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MOLECULAR GENETIC ANALYSIS OF JASMONATE SIGNALING IN TOMATO (*LYCOPERSICON ESCULENTUM*)

presented by

Lei Li

has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Genetics

Major Professor's Signature

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Date

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MOLECULAR GENETIC ANALYSIS OF JASMONATE SIGNALING IN TOMATO (*LYCOPERSICON ESCULENTUM*)

By

Lei Li

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ABSTRACT

MOLECULAR GENETIC ANALYSIS OF JASMONATE SIGNALING IN TOMATO (*LYCOPERSICON ESCULENTUM*)

By

Lei Li

Synthesized from polyunsaturated fatty acids via the octadecanoid pathway, jasmonic acid (JA) and its cyclic precursors and derivatives, collectively called jasmonates, play critical roles in regulating many plant defensive and developmental processes. Extensive studies of the wound signaling pathways in tomato have led to a proposed model in which systemin, an 18-amino-acid polypeptide, acts as a mobile signal to evoke *de novo* synthesis of JA, which in turn activates the expression of defense-related genes. A major gap in our understanding of the function of jasmonates concerns how jasmonate perception is coupled to transcriptional activation of jasmonate-inducible genes in response to developmental and environmental cues. The focus of this dissertation research was to dissect the role of jasmonates in tomato defense and developmental processes by isolating and characterizing mutants with impaired responses to exogenous JA. To this end, a fast neutron-mutagenized tomato population was screened for plants that were deficient in methyl-JA-induced accumulation of polyphenol oxidase and proteinase inhibitor-II, two jasmonate-regulated defensive proteins. One recessive mutant (called *JA-insensitive1-1*) was isolated that was completely defective in jasmonate signaling in roots, leaves, and flowers. Failure of *jai1-1* plants to express jasmonate-regulated genes was correlated with increased susceptibility to herbivores. Reciprocal grafting experiments using *jai1* and *spr2*, a tomato mutant defective in JA

biosynthesis, showed that *spr2* plants are defective in the production, but not recognition, of a graft-transmissible wound signal, whereas *jai1* plants are compromised in the recognition but not the production of this signal. These results indicate that JA or a related jasmonate species is an essential component of the long-distance wound signal. Plants homozygous for the *jai1-1* mutation exhibited several novel development phenotypes, including female sterility and impaired glandular trichome development. These findings extend the role of the jasmonate signaling pathway to developmental processes in tomato that have not been previously associated with jasmonates. In a separate screen for ethyl methane sulfonate-induced mutations that suppress prosystemin-mediated responses, a mutant (called *jai1-2*) that was unresponsive to wounding and methyl-JA was shown to be allelic to *jai1-1*. The *jai1* mutants were determined to harbor mutations in a gene that is homologous to the Arabidopsis *CORONATINE INSENSITIVE1* (*COI1*), which encodes an F-box protein involved in ubiquitin-dependent protein degradation. Stable transformation of *jai1-1* plants with the tomato *JAI1/COI1* cDNA restored jasmonate-induced expression of defense genes, fertility, and trichome development. We conclude that *JAI1/COI1* is a key regulator of the jasmonate signaling pathway in tomato and anticipate that the *jai1* mutants will be useful for future investigations aimed at elucidating in greater details the function of the jasmonate signaling pathway in defense and development.

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CHAPTER 1

Introduction: Jasmonate Biosynthesis, Action, and Function

Plants and insects have coexisted for over 350 million years, when the earliest forms of land plants and insects existed (Gatehouse, 2002). Although some of the relationships between the two phyla, such as pollination and seed dispersal, are mutually beneficial, the most common interaction involves insect feeding on plants, and plant defenses against herbivorous insects (Gatehouse, 2002; Pichersky and Gershenzon, 2002). Paleobotanic studies indicated that predation of angiosperm plants by insects can be dated as far as 97 million years ago (Labandeira et al., 1994). According to the co-evolutionary theory developed by Ehrlich and Raven (1964), insect predation on plants has been a determining factor in increasing species diversity in both herbivores and their plant hosts (Harborne, 1988; Gatehouse 2002).

