

The Tomato Homolog of CORONATINE-INSENSITIVE1 Is Required for the Maternal Control of Seed Maturation, Jasmonate-Signaled Defense Responses, and Glandular Trichome Development^W

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Jasmonic acid (JA) is a fatty acid–derived signaling molecule that regulates a broad range of plant defense responses against herbivores and some microbial pathogens. Molecular genetic studies in *Arabidopsis* have established that JA also performs a critical role in anther and pollen development but is not essential for other developmental aspects of the plant's life cycle. Here, we describe the phenotypic and molecular characterization of a sterile mutant of tomato (*jasmonic acid-insensitive1 [jai1]*) that is defective in JA signaling. Although the mutant exhibited reduced pollen viability, sterility was caused by a defect in the maternal control of seed maturation, which was associated with the loss of accumulation of JA-regulated proteinase inhibitor proteins in reproductive tissues. *jai1* plants exhibited several defense-related phenotypes, including the inability to express JA-responsive genes, severely compromised resistance to two-spotted spider mites, and abnormal development of glandular trichomes. We demonstrate that these defects are caused by the loss of function of the tomato homolog of CORONATINE-INSENSITIVE1 (COI1), an F-box protein that is required for JA-signaled processes in *Arabidopsis*. These findings indicate that the JA/COI1 signaling pathway regulates distinct developmental processes in different plants and suggest a role for JA in the promotion of glandular trichome–based defenses.

INTRODUCTION

Plant oxylipins constitute a group of bioactive fatty acid derivatives that perform several important roles in growth and development. A large body of research has focused on the jasmonate family of oxylipins, which includes jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA). These signaling compounds, collectively referred to as JAs, are ubiquitous in the plant kingdom and are well characterized with respect to their role in regulating defense responses against herbivore attack and infection by some pathogens (Kessler and Baldwin, 2002; Turner et al., 2002; Wasternack and Hause, 2002; Weber, 2002). JAs also are implicated in the control of plant responses to abiotic stimuli such as mechanical stress (Weiler et al., 1993), salt stress (Dombrowski, 2003), UV irradiation (Conconi et al., 1996), and ozone exposure (Rao et al., 2000). The ability of JAs to regulate gene expression in plant, insect (Li et al., 2002c), and mammalian (Rotem et al., 2003) cells indicates that some components of this lipid-based signaling system may be conserved in diverse biological systems.

Exogenous JAs exert numerous inductive and inhibitory effects on plant developmental processes (Creelman and Mullet, 1997; Wasternack and Hause, 2002). Correlations between endogenous JA levels in specific tissues and the effects of the applied hormone have provided evidence that JAs have a role in promoting senescence, fruit ripening, embryo development, and the accumulation of storage proteins (Staswick, 1990; Wilen et al., 1991; Creelman and Mullet, 1997; He et al., 2002). However, because exogenous JAs do not target specific cell types and often are administered at nonphysiological concentrations, confirmation of these roles requires genetic manipulation of either endogenous JA levels or the signal transduction steps that couple JA production to the physiological response.

Genetic analysis has been instrumental in elucidating the function of JA in *Arabidopsis* development. Initial insight into the role of JA in flower development came from the observation that the *coronatine-insensitive1 (coi1)* mutant that is insensitive to JAs is male sterile (Feys et al., 1994). The *COI1* gene encodes an F-box protein that participates in the formation of an E3 ubiquitin ligase complex involved in ubiquitin-dependent proteolysis (Xie et al., 1998; Devoto et al., 2002; Xu et al., 2002). The critical requirement for JA in male fertility was established by the characterization of an *Arabidopsis* mutant that fails to produce linolenic acid, the fatty acid precursor of JA (McConn and Browse, 1996). Subsequently, mutations that disrupt other steps in the JA biosynthetic pathway also were shown to cause

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male sterility (Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002). Reproductive dysfunction in JA-deficient *Arabidopsis* plants results from a combination of defects in anther filament elongation, anther dehiscence, and pollen maturation. Based on the temporal and spatial expression pattern of the *DAD1* gene, which is required for JA biosynthesis and male fertility, it was proposed that JA-regulated water transport within floral tissues may promote synchronous pollen maturation, anther dehiscence, and flower opening (Ishiguro et al., 2001). The molecular mechanism by which JA promotes male reproductive development remains unknown. Recent studies support the hypothesis that JA controls the expression of genes that are required for normal anther development and pollen maturation (Mandaokar et al., 2003).

A more complete understanding of the physiological function of JA in plant growth and development would be facilitated by the identification of JA signaling mutants in diverse plant species. Toward this goal, we previously reported the identification of tomato mutants that are insensitive to JAs (Li et al., 2001). The recessive *jasmonic acid-insensitive-1-1* (*jai1-1*) mutant was isolated in a genetic screen for plants that fail to accumulate defense-related proteins in response to volatile MeJA. A second, allelic mutation (*jai1-2*) was identified as a suppressor of the systemin-mediated signaling pathway that requires JA biosynthesis and action to promote anti-herbivore defense responses in tomato (Howe and Ryan, 1999; Li et al., 2002b). In contrast to JA signaling mutants of *Arabidopsis*, reciprocal crosses showed that *jai1* plants are male fertile and female sterile (Li et al., 2001). This observation suggested that JA has different roles in the reproductive development of different plant species. On the other hand, several defense-related phenotypes of *jai1* plants are similar to those of *Arabidopsis coi1* mutants, including loss of expression of defense-related genes in response to wounding and MeJA, insensitivity to the phytotoxin coronatine (COR), and increased resistance to virulent strains of *Pseudomonas syringae* (Li et al., 2001; Zhao et al., 2003).

Here, we report that *jai1-1* and *jai1-2* correspond to mutations in the tomato homolog of the *Arabidopsis COI1* gene. These results demonstrate that this gene is essential for JA-regulated gene expression and anti-herbivore defense. We also show that tomato COI1 plays a role in the maternal control of seed maturation and the development of glandular trichomes. The potential involvement of JA in the regulation of these developmental processes is discussed.

RESULTS

jai1-1 Plants Are Insensitive to Exogenous Jasmonates

The *jai1-1* mutant was identified in a screen of a fast-neutron-mutagenized population of Micro-Tom plants for individuals that are deficient in the accumulation of polyphenol oxidase (PPO) and the Ser proteinase inhibitor (PI), PI-II, in response to exogenous MeJA (see Methods) (Li et al., 2001). With the exception of the overt phenotypes described below, *jai1-1* homozygotes exhibited normal vegetative morphology and growth (Figure 1A). F1 plants derived from a cross between *jai1-1* pollen

and a wild-type female parent accumulated normal levels of leaf PPO and PI-II in response to MeJA treatment. Semiquantitative analysis of leaf PPO activity in 687 adult F2 plants (Figure 1B) showed that the ratio of MeJA-responsive to MeJA-nonresponsive plants was 519:168 ($\chi^2 = 0.12$, $P = 0.72$ for the 3:1 hypothesis), indicating that *jai1-1* is a single recessive mutation.

To determine whether *jai1-1* affects seedling responses to JAs, we treated 4-day-old wild-type and *jai1-1* F2 seedlings ($n = 132$) with a 1-mM solution of MeJA. All MeJA-treated wild-type seedlings showed inhibition of root elongation, reduced hypocotyl growth, and accumulation of anthocyanin in the hypocotyl (Figure 1C). Approximately three-fourths of the F2 population (104 seedlings) exhibited this MeJA-sensitive pheno-

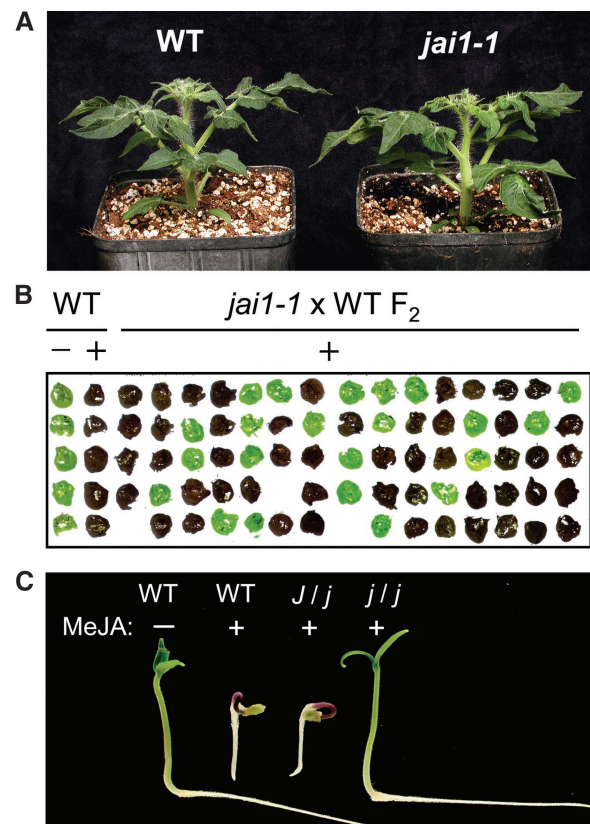


Figure 1. *jai1-1* Plants Are Insensitive to MeJA.

(A) Morphology of 4-week-old Micro-Tom (wild type [WT]; left) and *jai1-1* (right) plants.

(B) Rapid assay for MeJA-induced PPO activity in tomato leaves. Leaf juice from individual leaflets of 15-day-old plants was expressed on a nitrocellulose membrane and assayed for PPO activity as described by Howe and Ryan (1999). Wild-type plants were treated with MeJA (+) or ethanol (-) as a control. F₂ plants derived from a cross between *jai1-1* and the wild type were treated with MeJA before testing for PPO activity. Dark brown staining indicates the presence of PPO activity.

(C) Response of germinating seedlings to MeJA. Wild-type, heterozygous (*J/j*), and homozygous (*j/j*) seeds were germinated on moist filter paper and exposed to MeJA (+) for 2 days. A wild-type seedling grown in the absence of MeJA (-) is shown as a control.

type, whereas the remaining one-fourth (28 seedlings) were unaffected by the hormone (Figure 1C). After transfer to soil and growth for an additional 3 weeks, we found that MeJA-treated leaf tissue from each of the JA-insensitive plants lacked detectable PPO and PI-II accumulation (data not shown). These results demonstrate that *jai1-1* disrupts multiple JA-signaled responses in seedlings and mature plants.

