr6 we described here represents an example showing the convenience of the candidate gene approach based on genetic linkage, thus avoiding the need for the labor-intensive fine mapping efforts. We speculate that this candidate gene approach will be helpful to our long-term goal of genetic dissection of the systemin/JA-signaled defense responses in tomato.

Materials and Methods

Plant materials and growth conditions

Tomato (<em>L. esculentum</em>) cv. Castlemart was used as the wild-type parent for all experiments involving comparison of wild-type and mutant plants. Tomato seedlings were grown in a growth chamber maintained under 16 h of light (200 μE m<sup>-2</sup> s<sup>-1</sup>) at 28°C and 8 h of dark at 18°C. Seeds of <em>L. pennellii</em> (LA0716) and the ILs (Eshed and Zamir 1994) were obtained from the C.M. Rick Tomato Genetic Resource Center (University of California at Davis, CA, USA).

Mutant isolation and genetic analysis

Seeds of a tomato line expressing a 35S:<em>prosystemin</em> transgene (McGurl et al. 1994) were mutagenized with EMS as previously described (Howe and Ryan 1999), and M<sub>1</sub> seeds were collected separately from individual M<sub>1</sub> plants. About 30 plants per M<sub>2</sub> family were screened for PPO activity using a rapid leaf squash staining assay as described (Howe and Ryan 1999). PPO-deficient (PPO<sup>–</sup>) plants were then tested for the accumulation of PI-II, another classical biochemical marker for tomato wound response. spr6 is one of the identified mutant lines deficient in both PPO and PI-II accumulation (PPO<sup>–</sup>PI-II<sup>–</sup>).

The original spr6 mutant in the 35S:<em>prosystemin</em> genetic background was backcrossed to cv. Castlemart to generate an F<sub>2</sub> population segregating for both 35S:<em>prosystemin</em> and spr6. To identify spr6 homozygotes that lack the transgene, F<sub>2</sub> plants that exhibit a wound-
non-responsive (PPO-PI-II) phenotype were identified. Genomic DNA from these plants was subjected to PCR screening using a primer set P1 (5’-GGGGATCCCTGGAATGACAAAGACATTCC-3’) and P2 (5’-GGGGATCCCGAATTCATTCTTTCTAAGGAGAGAC-3’) that specifically amplifies a 200 bp fragment corresponding to the 35S::prosystemin transgene and a 1 kb fragment corresponding to the endogenous Prosystemin gene. DNA gel blot analysis was used also to confirm the absence of the transgene in selected spr6 homozygotes. One such line was used as the female parent for two successive backcrosses to the wild-type.

The identified homozygous spr6/spr6 plants were crossed to the wild-type, and F1 plants were allowed to self-pollinate. The wound response phenotype of F1 and F2 plants was scored by measuring PI-II accumulation in response to mechanical wounding. spr6 mutants were also crossed to several other mutants such as spr2 (Howe and Ryan 1999, Li et al. 2003), acx1/JL1 (Lightner et al. 1993, Li et al. 2005), def1/JL5 (Lightner et al. 1993, Howe et al. 1996, C. Li et al. 2002) and jai1-1 (Li et al. 2004) for allelic tests. The wound response phenotypes of the resulting F1 or F2 progeny were scored 16 d after germination.

PI-II accumulation assay

The wound response of tomato plants was determined by PI-II accumulation assay in response to mechanical wounding. Briefly, at 16–18 days of age, young tomato plants were wounded by crushing with a hemostat across the midrib of all leaflets of a single basipetal leaf. After a 3 h interval, this wounding was repeated on the same leaflet closer to the petiole. The plants were then incubated in a growth chamber for 24 h before PI-II assay. The wounded leaf (local) and the upper unwounded leaf (systemic) were collected and crushed to obtain leaf juice which was assayed for PI-II accumulation by radial immunodiffusion as described (Ryan 1967, Trautman et al. 1971). Systemin and JA feeding experiments were performed using two-leaf-age tomato seedlings (16–18 days old) as previously described (Lee and Howe 2003, Li et al. 2003). Plants were excised at the base of the stem and placed in 0.5 ml microfuge tubes containing 300 µl of the inducing compound. When >90% of the elicitor solution had been imbibed (approximately 2 h), plants were transferred to glass vials containing 20 ml of water, and incubated in a Lucite Box under continuous light for 24 h. PI-II levels in leaves were measured by radial immunodiffusion assay (Ryan 1967). JA was purchased from Sigma. Systemin was commercially synthesized by Shanghai Sangon Biological Engineering & Technology and Service Co. Ltd (Shanghai, PR China).