In the face of this long-standing relationship, it is not surprising that plants have developed diverse and sophisticated means to resist or evade their insect predators. Sessile as they are, plants seek to minimize herbivore damage through rapid growth and development, dispersion, or choice of habitat (Gatehouse, 2002). They also develop morphological structures that physically repel or trap insect predators. But often times, plants can accumulate high levels of pre-formed compounds which function as biochemical defenses through their toxicity, or their physical properties (Wittstock and Gershenzon, 2002). This defense mechanism can be described as constitutive, in contrast to induced defenses in which the synthesis of defensive compounds is triggered by insect attack (Harborne, 1988; Ryan, 2000; Walling, 2000). Since the latter mechanism cannot come into play until plants are attacked, it does not involve the commitment of plant resources to the synthesis of defensive compounds that must be accumulated and stored (Gatehouse, 2002). Thus, the fitness cost of induced resistance is less than that involved

in constitutive defense (Simms and Fritz, 1990; Baldwin, 1998; Gatehouse, 2002; Heil and Baldwin, 2002).

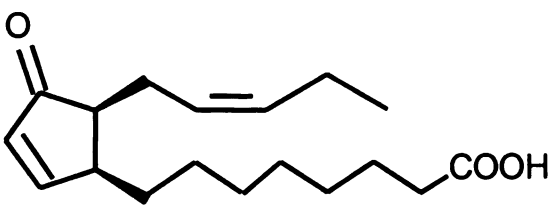
An important aspect of many induced defense responses is their occurrence not only at the site of damage but in undamaged tissues located distal to the site of attack (Karban and Baldwin, 1997). Wound-inducible proteinase inhibitors (PIs) in tomato (*Lycopersicon esculentum*), which are expressed within ~ 2 h after mechanical wounding or herbivory, represent one of the best examples of this phenomenon (Karban and Baldwin, 1997; Howe et al., 2000; Ryan 2000). In their landmark study of wound-inducible PIs, Green and Ryan (1972) proposed that specific signals generated at the wound site travel through the plant and activate *PI* expression in undamaged responding leaves. Several chemical and physical signals have since been implicated in the systemic wound response (reviewed by Ryan, 2000; Walling, 2000; León et al., 2001). One of these signals is the fatty acid-derived hormone jasmonates. There is an ever-increasing body of evidence indicating that jasmonates are essential signals for the control of defense responses, and partitioning of metabolic resources between growth and defense (Creelman and Mullet, 1997; Walling, 2000; Berger, 2002; Turner et al., 2002; Wasternack and Hause, 2002; Weber, 2002).

I. Biosynthesis of Jasmonates: The Octadecanoid Pathway

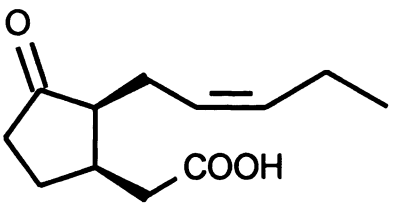
Jasmonic acid (JA) is representative of a family of plant signaling molecules derived from fatty acids. These compounds are notably similar to the eicosanoid family of animal hormones (Bergey et al., 1996). The methyl ester of JA (MeJA) was first identified as a major fragrance in the essential oil of jasmine plants (Demole et al., 1962), and JA was later obtained from a culture filtrate of the fungus *Botryodiplodia theobromae* (Aldridge et al., 1971). It is now believed that JA and its cyclic precursors and derivatives (Figure 1.1), collectively referred to as jasmonates, occur ubiquitously in the plant kingdom (Sembdner and Parthier, 1993; Creelman and Mullet, 1997; Wasternack and Hause, 2002).