Reproductive Phenotypes of *jai1-1* Plants

The general morphology and timing of development of *jai1-1* flowers were very similar to those of wild-type flowers (Figures 2A and 2B). However, the tip of mature *jai1-1* anther cones exhibited tissue collapse and browning, and the stigmas of mutant flowers protruded from the anther cone during later stages of flower development. Despite these anomalies, *jai1* flowers readily initiated fruit development upon pollination. The size of immature *jai1* fruit was similar to that of the wild type (Figures 2C and 2D), as was the general timing of fruit ripening. However, the size and mass of mature ripened *jai1-1* fruit were significantly less than those of mature wild-type fruit (Figure 2E). In one set of measurements using plants grown under identical conditions, the average weight of ripe fruit from wild-type and

jai1-1 plants was 5.4 ± 1.1 g and 2.9 ± 0.4 g, respectively (mean \pm SD; $n = 100$ fruit/genotype; $P < 0.0001$). Wild-type (Micro-Tom) fruit yielded ~ 30 seeds, each having a dry weight of ~ 2.5 mg. Ripe *jai1-1* fruit contained small, undeveloped seeds weighing < 0.7 mg dry weight (Figure 2F). Although the vast majority ($> 99\%$) of these were not viable, a few viable seeds were recovered from *jai1-1* fruit (Li et al., 2001) (data not shown). We estimated that the number of viable seeds produced by *jai1-1* plants was $< 0.1\%$ of the viable seed yield from wild-type plants grown under identical conditions, which for all practical purposes constitutes a sterile phenotype. Examination of several hundred fruit-bearing *jai1-1* homozygotes obtained from three successive backcrosses showed that the sterile phenotype strictly cosegregated with the JA-insensitive phenotypes in leaves (data not shown).

Reciprocal crosses between wild-type and *jai1-1* flowers showed that the mutation disrupts the maternal control of seed maturation (Li et al., 2001). We considered the possibility that this defect might be caused by an interaction between *jai1* and one of the mutations responsible for the dwarf stature of Micro-Tom (Meissner et al., 1997). However, after four successive backcrosses of *jai1-1* pollen to the standard Castlemart cultivar, *jai1-1* homozygotes selected from a resulting F2 popula-

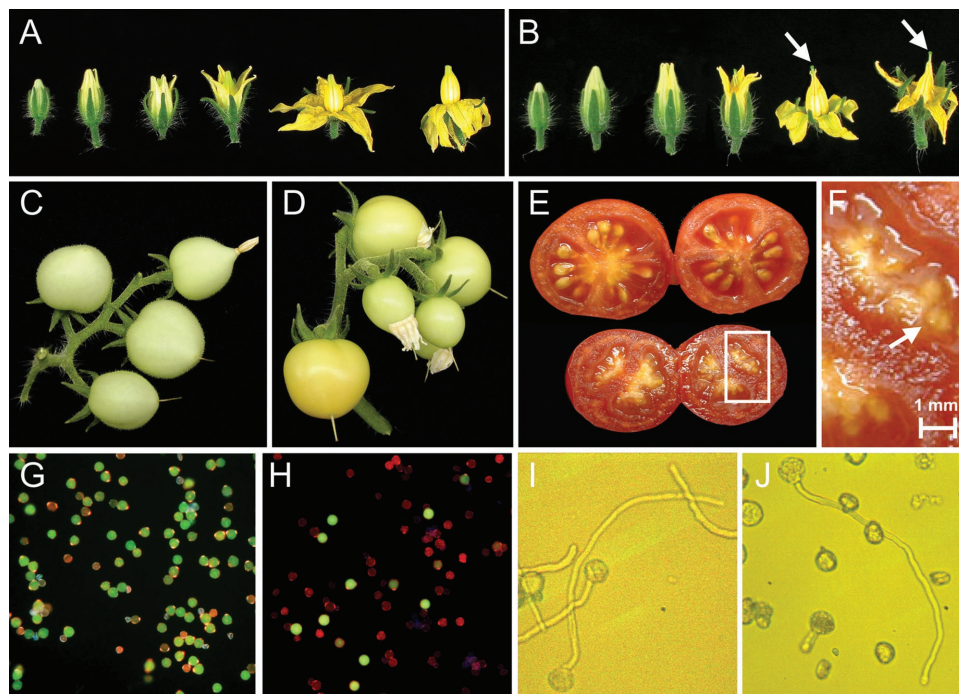


Figure 2. Reproductive Phenotypes of *jai1-1* Plants.

(A) and (B) Developmental progression of wild-type (A) and *jai1-1* (B) flowers. Arrows in (B) show the stigma protruding from the anther cone.

(C) and (D) Developing fruit on wild-type (C) and *jai1-1* (D) plants.

(E) Mature wild-type (top) and *jai1-1* (bottom) fruit.

(F) Enlargement of *jai1-1* fruit in (E), showing small undeveloped seeds.

(G) and (H) Fluorescein diacetate/propidium iodide costaining showing viable (green) and nonviable (red) pollen from wild-type (G) and *jai1-1* (H) anthers.

(I) and (J) Light microscopic images ($\times 100$ magnification) of germinated wild-type (I) and *jai1-1* (J) pollen.

tion remained sterile (data not shown). Thus, we conclude that *jai1-1* is sufficient to disrupt the maternal control of seed development.

The expression of PI-I and PI-II in tomato and potato leaves is induced in response to wounding and JA but is constitutive in flowers and tubers (Ryan, 1973; Peña-Cortés et al., 1991). The absence of PI-II accumulation in MeJA-treated *jai1-1* leaves suggested that the mutant also might be deficient in the constitutive expression of this protein in flowers. Using a radial immunodiffusion assay, we determined that the PI-II content of newly opened flower buds from wild-type plants was 218 ± 52 $\mu\text{g}/\text{mL}$ total soluble protein ($n = 15$ flowers). By contrast, PI-II accumulation in similarly staged *jai1-1* flowers was undetectable. This finding indicates that *jai1-1* abrogates constitutive PI-II expression in flowers, which is correlated with female sterility.

Because Arabidopsis mutants that are impaired in JA synthesis or JA perception are male sterile (Feys et al., 1994; McConn and Browse, 1996), we examined *jai1-1* plants for possible defects in pollen development. Staining of pollen grains with Alexander's triple stain showed that the proportion of nonaborted pollen grains (i.e., containing cytoplasm) from similarly staged wild-type and *jai1-1* anthers was 94 and 67%, respectively (data not shown). Fluorescein diacetate/propidium iodide co-staining further showed a significant reduction ($P < 0.005$) in the viability of *jai1-1* pollen (28% viability) compared with wild-type pollen (82% viability; Figures 2G and 2H). The general trend toward reduced vigor of *jai1-1* pollen was reflected in measurements of in vitro germination rates. In three independent experiments, the germination rate of *jai1-1* was $\sim 10\%$, whereas that of wild-type pollen was $>55\%$. The morphology and tube length of germinated *jai1-1* pollen grains were similar to those of wild-type pollen (Figures 2I and 2J). Together with the results from reciprocal cross experiments (Li et al., 2001), these findings indicate that viable pollen production in *jai1-1* plants is reduced but nevertheless sufficient for male reproductive function.

***jai1-1* Affects the Development of Glandular Trichomes**

Epidermal cells of immature tomato fruit give rise to various types of trichomes, which disappear upon fruit maturation. We observed two morphologically distinct multicellular trichome types on immature (green) wild-type fruit (Figures 3A and 3D): the hair-like glandular type-I trichome consisting of an elongated multicellular stalk and a small unicellular vesicle at the tip, and the well-characterized type-VI glandular trichome containing a short stalk and a four-celled glandular head (Luckwill, 1943; Kennedy, 2002). A striking feature of developing *jai1-1* fruit was the complete absence of both trichome types, resulting in a smooth and glabrous appearance (Figures 3B and 3E). We also observed a significant reduction in the density of type-VI trichomes on *jai1-1* leaves and sepals (Figures 3F to 3I). Quantification of trichome density on leaflets of 6-week-old wild-type plants (containing at least seven leaves) showed that densities ranged from ~ 35 type-VI trichomes/ mm^2 on terminal leaflets of young leaves to ~ 3 type-VI trichomes/ mm^2 on leaflets from older leaves. Analysis of comparable *jai1-1* leaflets showed that, irrespective of leaf age, type-VI trichome density on the mutant was 25 to 35% of that of wild-type leaflets. Simi-

larly, the density of type-VI trichomes on wild-type and *jai1* sepals of the same developmental stage was 20 ± 3 and 7 ± 3 per mm^2 , respectively ($P < 0.001$).

To determine whether *jai1-1* affects the production of compounds that are synthesized in trichome glands, we used gas chromatography to measure the content of volatile terpenes in exudates obtained by brief extraction of tissue with *tert*-methyl butyl ether (MTBE). Six compounds were detected in exudates from wild-type fruit. Four of these were identified as the monoterpenes α - and β -pinene, limonene, and *cis*- β -ocimene (Figure 4A), which were shown previously to be volatile components of tomato leaf aroma (Andersson et al., 1980; Buttery et al., 1987). Analysis of extracts obtained either from wiped wild-type fruit (to remove trichomes) or directly from type-VI glands demonstrated that the monoterpenes were derived from glandular trichomes (Figures 4E and 4F). Monoterpene accumulation was not detected in extracts from *jai1* fruit (Figure 4B), consistent with the lack of trichomes on this tissue. The monoterpene content of trichome exudates from wild-type sepals was comparable to that of developing fruit (Figure 4C). By contrast, the monoterpene level in *jai1* sepals was reduced significantly compared with that in wild-type sepals (Figure 4D).

***jai1-1* Plants Are Defective in JA-Induced Gene Expression**

RNA gel blot analysis was used to determine the effect of *jai1-1* on the MeJA-induced expression of several defense-related genes that are induced either "early" or "late" in response to wounding of tomato leaves (Ryan, 2000; Lee and Howe, 2003). Late gene transcripts, including *PI-I*, *PI-II*, *CATHEPSIN D INHIBITOR*, and *THREONINE DEAMINASE*, began to accumulate within 2 to 4 h of MeJA treatment and reached maximum levels 12 to 24 h after treatment. With the exception of very low levels of mRNA accumulation ($<2\%$ of wild type) at the 24-h time point, the expression of late genes in MeJA-treated and untreated control *jai1-1* plants was undetectable. Treatment of wild-type plants with MeJA caused moderate and transient expression of early wound response genes, including those that encode the octadecanoid pathway enzymes LIPOXYGENASE D (*LoxD*; Heitz et al., 1997), ALLENE OXIDE SYNTHASE2 (*AOS2*; Howe et al., 2000), 12-OXO-PHYTODIENOIC ACID REDUCTASE3 (*OPR3*; Strassner et al., 2002), and PROSYSTEMIN (*PSYS*; Jacinto et al., 1997). In *jai1-1* plants, MeJA treatment either reduced (e.g., *LoxD*) or did not significantly affect (e.g., *PSYS*) the accumulation of early gene transcripts (Figure 5). It also was apparent that, unlike the late genes, *jai1-1* plants maintained a basal level of expression of early response genes.