Measurement of JA

Leaves of 3-week-old tomato plants were wounded with a hemo- stat as described above. Wounded leaf tissue (300 mg fresh weight) from four different plants was pooled and immediately frozen in liquid nitrogen. Leaf tissue was also harvested from unwounded plants as a control. JA was extracted and quantified by GC–MS with 190 ng of [1, 2-13C]JA (kindly provided by Ian T. Baldwin, Max-Planck Institute of Chemical Ecology, Jena, Germany) as internal standards, as described by Heidel and Baldwin (2004). The concentrations of JA were expressed as ng g−1 of fresh leaf.

Genetic mapping of the Spr6 locus

In order to determine the chromosomal location of the Spr6 locus, a BC1 mapping population segregating for the spr6 phenotype was constructed and the wound response phenotype of individual BC1 plants was scored by measuring PI-II protein accumulation in response to mechanical wounding. DNA was isolated from fresh leaves as described by McCouch et al. (1988).Bulked segregant analysis (Michelmore et al. 1991) was used to identify molecular markers linked to Spr6. The chromosomal location of Spr6 was determined by mapping a linked SSR marker Tom152–153 to chromosome 5–3 (Suliman-Pollatschek et al. 2002) using a tomato ILs population (Eshed and Zamir 1994). Primers for SSR marker Tom152–153 are (5’-AAAT CAA GGA ACT TTT AGC TCC-3’) and (5’-TGC ATT GAT GTT CAT AAA TGA-3’), and PCRs were performed as described (Suliman-Pollatschek et al. 2002).

Chromosomal localization of systemin/JA-related genes using tomato introgression lines

A cDNA fragment corresponding to a particular gene was used as probe to do a survey blot to find proper RFLPs between genomic DNAs of cultivated tomato var. MS2 (L. esculentum) and LA0716 (L. pennellii) digested with different restriction enzymes (DraI, EcoRI, EcoRV, HaeIII and HindIII). Once a polymorphism was identified in the survey blot, the probe–enzyme combination was employed to screen the whole set of 50 ILs to determine which IL shows the same hybridization pattern as L. pennellii and, as a consequence, the chromosome region defined by this IL represents the chromosomal location of the particular gene.

Sequence analysis

LeCO1-specific primers P3 (5’-GGGGATCCCTGGAATGACAAAGACATTCC-3’) and P4 (5’-CGGAGCTCATAATGGGACACACCT3’) were used to obtain full-length cDNA from both wild-type and spr6 plants by RT–PCR using total RNA isolated from seedlings. DNA sequences from wild-type and mutant were compared to identify the mutation. Part of the LeCO1 gene was amplified from genomic DNAs of both wild-type and spr6 alleles using PCR primer pair P5 (5’-ATGTTGGTGAGGATAGGATTTGG-3’) and P6 (5’-ATTGCCGACATACTGAGACC-3’). The G to T mutation in spr6 destroys a MwoI recognition site that is used as a CAPS marker to distinguish the mutant from the wild-type.

Nucleic acid gel blot analysis

Total RNA was isolated from tomato leaf tissue and analyzed by gel blot hybridization, as previously described (Li et al. 2003). A 5 µg aliquot of total RNA was also electrophoresed and stained with ethidium bromide before gel blotting to ensure equal loading of samples. Hybrid-N’ filters were hybridized to the following tomato cDNAs labeled with a T7 Quickprime Kit (Pharmacia Biotech): PI-II (Graham et al. 1985) and a translation initiation factor eIF4A mRNA (Taylor et al. 1993).

Genomic DNA digestion, Southern blot and hybridization were performed according to standard protocols (Sambrook et al. 1989). Briefly, 5 µg of genomic DNA was digested with restriction enzymes, electrophoresed on a 0.9% (w/v) agarose gel and blotted onto a Hybond-N’ membrane (Pharmacia Biotech) as suggested by the manufacturer. Blots were hybridized with the 32P-labeled probes at 65°C. DNA probes were prepared using a T7 Quickprime Kit (Pharmacia Biotech).

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