The octadecanoid pathway for jasmonate biosynthesis was first proposed by Vick and Zimmerman (1984) and has since been elucidated in detail in Arabidopsis and a few other plants species (Schaller, 2001). The pathway starts with the release of α-linolenic acid (α-LA; 18:3) from membrane lipids. Oxygenation of free α-LA by a 13-lipoxygenase (LOX) generates (9Z,11E,15Z,13S)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13S-HPOT) that serves as a substrate for allene oxide synthase (AOS). AOS then converts 13S-HPOT to 12,13(S)-epoxy-9(Z),11,15(Z)-octadecatrienoic acid (12,13-EOT), which is cyclized by allene oxide cyclase (AOC) to the first cyclic and biologically active compound of the pathway, 12-oxo-10,15(Z)-phytodienoic acid (OPDA). Reduction of the 10,11-double bond in OPDA by OPDA reductase (OPR) then yields 3-oxo-2(2'(Z)-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0), which undergoes three rounds of β-oxidation to produce JA (Figure 1.2; Table 1.1).

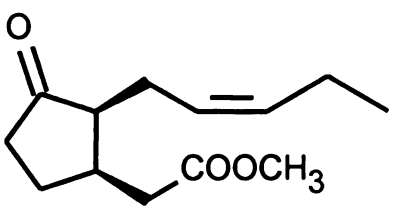
Figure 1.1. Structure of representative jasmonates and coronatine. OPDA, 12-oxo-10,15(Z)-phytodienoic acid; JA, jasmonic acid; MeJA, methyl jasmonate; coronatine, a phytotoxin, produced by *Pseudomonas syringae*, that has biological activity similar to jasmonates (Feys et al., 1994).



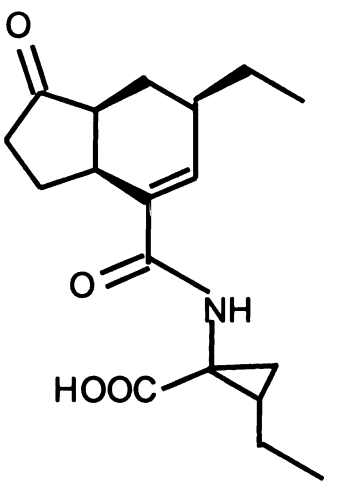
OPDA



JA



MeJA



coronatine

Figure 1.1

Figure 1.2. The octadecanoid pathway for jasmonate biosynthesis. The pathway originates with the release of α-LA from chloroplast membrane by DAD1, a phospholipase A_1. α-LA is then converted in the chloroplast to OPDA by the sequential action of LOX, AOS, and AOC. Reduction of the cyclopentenone ring and subsequent β-oxidations take place in the peroxisome. The spatial separation of OPDA and JA formation implies that OPDA is transferred from the chloroplast to the peroxisome to be further metabolized. JA can be methylated by JMT to the volatile MeJA in the cytosol. JA can also be adenylated in the cytosol by JAR1, a process that might lead to the formation of JA conjugates. Enzymatic steps and fluxes of the intermediates are shown. Question marks indicate the steps without explicit experimental evidence. See text for abbreviations.