DNA microarray analysis was used to further examine the effect of *jai1-1* on MeJA-induced gene expression. These experiments used a microarray slide containing 607 tomato cDNAs corresponding to ~ 500 unique genes involved in various aspects of herbivore and pathogen defense, signal transduction, lipid metabolism, and hormone synthesis (Zhao et al., 2003). A complete list of these genes together with the expression data for each is provided in the supplemental data online. To identify jasmonate-responsive genes among this collection of sequences, slides were hybridized simultaneously to probes that were prepared from plants treated for 8 h with MeJA or a mock

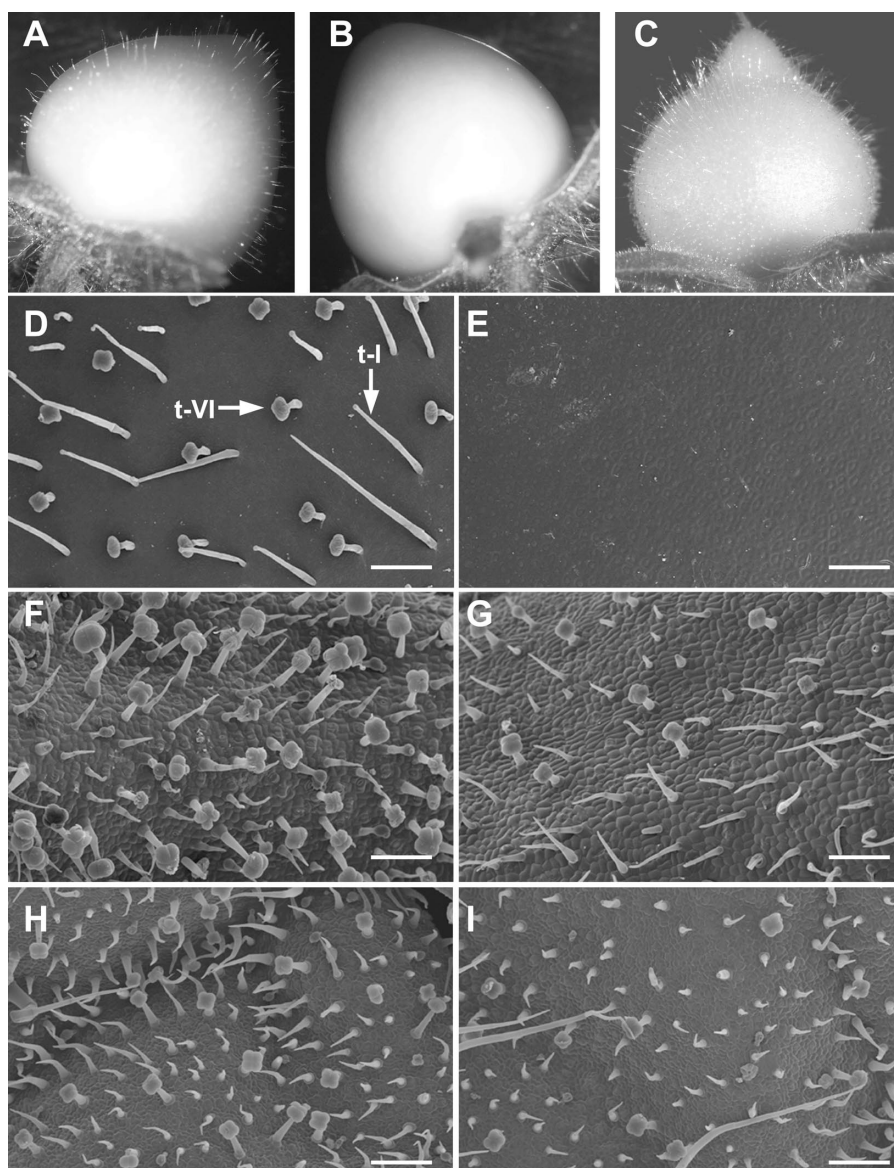


Figure 3. *jai1-1* Plants Exhibit Defects in Trichome Development.

(A) to (C) Photographs of developing green fruit from wild-type (A), *jai1-1* (B), and *35S-LeCoi1*-complemented *jai1-1* (C) plants.

(D) and (E) Scanning electron micrographs of the surface of developing green fruit from wild-type (D) and *jai1-1* (E) plants. Arrows in (D) denote type-VI (t-VI) and type-I (t-I) trichomes. The tissue fixation procedure used for scanning electron microscopy affected the structure of type-I trichomes, which were 1 to 2.5 mm long on intact tissue (see [A] and [D]).

(F) and (G) Scanning electron micrographs of wild-type (F) and *jai1-1* (G) sepals.

(H) and (I) Scanning electron micrographs of the adaxial (upper) side of young leaves from wild-type (H) and *jai1-1* (I) plants.

Bars in (D) to (I) = 0.2 mm.

control (ethanol). This relatively brief exposure time was chosen to minimize possible secondary effects resulting from MeJA application and because both early and late genes are upregulated at this time point (Figure 5). Analysis of data from two independent biological replicates showed that 37 genes were upregulated >2.5-fold in MeJA-treated wild-type plants (Table 1; see also supplemental data online). This list included the four

early genes and four late genes analyzed by RNA gel blot analysis (Figure 5) as well as other previously identified JA- and wound-responsive genes, including *LEUCINE AMINOPEPTIDASE (LAP)*; Chao et al., 1999), *METALLOCARBOXYPEPTIDASE INHIBITOR (MCPI)*; Villanueva et al., 1998), *ALLENE OXIDE CYCLASE (AOC)*; Hause et al., 2000), *LIPOXYGENASE A (LOXA)*; Beaudoin and Rothstein, 1997), *POLYPHENOL OXI-*

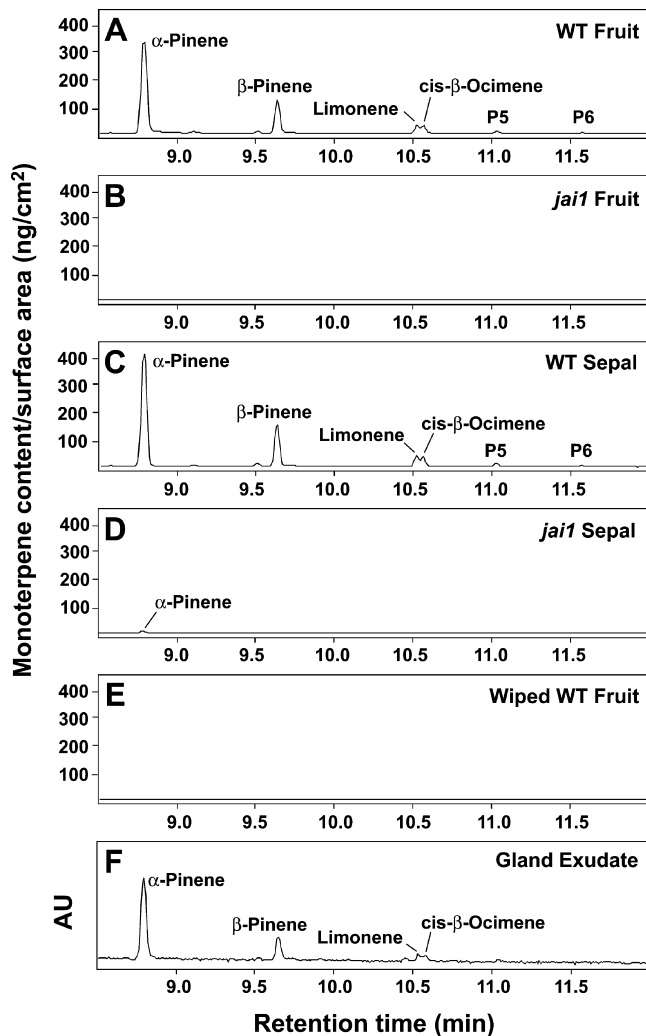


Figure 4. Monoterpene Content in Trichome Exudates.

Trichome exudates obtained by brief extraction of tissues with MTBE were analyzed for monoterpene content by gas chromatography–mass spectrometry.

- (A) Wild-type (WT) green fruit.
 (B) *jai1-1* green fruit.
 (C) Wild-type sepal.
 (D) *jai1-1* sepal.
 (E) Before extraction with MTBE, wild-type fruit was wiped with a cotton swab to remove trichome contents.
 (F) Exudate collected directly from type-VI trichome glands of wild-type green fruit. The y axis indicates arbitrary absorbance units (AU).

DASE-F (*PPO-F*; Constabel et al., 1995), a wound-inducible *SERINE CARBOXYPEPTIDASE* (*CP*; Moura et al., 2001), *CY-STATIN* (Schaller et al., 1995), and *AOS1* (Sivasankar et al., 2000). Remarkably, none of these 37 genes was upregulated differentially in MeJA-treated *jai1-1* plants (Table 1; see also supplemental data online). This finding indicates that the gene defined by *jai1* plays a major role in promoting the expression of the JA-regulated transcriptome in tomato.

jai1-1 Causes Susceptibility to Spider Mites and Modulates Mite Behavior

The lack of MeJA-induced gene expression in *jai1-1* plants suggested that the mutant might be compromised in resistance to herbivore attack. To test this idea, we used various biological assays to compare the interaction of wild-type and *jai1* genotypes with the two-spotted spider mite, a cell-content-feeding arachnid that is a major pest of many crop plants. Two days after challenge with adult female mites, small (<0.5 mm) chlorotic lesions indicative of mite feeding were evident on *jai1-1* but not on wild-type leaves (Figures 6A and 6B). Localized collapse of *jai1-1* tissue was apparent 6 days after infestation, with total collapse and desiccation of the leaf occurring by 11 days (Figures 6C and 6D). Longer-term feeding trials, in which spider mites dispersed to aerial tissues, resulted in severe damage to and eventual death of *jai1-1* plants (Figure 6F). Wild-type plants showed relatively few signs of macroscopic damage during the course of these feeding trials (Figures 6C and 6E). These results indicate that Micro-Tom plants have a relatively high level of natural resistance to the two-spotted spider mite and that this resistance is severely compromised by *jai1-1*.