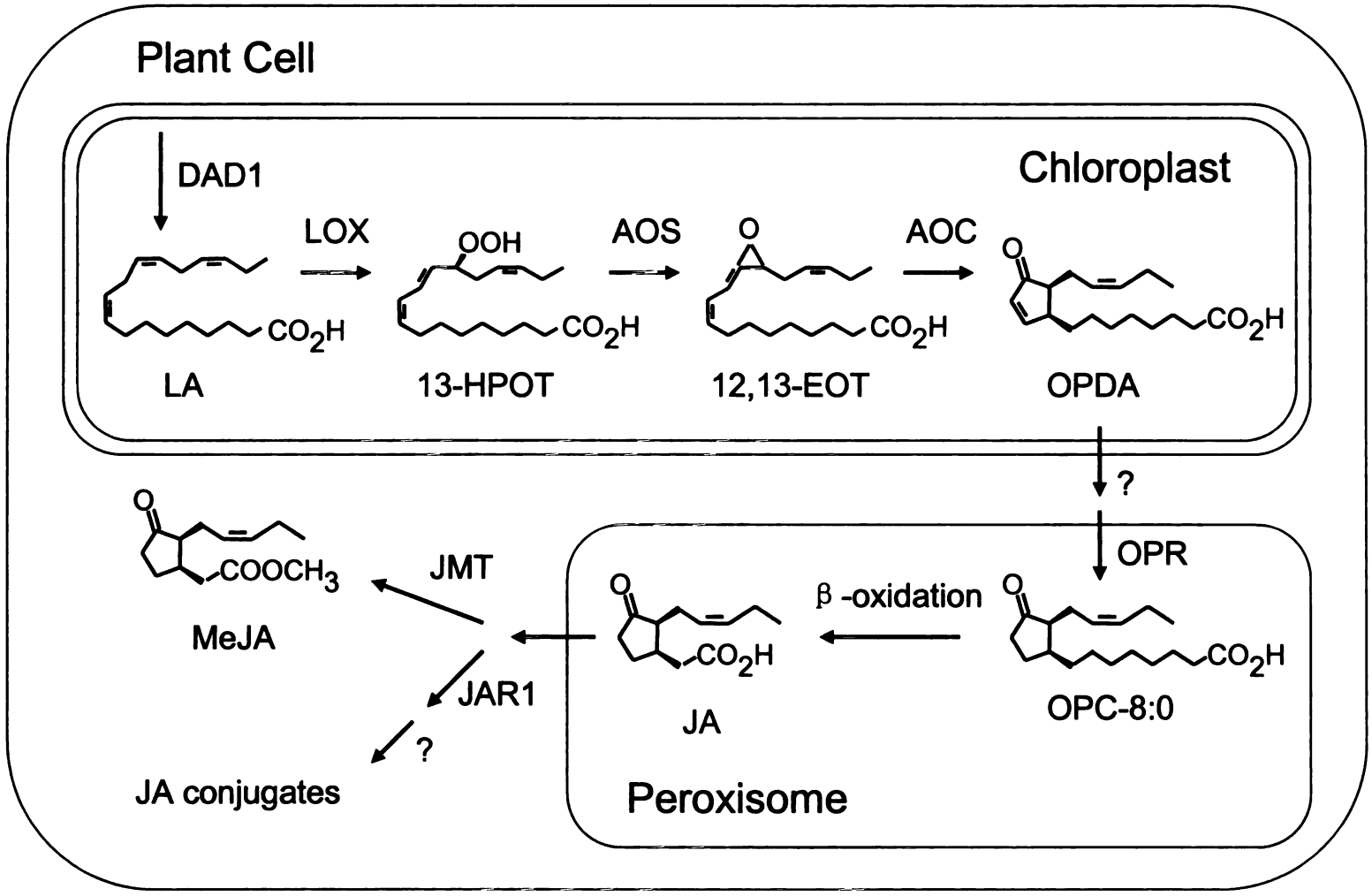

Plant Cell
Chloroplast
DAD1
LOX
AOS
AOC
OOH
O
CO_2H
LA
13-HPOT
12,13-EOT
OPDA
?
OPR
JMT
β -oxidation
$COOCH_3$
MeJA
JAR1
?
JA conjugates
JA
OPC-8:0
Peroxisome

Figure 1.2

I.1. Release of α-linolenic acid from membrane lipids

The octadecanoid pathway originates from the release of α-LA from membrane lipids (Vick and Zimmerman, 1984). The critical requirement of α-LA in JA biosynthesis was explicitly demonstrated by the analysis of an Arabidopsis "triple" fatty acid desaturase mutant (*fad3-2/fad7-2/fad8*). The mutant contained negligible levels of trienoic fatty acids. Although photosynthesis and vegetative growth of the mutant were unaffected, the triple mutant was male sterile. Application of α-LA and JA restored fertility (McConn and Browse, 1996). When wounded, free α-LA levels in plants doubled within 1 h after wounding, while JA levels increased 10-fold (Conconi et al., 1996). Given that the level of free α-LA measured before wounding was many times higher than the maximum JA accumulated after wounding, the wound-induced increase in JA levels could have resulted from release of α-LA from lipids, or the utilization of α-LA present before wounding (Conconi et al., 1996).