To further characterize the effect of *jai1-1* on spider mite performance, we used a two-choice assay to assess the relative preference of the herbivore for wild-type and *jai1-1* leaves. Within 1 h of initiating the assay, >94% of the mites were found either on or under one of the two leaflets. However, the mites selected *jai1* over wild-type leaves in 99 of 140 plant visits (Figure 6G) ($P < 0.05$). Even distributions of mites were observed when leaflet pairs of the same genotype were used, with no significant difference in the number of visitations to either genotype (data not shown). This observation indicates that neither genotype is particularly repellent to mites, but rather that *jai1* plants are more attractive than are wild-type plants. The ability of the herbivore to colonize the host was examined by measuring the fecundity of female mites on wild-type and *jai1* leaves. The results showed that the number of eggs laid on *jai1* leaves during the 4-day time course was significantly greater than that on wild-type leaves (Figure 6H).

jai1 Disrupts the Function of the Tomato Homolog of COI1

The effect of *jai1-1* on MeJA-induced gene expression, resistance to herbivores, and responses to COR (Zhao et al., 2003) suggested that the mutant might be defective in the tomato homolog of the COI1 protein that is required for JA-signaled processes in Arabidopsis (Xie et al., 1998). To test this possibility, we isolated a cDNA that encodes the tomato homolog, designated LeCOI1 (*Lycopersicon esculentum* COI1; see Methods). The full-length cDNA contained a 1812-nucleotide open reading frame predicted to encode a 603-amino acid protein. A BLAST (Basic Local Alignment Search Tool) search (Altschul et al., 1990) of this sequence against the NCBI nonredundant protein database identified unambiguous matches (E value = 0) to Arabidopsis COI1 and a putative homolog predicted from the rice genome sequence. Amino acid sequence alignments showed that LeCOI1 is 68 and 57% identical to the Arabidopsis and rice proteins, respectively (Figure 7). Similar to Arabidopsis COI1 (Xie et al., 1998), the predicted COI1 proteins in tomato

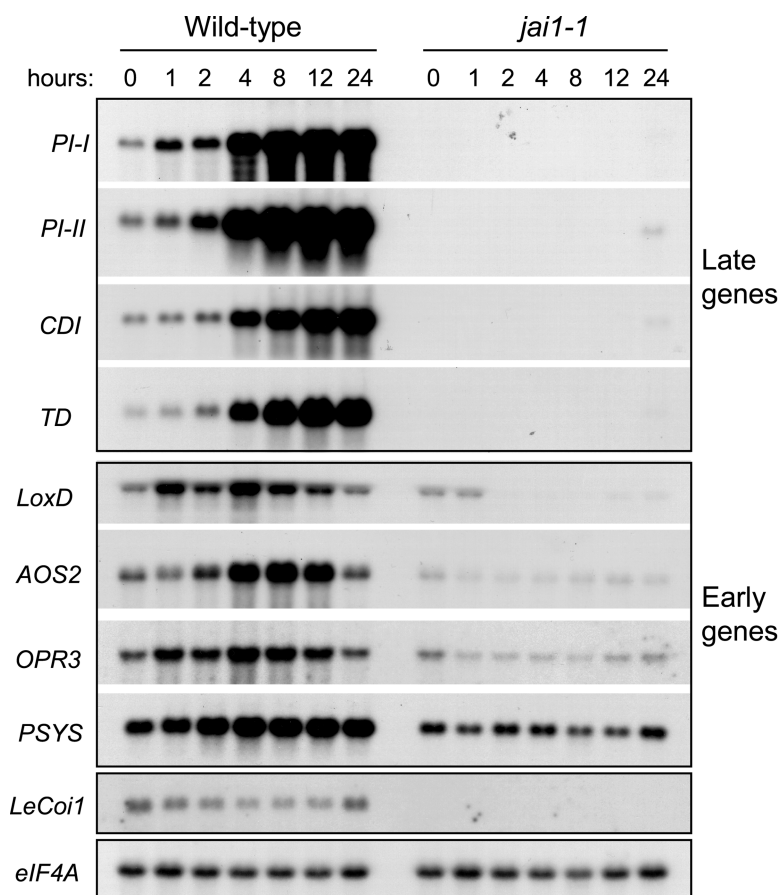


Figure 5. MeJA-Induced Gene Expression in Wild-Type and *jai1-1* Plants.

Three-week-old Micro-Tom and *jai1-1* plants were exposed to MeJA vapor for various lengths of time (hours) in an enclosed box. Leaves from similarly treated plants of the same genotype were pooled for RNA extraction. RNA isolated from untreated plants (0-h time point) also was analyzed as a control. RNA gel blots were hybridized to cDNA probes representing four late genes (*PI-I*, *PI-II*, *CATHEPSIN D INHIBITOR [CDI]*, and *THREONINE DEAMINASE [TD]*) and four early genes (*LIPOXYGENASE D [LoxD]*, *ALLENE OXIDE SYNTHASE2 [AOS2]*, *OPDA REDUCTASE3 [OPR3]*, and *PRO-SYSTEMIN [PSYS]*). Blots also were hybridized to the *LeCoi1* cDNA and, as a loading control, to an *eIF4A* probe.

and rice contain an N-terminal F-box domain and 16 imperfect Leu-rich repeats (LRRs). To obtain additional evidence for homology between tomato and Arabidopsis COI1, we determined the DNA sequence of the tomato gene (*LeCoi1*) that was identified on a BAC clone. Comparison of the genomic and cDNA sequences showed that *LeCoi1* comprises three exons and two introns that span ~6 kb (Figure 8A). The positions of the two introns within the gene, together with the coding capacity of individual exons, were identical to those of the Arabidopsis and rice genes (Figure 8B). Intron size accounted for the major differences between the three genes; on average, the size of introns in *LeCoi1* was threefold greater than the corresponding introns in the Arabidopsis and rice genes (Figure 8B).

Genomic DNA gel blot analysis was used to determine whether the fast-neutron bombardment used to create *jai1-1* plants caused a deletion mutation at the *LeCoi1* locus. The full-length *LeCoi1* cDNA hybridized to three BglIII restriction fragments (~6.5, 4.5, and 1.3 kb) in wild-type genomic DNA but only a single 5.5-kb fragment in DNA from a *jai1-1* homozygote

(Figure 8C). Hybridization experiments using cDNA end-derived probes further indicated that genomic DNA from *jai1-1* plants contains the 5' end but not the 3' end of *LeCoi1* (Figure 8C). To define the size of this deletion polymorphism more precisely, thermal asymmetric interlaced PCR was used to isolate a fragment of genomic DNA from *jai1-1* plants that spans the deletion end points. Comparison of the sequence of a resulting 1.3-kb PCR product with the *LeCoi1* genomic sequence showed that the deletion spans 6243 bp and includes exons 2 and 3 (Figure 8A). The 3' end of the deletion was mapped 2.3 kb downstream of the *LeCoi1* stop codon. Sequence analysis using BLAST searches indicated that the deletion does not affect genes other than *LeCoi1*, including a putative MYB factor gene located ~0.8 kb downstream of the deletion end point. RNA gel blot analysis demonstrated that *LeCoi1* transcripts, which accumulate in all wild-type tissues examined, do not accumulate in *jai1-1* plants (Figures 5 and 8D).

To determine whether the ethyl methanesulfonate-induced *jai1-2* mutation corresponds to a lesion in *LeCoi1*, we used re-

Table 1. Microarray Analysis of Genes Induced by MeJA in Wild-Type and *jai1-1* Plants

Accession No.	Gene Product (Best BLAST Hit)	Expression Ratio	
		Wild Type	<i>jai1</i>
AI485116	Thr deaminase	41.9	– ^a
Q10712	Leu aminopeptidase A (LAP-A)	33.0	1.3
AI485529	Putative acyltransferase	25.9	0.7
AI486173	Protein translation inhibitor	19.8	– ^a
AI897750	Kunitz-type trypsin inhibitor	14.8	0.8
AW037833	Metalloproteinase inhibitor	12.7	– ^a
AI487422	Pto-responsive gene 1 protein	12.5	1.0
AI488657	Cathepsin D inhibitor protein (CDI)	12.4	0.7
K03291	Proteinase inhibitor II (PI-II)	11.2	– ^a
AW649914	Leu aminopeptidase N (LAP-N)	11.0	1.3
AW624058	Allene oxide cyclase (AOC)	10.8	1.4
U09026	Lipoxygenase A (LOXA)	9.2	0.8
Z12838	Polyphenol oxidase F (PPO-F)	8.4	1.3
K03290	Proteinase inhibitor (PI-I)	8.2	1.0
AW092579	Nucleoside diphosphate kinase	5.7	1.0
AI897184	Glyoxylase family protein	5.5	0.9
AI490318	NAC domain protein	5.5	0.8
AI486025	4-Coumarate:CoA ligase	5.2	1.1
AW040669	Thioredoxin M-type 3 chloroplast precursor	5.1	0.9
AI486916	Kunitz-type enzyme inhibitor	4.5	1.1
AI486546	Wound-inducible carboxypeptidase (WIC)	4.5	1.1
AF198389	Cystatin	4.4	1.1
AI489221	Wound-inducible WRKY transcription factor	4.3	1.0
AI483527	Kunitz-type trypsin inhibitor	4.2	0.7
Z21793	DAHPh-synthase 2	4.2	1.2
AW034958	12-Oxo-phytyldienoate reductase3 (OPR3)	4.2	1.1
AI771886	RD2 auxin-regulated protein	4.0	1.1
AW032472	Unknown protein, similar to PnFL-2	4.0	0.9
AW220064	Glutathione S-transferase	3.9	1.2
AF230371	Allene oxide synthase2 (AOS2)	3.8	1.0
AW648326	Adenosylmethionine decarboxylase	3.5	1.1
U37840	Lipoxygenase D (LOXD)	3.4	1.0
AI895589	Allene oxide synthase1 (AOS1)	3.0	0.9
M84800	Prosystemin	2.9	1.2
AI897620	Putative chorismate mutase	2.9	1.2
AI483536	TMV response-related gene product	2.7	0.9
BE459901	Putative caffeoyl-CoA O-methyltransferase	2.6	0.7

Three-week-old Castlemart (wild-type) and *jai1-1* plants were treated with either MeJA or ethanol (mock control) for 8 h. Leaf tissue was harvested for RNA isolation after the treatment. A custom cDNA microarray slide representing ~500 tomato genes was hybridized simultaneously to probes derived from RNA isolated from ethanol- and MeJA-treated plants of the same genotype (i.e., wild type or *jai1-1*). Numbers represent the mean expression ratio (MeJA:ethanol) of two independent biological replicates for each experiment. Genes (GenBank accession number and putative function) that were differentially regulated by >2.5-fold in response to MeJA in wild-type plants are listed.

^aExpression was not detectable, indicating that the microarray signal was below background levels.

verse transcription-PCR to obtain full-length cDNAs from *jai1-2* homozygotes. DNA sequencing of these clones revealed a single G-to-T transversion that results in the replacement of Gly-261, which is located between the sixth and seventh LRRs, by a Cys (Figure 7). This polymorphism was verified by sequencing the corresponding genomic region from wild-type and *jai1-2* plants.

To confirm that *jai1*-associated phenotypes result from the loss of function of LeCOI1, we used *Agrobacterium tumefaciens* to transform *jai1-1* plants with the *LeCoi1* cDNA under the control of the 35S promoter of *Cauliflower mosaic virus*. From 28 independent 35S-*LeCoi1* transgenic plants confirmed by genomic PCR, nine primary lines (T1) exhibited trichome development on immature fruit (Figure 3C) and normal seed production. Gas chromatographic analysis of trichome exudates from three of these lines showed that monoterpene accumulation was restored (data not shown). Additional phenotypic characterization was performed on T2 progeny derived from two primary lines (T2-08 and T2-13), which by DNA gel blot analysis were shown to harbor one or two copies of the transgene (data not shown). RNA gel blot experiments demonstrated that T2 plants expressing 35S-*LeCoi1* accumulated *PI-II* mRNA in response to exogenous MeJA, whereas T2 siblings lacking the transgene did not (Figure 9). The results of this and other (Figure 5) RNA gel blot hybridization experiments also showed that exogenous MeJA reduced the abundance of *LeCoi1* transcripts in wild-type plants. This effect was not observed in the transgenic lines in which *LeCoi1* was expressed from the 35S promoter. Consistent with the restoration of JA-induced *PI* expression in 35S-*LeCoi1*-complemented lines, we also found that the fecundity of female spider mites on these lines was reduced to the level observed on wild-type plants (Figure 6H). These genetic complementation studies demonstrate that *Jai1* and *LeCoi1* are equivalent and that this gene promotes multiple processes in tomato, including sensitivity to JAs, resistance to herbivores, glandular trichome development, and seed maturation.

DISCUSSION

Identification of a Tomato Homolog of COI1

We report here the phenotypic and molecular characterization of the tomato *jai1* mutant that was identified in a genetic screen for mutants that fail to produce PPO and PI-II in response to MeJA. Using a candidate gene approach that relied on previous knowledge of JA signaling in Arabidopsis, we demonstrate that *jai1* disrupts the function of a tomato homolog (LeCOI1) of COI1. The role of *Jai1/LeCoi1* (henceforth referred to as *LeCoi1*) in promoting the responsiveness of roots to JA, JA-induced gene expression in leaves, and the accumulation of JA-regulated PIs in flowers indicates that this gene mediates JA-signaled responses in most, if not all, tissues of tomato. This conclusion is supported by the accumulation of *LeCoi1* transcripts in all major organs of wild-type plants (Figure 8D).

Recent studies with Arabidopsis have established that COI1 is part of a multiprotein complex that functions as an E3-type ubiquitin ligase (Xie et al., 1998; Devoto et al., 2002; Xu et al., 2002). This assembly of proteins, referred to as SCF^{COI} (for Skp1, Cullin, and F-box protein) is proposed to work together

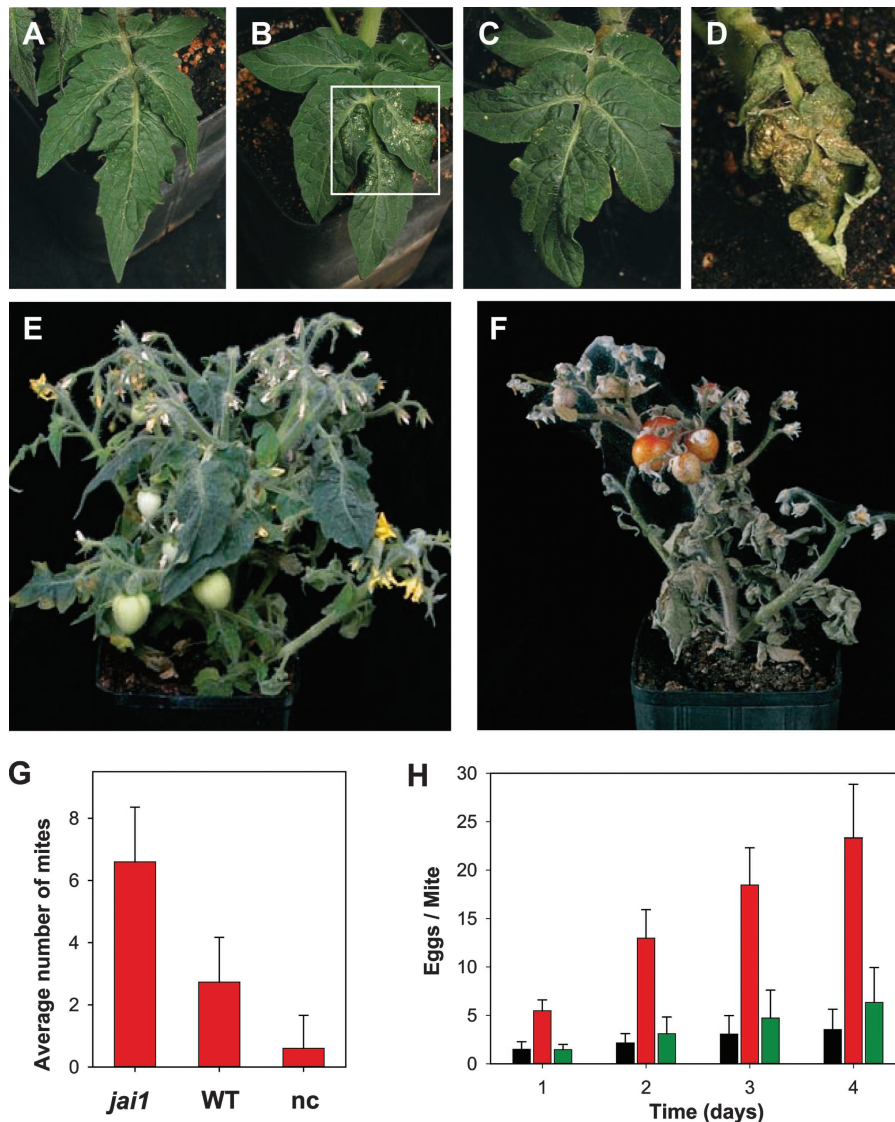


Figure 6. Resistance of *jai1-1* Plants to the Two-Spotted Spider Mite Is Severely Compromised.

(A) to (D) Fifteen adult female mites were transferred to a single leaf on 3-week-old wild-type (A) and (C) and *jai1-1* (B) and (D) plants. Photographs of the infested leaves were taken 6 days (A) and (B) and 11 days (C) and (D) after challenge. The boxed area in (B) shows the initial effects of feeding damage on *jai1-1*.

(E) and (F) Photographs of representative 7-week-old wild-type (E) and *jai1-1* (F) plants at 30 days after infestation.

(G) Two-choice assay to measure the preference of spider mites for wild-type (WT) or *jai1-1* plants. Ten adult female spider mites were placed in an arena equidistant from wild-type and *jai1-1* leaflets. The number of mites that moved to one or the other of the leaflets was determined 1 h after initiating the assay, as was the number of mites that failed to make a choice (nc). Data represent means and standard errors from 16 repetitions (total of 160 mites).

(H) Fecundity of two-spotted spider mites on wild-type (black bar), *jai1-1* (red bar), and 35S-*LeCoi1*-complemented *jai1-1* (green bar) plants. Data represent means and standard deviations from 12 experimental repetitions in which five teneral female mites were reared for 4 days on leaf discs of the indicated host genotype.

with ubiquitin-activating (E1) and ubiquitin-conjugating (E2) enzymes to attach ubiquitin to a protein substrate that is recruited to the complex via interaction with the LRR domain of COI1 and subsequently degraded by the 26S proteasome (Creelman and Rao, 2002; Turner et al., 2002). Ubiquitin-dependent pro-

teolysis plays an important role in modulating the abundance of regulatory proteins and recently emerged as a major theme in many aspects of plant signaling (Hare et al., 2003). Based on the strong JA-insensitive phenotypes of tomato *jai1* and Arabidopsis *coi1* mutants, we conclude that COI1 performs a similar

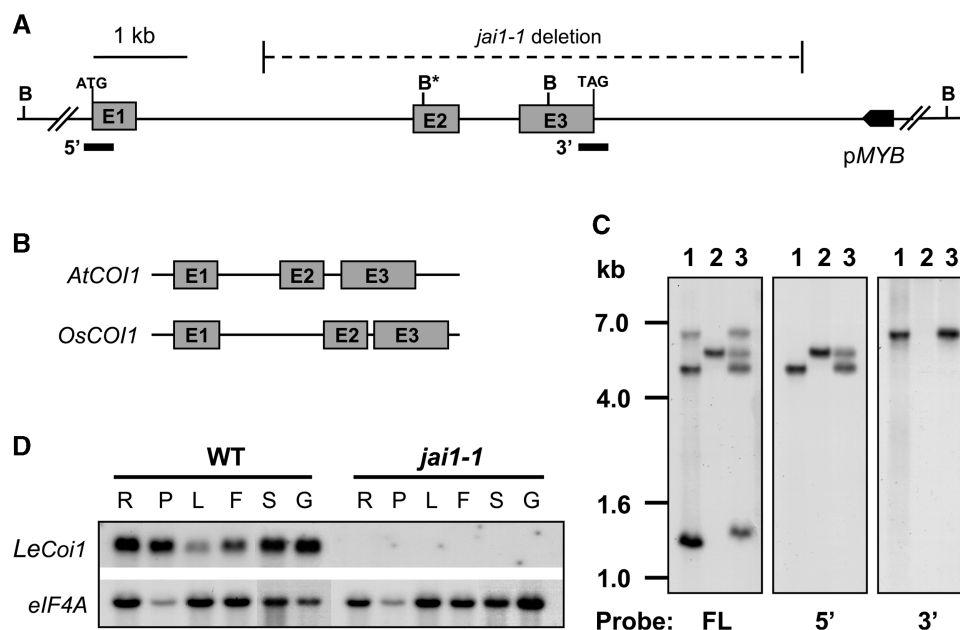


Figure 8. The *jai1-1* Mutation Is a 6.2-kb Deletion in *LeCOI1*.