By analogy with mammalian eicosanoid biosynthesis, a phospholipase A may catalyze the release of α-LA from membrane lipids in plants. This has recently been confirmed for JA biosynthesis in flowers by characterizing the male-sterile Arabidopsis mutant *defective anther dehiscence1* (*dad1*, Ishiguro et al., 2001). The *dad1* mutant was isolated from a transposon-tagged population on the basis of its male sterility, which could be rescued by α-LA or JA application. DAD1 was shown to be a lipase that hydrolyses phosphatidylcholine at the sn-1 position, and targeted to the chloroplasts (Ishiguro et al., 2001). The *DAD1* promoter was strongly activated in filaments of stamens prior to the stage at which JA is required for development of the filament, maturation of pollen grains, and dehiscence of the anther (Ishiguro et al., 2001). The

involvement of DAD1 in wound-induced JA synthesis in leaves is less clear. The *DAD1* transcript was wound-inducible but, significantly, *dad1* plants were competent for wound-induced JA formation in leaves (Ishiguro et al., 2001). Therefore, *DAD1* is required for developmentally regulated production of JA for stamen development, but not for wound-induced JA accumulation in leaves.

On the other hand, there is evidence suggesting that a phospholipase D (PLD) is required for wound-induced JA formation in both Arabidopsis and tomato leaves. For example, wounding of wild-type plants promoted a substantial increase in α-LA levels, whereas transgenic Arabidopsis plants in which *PLDα* was suppressed by the antisense technique showed no significant increase in α-LA (Zien et al., 2001). Antisense suppression of Arabidopsis *PLDα* also reduced wound-induced JA and the accumulation of JA-inducible transcripts (Wang et al., 2000). These transgenic plants were male fertile (Wang et al., 2000), suggesting that *PLDα* is required for wound-induced JA biosynthesis but not for JA biosynthesis in stamen development. However, McConn and Browse (1996) observed that the threshold level of α-LA for male fertility was less than 5% of the α-LA levels in wild-type plants. Therefore, it is possible that the flower JA content was reduced in *PLDα* antisense plants, but not to a level to cause male sterility.

I.2. Lipoxygenase

LOXs constitute a family of dioxygenases that catalyze the oxygenation of fatty acids to their corresponding hydroperoxy derivatives. Plant LOXs oxygenate α-LA at the

9 or 13 position to give 9- or 13- hydroperoxy-octadecatrienoic acid (HPOT). Those involved in JA biosynthesis produce 13-HPOT and are called 13-LOXs (Feussner and Wasternack, 2002). The role of 9-hydroperoxides and their catabolites in plants is unclear, though studies in potato suggested a role for these compounds in defense responses against fungal pathogens (Gobel et al., 2001). Generally speaking, higher plants contain multiple isoforms of LOXs (Feussner and Wasternack, 2002). Consequently, the specific role of a given LOX in the regulation of JA synthesis has been difficult to analyze because different LOX isoforms may have different enzymatic specificity and may be located in different cell compartments (Feussner and Wasternack, 2002).

The involvement of 13-LOXs in JA biosynthesis was apparent from the observations that plants treated with LOX inhibitors (Peña-Cortés et al., 1993), or transgenic plants with suppressed LOX activity, exhibit reduced ability to synthesize JA (Bell et al., 1995; Royo et al., 1999). The stroma-localized plastidial LOX2 appears to be responsible for the wound-induced biosynthesis of JA in Arabidopsis. *LOX2* mRNA levels were high in leaves and inflorescences but low in seeds, roots, and stems. Suppression of *LOX2* expression by the antisense technique caused reduction in the wound-induced accumulation of JA in leaves. However, no obvious changes in the growth and fertility of the antisense plants were observed (Bell et al., 1995). Therefore, these results suggested that *LOX2* is required for wound-induced JA formation, but may not be required for JA production during pollen and stamen development.