(A) Scheme of a 9.4-kb region of genomic DNA containing *LeCOI1* and a gene encoding a putative MYB transcription factor (pMYB). The three exons (E1 to E3) and intervening sequences that constitute *LeCOI1* are drawn to scale. Only the translated portion of the first and last exons are shown, together with the start (ATG) and stop (TAG) codons within the respective exons. The 6.2-kb region of DNA that is deleted in *jai1-1* plants is shown by the horizontal hatched line. B indicates *Bgl*III restriction sites used for the DNA gel blot analysis shown in (C), and B* indicates the presence of two *Bgl*III sites that are separated by 24 bp. cDNA probes used to detect the 5' and 3' ends of the gene in (C) are shown as thick horizontal bars.

(B) Structures of the Arabidopsis (*AtCOI1*) and rice (*OsCOI1*) homologous genes drawn to the same scale as *LeCOI1* in (A).

(C) DNA gel blot analysis of *Bgl*III-restricted genomic DNA from the wild type (lanes 1), *jai1-1* (lanes 2), and an F1 hybrid produced from a cross between the wild type and *jai1-1* (lanes 3). Three identical blots were hybridized to a full-length (FL) *LeCOI1* cDNA probe or to probes corresponding to the 5' and 3' ends of the cDNA (see [A]). Numbers at left indicate the positions of DNA size standards (in kb).

(D) RNA gel blot analysis of *LeCOI1* transcript levels in wild-type (WT) and *jai1-1* plants. Five micrograms of total RNA from root (R), petiole (P), leaf (L), unopened flower bud (F), sepal (S), and immature green fruit (G) was immobilized to a membrane and hybridized to a *LeCOI1* cDNA probe (top gel). A duplicate blot was hybridized to an *eIF4A* cDNA probe as a loading control.

requires JA (Ryan, 2000; Lee and Howe, 2003; Li et al., 2003). The Gly-to-Cys amino acid substitution caused by *jai1-2* is located in a region of the LRR domain that was shown previously to be important for JA signaling in Arabidopsis as well as for interaction between COI1 and putative SCF^{COI1} substrates (Devoto et al., 2002; Ellis and Turner, 2002). Notably, however, this substitution is located within an eight-amino acid segment that is missing from both the Arabidopsis and rice sequences (Figure 7). Because the mutation causes the loss of JA responsiveness, it is possible that this region of LeCOI1 plays a role in recruiting protein substrates for ubiquitination. An alternative explanation is that the Cys created by *jai1-2* forms a disulfide linkage that disrupts the structure and function of the protein.

COI1-Regulated Gene Expression

PIs and PPOs constitute an important anti-herbivore defense system in tomato (Duffey, 1986; Ryan, 2000). The inability of *jai1* plants to express PIs and PPO in response to wounding or JA demonstrates that LeCOI1 is essential for this line of chemical defense. Interestingly, genes that encode PPO and PI-II are

not present in the Arabidopsis genome (Allen, 2002; Van der Hoeven et al., 2002). This observation indicates that although components of the JA signaling pathway (i.e., COI1) are conserved in different plant species, many of the target genes regulated by the pathway are specific to a particular plant species or family. This idea is supported by numerous examples of JA-regulated phytochemical defense systems that operate in specific plant lineages (Heil et al., 2001; Memelink et al., 2001; Kessler and Baldwin, 2002; Wasternack and Hause, 2002; Goossens et al., 2003). Additional insight into the role of LeCOI1 in regulating the JA transcriptome in tomato was obtained by cDNA microarray analysis. These results showed that the MeJA-induced expression of each of the 37 genes identified in wild-type plants was dependent on LeCOI1 (Table 1), which demonstrates a central role for this F-box protein in the expression of JA-responsive genes in tomato. It should be noted that we identified two genes (encoding putative glucosyltransferases) that were induced by MeJA in *jai1-1* plants (see supplemental data online). However, these genes were not regulated differentially in MeJA-treated wild-type plants and thus were not considered to be COI1-dependent genes.

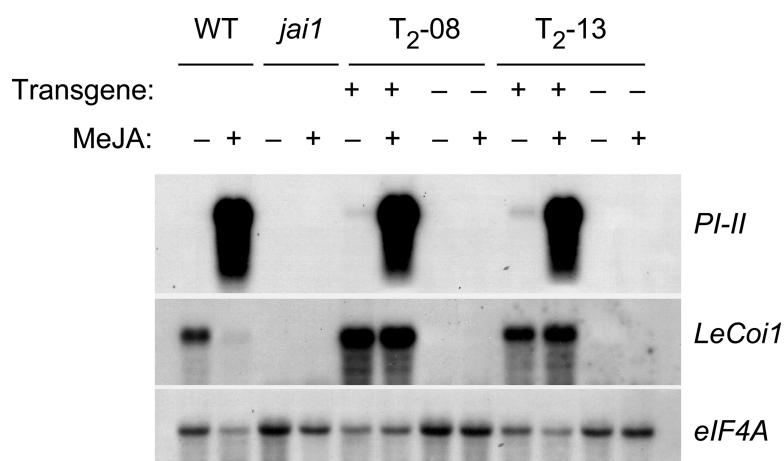


Figure 9. MeJA-Induced Gene Expression in 35S-*Coi1*-Complemented Transgenic Lines.

Total RNA was prepared from wild-type (WT), *jai1-1* (*jai1*), and T2 siblings from two 35S-*Coi1*-complemented lines (T2-08 and T2-13) either before (-) or after (+) exposure to MeJA vapor for 12 h. For each transgenic line tested, a PCR assay was used to identify siblings that either harbor (+) or lack (-) the 35S-*LeCoi1* transgene. RNA gel blots were hybridized to cDNA probes for *PI-II* and *LeCoi1* and to *eIF4A* as a loading control.

Although our results demonstrate that LeCOI1 is essential for JA-induced gene expression in tomato, RNA gel blot analysis showed that the JA/COI1 signaling pathway performs different roles in controlling the expression level of early and late wound response genes. For example, both the basal and JA-induced expression of late genes (e.g., *PIs*) was abrogated in *jai1-1* plants (Figure 5). By contrast, significant basal expression of early genes was maintained in *jai1* plants. Furthermore, the expression of some of these genes (e.g., *LoxD*) decreased in *jai1* plants in response to MeJA (Figure 5). This phenomenon may reflect the existence of a compensatory COI1-independent mechanism to restrict the expression of some wound/JA-responsive genes. The transient nature of the increase in early gene expression in MeJA-treated wild-type plants supports this view. The downregulation of *LeCoi1* transcript levels in MeJA-treated wild-type plants (Figures 5 and 9) provides additional evidence that control points within the JA signaling pathway may be negatively regulated by JA.

Because *jai1-1* is a null mutation in *LeCoi1*, we conclude that the expression of early genes is controlled by both COI1-dependent and COI1-independent mechanisms. This conclusion is consistent with previous studies showing that *PSYS* (an early gene) is expressed constitutively in unwounded tomato leaves and induced further in response to wounding and MeJA treatment (Jacinto et al., 1997). Similarly, JA biosynthetic enzymes, which also are encoded by early wound response genes, accumulate constitutively in unwounded tomato leaves (Stenzel et al., 2003). The COI1-independent basal expression of early genes suggests that the respective gene products serve an important function in the absence of stress conditions that trigger JA signaling. For example, uncoupling of the basal expression level of JA biosynthetic enzymes and prosystemin from the JA/COI1 pathway might provide a mechanism to ensure that the amplitude and timing of JA biosynthesis in response to herbivore attack is sufficient to activate downstream target genes.

This hypothesis is consistent with evidence indicating that wound-induced JA synthesis in tomato and tobacco does not depend on the induced expression of JA biosynthetic genes (Miersch and Wasternack, 2000; Ziegler et al., 2001).

The critical role of LeCOI1 in JA-regulated gene expression is relevant to the observation that *jai1-1* plants are insensitive to COR and highly resistant to COR-producing strains of *P. syringae* that cause bacterial speck disease on tomato (Zhao et al., 2003). Because these phenotypes of *jai1-1* are essentially identical to those of Arabidopsis *coi1* mutants (Feys et al., 1994; Kloek et al., 2001), the mechanism by which COI1 promotes responsiveness to COR and susceptibility to *P. syringae* likely is conserved between tomato and Arabidopsis. Studies conducted with Arabidopsis have shown that COR acts both to inhibit salicylic acid-mediated host defense responses and to enhance the formation of lesions (Kloek et al., 2001). Recent work performed with tomato supports this conclusion and further indicates that COR, acting as a JA mimic, promotes host susceptibility by specifically targeting the JA/COI1 signaling pathway (Zhao et al., 2003). In support of this view, comparison of the microarray results reported here with those presented by Zhao et al. (2003) shows that 36 of the 37 JA/COI1-responsive genes (Table 1) were induced in a COI1-dependent manner during infection of wild-type plants with a virulent strain of *P. syringae*. The similar pattern of COI1-dependent gene expression in MeJA-treated and *P. syringae*-infected tomato leaves is consistent with the observation that exogenous MeJA complements the loss of virulence of a COR-deficient strain of *P. syringae* (Zhao et al., 2003) and the idea that COR functions as a molecular mimic of JAs.

Role of COI1 in Tomato Reproduction

jai1 plants fail to produce viable seeds as a result of a defect in female reproductive development (Li et al., 2001). Functional complementation of the sterile phenotype with 35S-*LeCoi1* in-

dicates that COI1 is essential for this aspect of tomato reproductive function. The production of small aborted seeds in *jai1* fruit (Figure 2F), together with the ability of *jai1* ovules to be fertilized (B. McCaig and G. Howe, unpublished results), suggest that sterility results from arrest in embryo/seed maturation. Because cell division and expansion in fruit tissues is dependent on normal embryo and seed development (Gillaspy et al., 1993; Giovannoni, 2001), this hypothesis would explain the reduced size of *jai1* fruit. A role for LeCOI1 in embryogenesis also is consistent with the abundance of JAs in tomato ovaries (Hause et al., 2000) as well as with previous studies implicating JA as an endogenous regulator of embryo development in oilseeds (Wilens et al., 1991; Hays et al., 1999). Whether endogenous JAs regulate reproductive function in tomato remains to be established. The fact that JA biosynthetic mutants of tomato do not exhibit female (or male) sterility does not disprove this hypothesis, because such mutants produce significant levels of JA in floral tissues (Li et al., 2003). Identification of tomato mutants that completely lack JA will be necessary to address this question. On the other hand, the disruption of either JA biosynthesis or COI1 function in Arabidopsis does not affect female reproductive development (Feys et al., 1994; McConn and Browse, 1996). We suggest that these differences in the hormonal control of seed development in tomato and Arabidopsis may be related to differences in fruit type: siliques of Arabidopsis and many other Brassicaceae species are dehiscent dry fruit, whereas tomato produces a nondehiscent fleshy fruit.

The behavior of *jai1* as a recessive sporophytic mutation (i.e., typical 3:1 Mendelian segregation) indicates that one or more COI1-dependent processes in maternal sporophytic tissue is required for seed maturation. It is possible that LeCOI1 regulates the synthesis of a vitamin or cofactor that requires transport from maternal tissues to the developing embryo, similar to the role of biotin synthesis in Arabidopsis embryogenesis (Patton et al., 1998). Alternatively, COI1 may promote the accumulation of proteins that supply reproductive tissues with sources of carbon or nitrogen. This hypothesis is consistent with the observation that the sterility of *jai1* flowers is associated with a loss of accumulation of PI-II. This finding, together with the fact that tomato flowers contain high levels of endogenous JAs (Hause et al., 2000), demonstrates that PI expression in tomato reproductive tissues is regulated primarily by the JA/COI1 signaling pathway. It is likely that PI-II represents one of many proteins that are deficient in *jai1* flowers. In this context, *jai1* plants will provide a useful tool for identifying other COI1-dependent processes that operate in reproductive tissues and may be used as a genetic background in which to assess the functions of various JA/COI1-regulated genes in reproductive development.

In addition to a defect in the maternal control of seed development, *jai1* plants also exhibited defects in male reproductive function. Most notable among these was a reduction in pollen viability and germination. It also was apparent that the stigma of *jai1* flowers protruded from the anther cone of mature flowers, which is known to reduce pollination efficiency in some genotypes and under some environmental conditions (Rick and Dempsey, 1969). Although stigma exertion did not appear to result from abnormal development of the anther cone, the tip of

jai1 anther cones did exhibit localized browning and tissue collapse. Despite these anomalies in *jai1* anther development, the mutant produced a sufficient amount of viable pollen to render it male fertile in genetic crosses (Li et al., 2001). Thus, we conclude that, unlike Arabidopsis, COI1 function is not absolutely required for male fertility in tomato.

Role of COI1 in Glandular Trichome Development and Anti-Herbivore Defense

Cultivated and wild species of tomato produce a variety of multicellular glandular and nonglandular trichomes that provide both physical and chemical (i.e., entrapment or toxicity) barriers against insect invaders (Duffey, 1986; Kennedy, 2002). In contrast to detailed knowledge about the molecular processes underlying the development of single-celled trichomes in Arabidopsis, relatively little is known about the genetic control of multicellular trichomes (Glover and Martin, 2000). Here, we provide genetic evidence that LeCOI1 performs an important role in the production of glandular trichomes on immature fruit and also modulates type-VI trichome density on leaves and sepals. Several questions concerning the effect of LeCOI1 on trichome development remain to be answered. For example, it is currently unclear whether the trichome phenotype of *jai1* fruit (i.e., hairlessness) results from a defect in the same COI1-mediated process that affects trichome density on leaves and sepals. Because *jai1* disrupts the developmental program leading to normal seed and fruit production, it is possible that the absence of fruit trichomes is a pleiotropic effect of abnormal fruit development. The normal growth and morphology of *jai1* leaves and sepals, on the other hand, suggests that LeCOI1 plays a more specific role in modulating the development of type-VI trichomes in these tissues.

A role for LeCOI1 in promoting glandular trichome-based defense is supported by several observations. First, the loss of LeCOI1 function appears to affect glandular trichomes (e.g., type VI) but not the nonglandular trichome types. This finding is consistent with evidence indicating that different types of multicellular trichomes are under the control of different developmental programs (Glover and Martin, 2000). Second, because Arabidopsis trichomes are exclusively of the unicellular nonglandular type, a specific role for LeCOI1 in the development of glandular trichomes might explain why *coi1* null mutants have not been reported to exhibit trichome-related phenotypes. Third, the reduced density of type-VI trichomes on *jai1* leaves was correlated with a profound loss of resistance to two-spotted spider mites. This observation is in agreement with studies showing that type-VI trichomes mediate the effective resistance of wild tomato species to spider mites and that the defense of cultivated tomato to this herbivore is regulated by endogenous JAs derived from the octadecanoid pathway (Kennedy, 2002; Li et al., 2002a). Finally, a role for LeCOI1 in glandular trichome function is supported by the effect of *jai1* on defense-related exudate chemistry, including the reduced accumulation of monoterpenes that exert defensive action against insect pests (Mahmoud and Croteau, 2002; Pichersky and Gershenzon, 2002). The monoterpene deficiency of *jai1-1* fruit is explained readily by the complete lack of glandular tri-

chomes on this tissue. The monoterpene content in *jai1-1* sepals, however, appeared to be significantly less than that predicted simply from the reduced density of type-VI trichomes on mutant sepals. Thus, it is possible that LeCOI1 positively regulates both glandular trichome development and the production of monoterpenes that are stored in these structures. This interpretation is in agreement with recent studies showing that terpene emission from tomato leaves is stimulated by exogenous MeJA and herbivore attacks that activate JA signaling (Faray and Paré, 2002; Thaler et al., 2002).

It also is noteworthy that *jai1* leaves are deficient in the MeJA-induced expression of PPO (Figure 1, Table 1), which constitutes >50% of the total protein in type-VI trichomes of tomato (Yu et al., 1992). The COI1-dependent expression of PPO is in agreement with previous studies showing that JA regulates PPO accumulation in tomato leaves, including the expression of specific PPO isoforms in type-VI trichomes (Constabel et al., 1995; Thipyapong et al., 1997; Thipyapong and Steffens, 1997). One interpretation of these observations is that type-VI trichome development is coordinated with the JA-regulated synthesis of compounds that are produced and stored in these glands. Consistent with this idea, Duffey (1986) observed a strong positive correlation between PPO activity and the density of type-VI trichomes in various *Lycopersicon* species and suggested that these patterns reflect genetic differences. The idea that biotic stress can influence the production of defense-related morphological structures fits with previous observations of increased trichome density in response to herbivory (Myers and Bazely, 1991; Karban and Baldwin, 1997) and the JA-induced formation of resin ducts involved in the synthesis of terpenoids in some conifers (Nagy et al., 2000; Martin et al., 2002; Hudgins et al., 2003). An important test of this hypothesis will be to determine whether changes in endogenous JA levels are sufficient to alter the development of defense-related morphological structures. The identification of LeCOI1 as a determinant of glandular trichome function indicates that the density and biosynthetic capacity of these secreting protuberances can be manipulated genetically through the altered expression of appropriate regulatory genes. Such an approach may be useful for enhancing plant resistance to herbivores or for increasing the production of the many trichome-derived natural products that have significant value to humans (Duke et al., 2000; Wang et al., 2001).

METHODS

Plant Material, Growth Conditions, and Isolation of *jai1-1*

Tomato (*Lycopersicon esculentum*) cv Micro-Tom was used as the "wild type" for all experiments except the cDNA microarray analysis, in which cv Castlemart was used as the wild type. Plants were grown in Jiffy peat pots (Hummert International, Earth City, MO) and maintained in growth chambers as described previously (Howe et al., 2000). Fast-neutron irradiation of Micro-Tom seeds was performed at the International Atomic Energy Agency (Seibersdorf, Austria). Calibrated doses between 12.7 and 17.8 Gy were used for mutagenesis, with the average dose being 15.9 Gy. M2 seeds were collected separately from 981 M1 plants. The appearance of visible phenotypes (chlorosis, variegation, albinism, and

dwarfism) in one or more M2 plants from 161 (16%) M1 families indicated that the mutagenesis was effective.

Twenty-five M2 plants per M1 family were tested for a deficiency in methyl jasmonate (MeJA)-inducible polyphenol oxidase (PPO) and proteinase inhibitor II (PI-II) accumulation as follows. Approximately 120 18-day-old seedlings were enclosed in a Lucite box (10 × 32 × 60 cm) containing 5 μ L of MeJA applied to cotton wicks that were spaced evenly within the box. Plants were exposed to MeJA vapor for 24 h and then incubated for an additional 24 h at ambient humidity in the absence of MeJA. A small piece of leaf tissue sampled from the lower leaf of each plant then was assayed for PPO activity as described previously (Howe and Ryan, 1999). Plants showing reduced PPO activity were tested immediately for PI-II accumulation using a radial immunodiffusion assay (Ryan, 1967; Zhao et al., 2003). The PI-II detection limit of this assay was \sim 10 μ g/mL tissue extract. From a total of 24,077 M2 plants tested, 18 putative mutants were identified that accumulated reduced levels of both PPO and PI-II in response to MeJA. Only one mutant (*jai1-1*; line 406A) exhibited undetectable PPO and PI-II accumulation. This line was backcrossed successively four times to both Micro-Tom and Castlemart. Micro-Tom and Castlemart were used as the recurrent pistillate parents in this series of crosses.

Selection of *jai1-1* Homozygotes

Homozygous *jai1-1* seedlings were selected from F2 populations as follows. Seeds were germinated on a piece of water-saturated filter paper in a closed Tupperware box in the dark at ambient temperature. After 4 to 5 days, when the emerging radical was \sim 1 cm in length, the filter paper was resaturated with a solution of 1 mM MeJA. This solution was prepared by mixing 2 μ L of pure MeJA (Bedoukian Research, Danbury, CT) with 75 μ L of ethanol, followed by dilution into 10 mL of sterile distilled water. Seedlings were grown in the dark for an additional 24 to 36 h, at which time they were scored for sensitivity to MeJA on the basis of the phenotypes depicted in Figure 1C. *jai1-1* homozygotes were transferred to peat pots and grown as described above. MeJA-sensitive seedlings (*Jai1/Jai1* and *Jai1/jai1-1*) that were transferred to soil resumed normal growth and development.

A PCR-based assay was used to distinguish the *jai1-1* deletion allele from the wild-type *LeCoi1* allele. The assay used an upper primer (5'-GTGGAGACGATATGTTGAGACTAA-3') that anneals to an intron-1 sequence present in both wild-type and *jai1-1* genomic DNA. PCR amplification in combination with a second primer (5'-CCATGGAGTCCA-TCACCTAACAGT-3') that anneals to a downstream intron-1 sequence (deleted in *jai1-1* plants) gave a 525-bp PCR product corresponding to the wild-type allele. The PCR assay also used a third primer (5'-GTG-GTCAGATCAGAGCCCTCTATT-3') that anneals to a region downstream of the 3' end point of the deletion and, in conjunction with the upstream primer, amplified a 777-bp fragment that is specific for *jai1-1*. PCR using genomic DNA from *Jai1/jai1-1* heterozygotes resulted in the amplification of both the 525- and 777-bp products.

Spider Mite Bioassays

General procedures for rearing and handling two-spotted spider mites (*Tetranychus urticae*) were as described previously (Li et al., 2002a). Two-choice assays were conducted by placing 10 mites within a 1-cm circle located equidistant (1 cm) between single leaflets from 4- to 6-week-old wild-type and *jai1-1* plants. A 10-cm Petri dish was placed over the arena to reduce ambient air currents and to prevent mites from escaping. All assays were performed at 26 \pm 2°C at 40 to 60% RH and were terminated at 1 h after initiation of the trial. Fecundity assays involved the transfer of five adult female mites from bean leaves to wild-type or *jai1-1* leaf discs (12 mm) that were placed on wetted cotton, as

described by Rodriguez et al. (1971, 1972). Mites and eggs were counted at 24-h intervals.

Pollen Viability and Germination Assays

Freshly collected pollen was incubated in germination medium (10% sucrose, 100 mg/L boric acid, 300 mg/L calcium nitrate, 200 mg/L magnesium sulfate, and 100 mg/L potassium chloride) for 2 h at room temperature and then analyzed for pollen tube formation. Pollen tube length was recorded with a digital video camera (model MDS100; Kodak). Pollen grains were considered germinated if the tube length was greater than the diameter of the grain. The germination rate was calculated as the average germination percentage from 10 arbitrarily selected microscopic fields. Fluorescein diacetate/propidium iodide staining was used to measure pollen viability as described previously (Oparka and Read, 1994). Alexander's triple staining procedure (Alexander, 1980) was used to determine the proportion of pollen containing active cytoplasm.

Analysis of Trichomes

Scanning electron microscopy was performed with a JEOL 6400V scanning electron microscope (Tokyo, Japan) at an accelerating voltage of 15 kV. To examine the general pattern of trichome distribution on leaves, sepals, and green fruit, small pieces of tissue (5 × 5 mm) were fixed in 4% glutaraldehyde in 20 mM sodium phosphate buffer, pH 7.4, dehydrated through an ethanol series, critical point dried in CO₂, and coated with gold using an EMSCOPE SC500 sputter coater (Ashford, UK). The density of type-VI trichomes on the adaxial surface of leaves and the lower surface of sepals (corresponding to the outer sepal surface for unopened flower buds) was determined by counting trichomes with a dissecting microscope equipped with a stage micrometer. All measurements were performed on wild-type and *jai1-1* plants grown side by side in the same growth chamber. Because the trichome density of tomato leaflets is highly dependent on position along the developmental axis of the leaflet as well as leaf age, care was taken to survey comparable tissues of both genotypes.

Trichome exudates from fresh young leaves, sepals, and fruit were obtained by dipping the tissues in 3 mL of tert-butyl methyl ether (MTBE) for 5 min, with gentle shaking. MTBE-extracted compounds were concentrated under nitrogen gas and analyzed by gas chromatography–mass spectrometry as described previously (Lewinsohn et al., 2001). Monoterpene content was normalized to the surface area of the tissue used for each extraction. A stretched glass pipette was used to collect exudate directly from type-VI glands on wild-type fruit. Collected material was dissolved in MTBE, concentrated as described above, and analyzed by gas chromatography–mass spectrometry. The monoterpenes α - and β -pinene, limonene, and *cis*- β -ocimene were identified by comparison with authentic standards. Compounds corresponding to P5 and P6 (Figure 4) were not identified. Toluene was used as the internal standard for the quantification of monoterpenes.

Nucleic Acid Blot Analysis

RNA and DNA gel blot analyses were conducted as described by Li and Howe (2001). Duplicate RNA gels were stained with ethidium bromide to verify RNA quality and to ensure equal loading. A cDNA for tomato translation initiation factor eIF4A (cLED1D24) was used as the loading control. Three-week-old plants were enclosed in a Lucite box (60 × 32 × 17 cm) containing 2 μ L of MeJA, which was dissolved in 0.25 mL of ethanol and distributed to several evenly spaced cotton wicks within the box. For each time point of sampling, five plants were removed from the box and leaf tissue was pooled for the extraction of RNA (Li and Howe, 2001).

cDNA Microarray Analysis

The microarray slide was composed of 607 tomato cDNAs that represent ~500 unique tomato genes (Zhao et al., 2003). Each cDNA was spotted in triplicate on the slide. A complete list of the clones on the array is provided in the supplemental data online. Microarray analysis involved two experimental comparisons: (1) 18-day-old wild-type plants (cv Castlemart) treated with MeJA or mock-treated with ethanol; and (2) 18-day-old *jai1-1* plants (in the Castlemart genetic background) treated with MeJA or mock-treated with ethanol. In each comparison, probes derived from MeJA-treated and ethanol-treated plants were considered as the test sample and reference sample, respectively. MeJA treatments were performed as described above. Mock-treated control plants were placed in a separate box in which an equivalent amount of ethanol was applied to cotton wicks. Plants were maintained in the box under standard growth conditions in the light for 8 h, after which time leaf tissue from six to eight plants of the same genotype was pooled for RNA isolation. Detailed procedures for probe preparation and labeling, hybridization and washing, and analysis of hybridization spot intensities are available at <http://www.prl.msu.edu/howe.shtml>.

Two biological replicate RNA samples were used for hybridization. Thus, four hybridization experiments were performed. In one of the two replicates, labeling of the two RNA samples with Cy5 or Cy3 dUTP was reversed to avoid potential dye-related differences in labeling efficiency. Spot intensities were quantified using GenePix Pro 3 image-analysis software (Axon, Foster City, CA). Ratio data were extracted and normalized to the set of spiking controls as described by Zhao et al. (2003). For data analysis, the ratios of the three replicate spots for each clone on the array were averaged. The ratio data for all 607 cDNAs on the array are provided in the supplemental data online. The final expression ratio was calculated as the average ratio from the two biological replicates (see supplemental data online). Only those genes with an expression ratio of >2.5 or <0.4 in both biological replicates were counted as being regulated differentially (see supplemental data online). The raw data for all hybridization experiments are available at <http://www.prl.msu.edu/howe.shtml>.

Cloning of *LeCoi1*

A BLAST (Basic Local Alignment Search Tool) search (Altschul et al., 1990) of the tomato EST database at The Institute for Genomic Research (<http://www.tigr.org/>) identified a single EST contig that was highly similar to Arabidopsis COI1 (TBLASTN score = 1085; E value = 2.1e-110). DNA sequence analysis of one clone (cLEB3P18) in this contig revealed a 1.1-kb insert that corresponds to the 3' end of the cDNA. A combination of 5' rapid amplification of cDNA ends (Gibco BRL) and Genome Walker (Clontech, Palo Alto, CA) technology was used to obtain additional sequence at the 5' end of the cDNA, including ~250 bp of DNA upstream of the putative ATG initiation codon. This sequence information was used to design gene-specific primers upstream (C1, 5'-CGG-GATCCCTCTCCTCATCTTCTCAA-3') and downstream (C2, 5'-CGAGCTCATACATATGGACAAGACACCT-3'); these were used to obtain a full-length cDNA by reverse transcription–PCR (RT-PCR). Reactions were performed with 5 μ g of total RNA from Micro-Tom leaves and the Enhanced Avian HS RT-PCR-20 Kit (Sigma) as recommended by the manufacturer. The resulting 2.0-kb PCR product was ligated into pGEM-T vector (Promega) to generate plasmid pGEM-COI1. The cDNA insert (designated *LeCoi1*) was sequenced in its entirety and deposited in GenBank. The presence of an in-frame stop codon (TGA) 36 bp upstream of the initiator ATG indicates that the cDNA encodes a full-length polypeptide.

The genomic sequence of *LeCoi1* was obtained from a BAC clone (249O9) that was identified by screening a tomato BAC library (Budiman et al., 2000) with EST clone cLEB3P18. To obtain the genomic sequence

for *LeCoi1*, we designed PCR primers (based on the cDNA sequence) that amplify several overlapping fragments that span the locus. This sequence was assembled into a 9.4-kb contiguous sequence that was deposited in GenBank. Thermal asymmetric interlaced PCR (TAIL-PCR) (Liu and Whittier, 1995) was used to clone a fragment of DNA that spans the *jai1-1* deletion end points and extends to the end of the 9.4-kb genomic sequence (Figure 8A). For this reaction, genomic DNA prepared from *jai1-1* plants was used as a template for TAIL-PCR involving three gene-specific primers (C5, 5'-GAGGCAATATGTGGATTTGATGGA-3'; C6, 5'-CCACACCGTGTTCCTTTGAAGTGGA-3'; C7, 5'-GGAGACGATATGTTGAGACTAAGT-3') that anneal to intron 1 of *LeCoi1* and a degenerate "AD2" primer [5'-NGTCGA(G/C)(A/T)GANA(A/T)GAA-3']. A 1.3-kb PCR fragment obtained from this reaction was cloned into pGEM-T and sequenced. Comparison of this sequence with the 9.4-kb genomic sequence identified the deletion end points.

Agrobacterium tumefaciens-Mediated Transformation

Plasmid pGEM-COI1 was digested with BamHI and SstI to release the 2.03-kb cDNA, which was cloned subsequently into BamHI and SstI sites of the binary vector pBI121 (Clontech). This construct was introduced into *Agrobacterium tumefaciens* strain AGLO and used to transform *jai1-1* cotyledon explants as described previously (Li et al., 2001, 2003). Cotyledon tissue was obtained from *jai1-1* homozygotes that were grown on sterile filter paper and selected from an F2 population using the root growth inhibition assay described above. The presence of the transgene in independent primary transformants (T1) was confirmed using the primer set C3 (5'-CTGCAAGTTAGGGCTGAAGATCTT-3') and C4 (5'-GGCCAAGCACTTCCAATCCTCTAT-3') in a PCR containing genomic DNA from each transformant. These primers amplify 1116- and 433-bp products corresponding to the endogenous *LeCoi1* and 35S-*LeCoi1* transgenes, respectively. Regenerated plants were transferred to the greenhouse for collection of T2 seeds. A PCR assay (see above) that detects both the *jai1-1* deletion allele and the wild-type allele was used to confirm that regenerated T1 plants were homozygous for *jai1-1*.

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact Gregg A. Howe, howeg@msu.edu.

Accession Numbers

The GenBank accession number for the *LeCoi1* genomic sequence is AY423549. Other accession numbers are as follows: Arabidopsis COI1, NP_565919; rice COI1, BAB84399; tomato COI1, AY423550.

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