I.3. Allene oxide synthase

AOS catalyzes the dehydration of 13-HPOT to an unstable epoxide, which is converted to OPDA by allene oxide cyclase (AOC). Tomato has at least two chloroplast-targeted 13-AOSs (Howe et al., 2000; Sivasankar et al., 2000), whereas there is only a single *AOS* gene in Arabidopsis (Kubigsteltig et al., 1999). Therefore, the Arabidopsis AOS must function in JA formation both in leaves and flowers. Analysis of AOS knockout mutants indeed indicated so. The mutant plants were completely abolished the wound-induced JA accumulation and the induction of wound response genes (Park et al., 2002; von Malek et al., 2002). Analysis of the mutant plants also confirmed the requirement of AOS in male reproductive development. The mutants displayed defects in filament elongation, anther dehiscence and pollen maturation that could be rescued by exogenous JA (Park et al., 2002; von Malek et al., 2002).

The reaction catalyzed by AOS is the first committed step in JA synthesis. Therefore, regulation of AOS expression and activity has been considered a major control point for JA biosynthesis. The Arabidopsis *AOS* promoter was shown to be activated by a variety of signals including JA, OPDA, wounding, and salicylic acid (SA; Laudert and Weiler, 1998). However, overexpression of *AOS* in transgenic Arabidopsis and tobacco did not alter the basal level of JA. Only when the transgenic plants were wounded did they produce a higher level of JA than wild-type plants (Laudert et al., 2000; Park et al., 2002). In Arabidopsis and in tobacco, therefore, it appears that AOS expression is limiting JA levels in wounded plants. In unwounded plants, on the other hand, availability of the AOS hydroperoxide substrate 13-HPOT might determine JA levels

(Park et al., 2002). By contrast, overexpression of the flax AOS in transgenic potato plants delivered a chloroplast-localized AOS protein, and increased the endogenous JA level. However, the JA-regulated *Pin2* gene was not up-regulated in these transgenic plants, indicating that the elevated JA in these transgenic plants was not biologically active (Harms et al., 1995).

I.4. Allen oxide cyclase

AOC catalyzes the stereo-specific cyclization of allene oxide to OPDA, thus establishing the stereochemistry of OPDA and JA. Because of the acute instability of the epoxide, AOS and AOC are probably functionally and physically connected (Vick and Zimmerman, 1984; Ziegler et al., 2000). Potato AOC combined with recombinant Arabidopsis AOS can indeed produce OPDA from 13-HPOT *in vitro* (Laudert et al., 1997). DNA gel blot analysis revealed a single *AOC* gene in tomato, whereas Arabidopsis appears to have four *AOC* genes encoding proteins that contain a chloroplast targeting transit peptide (He et al., 2002; Wasternack and Hause, 2002).

Immunohistochemical methods showed that the tomato AOC is localized to the chloroplast by an N-terminal transit peptide (Ziegler et al., 2000). *AOC* was found to express at low levels in stems, young leaves, and young flowers, contrasting with a high accumulation of the transcript in flower buds, flower stalks, and roots. *AOC* transcript was transiently induced in tomato leaves when the plant was wounded or treated with JA or systemin (an 18-amino-acid peptide signal molecule, see section II.3), where its

expression was primarily confined to the vascular bundle tissues (Hause et al., 2000; Stenzel et al., 2003). It should be noted that the vascular bundle-specific localization of tomato *AOC* transcript and activity coincides with the spatial expression of the prosystemin gene, suggesting that vascular bundle tissues are the site of active JA biosynthesis in tomato (Jacinto et al., 1997, 1999; Ryan, 2000; Stenzel et al., 2003).

I.5. 12-oxo-phytodienoic acid reductase

OPRs belong to the flavin-dependent oxidoreductase family and catalyze the reduction of cyclopentenones in JA biosynthesis. Three OPR isoforms have been characterized in both tomato and *Arabidopsis*. However, only the isoform OPR3 was found to participate directly in the octadecanoid pathway for JA biosynthesis, as only OPR3 reduces the 9*S*,13*S*-stereoisomer of OPDA, the biological precursor for JA formation (Schaller and Weiler, 1997; Biesgen and Weiler, 1999; Müssig et al., 2000; Strassner et al., 2002). The Arabidopsis *dde1* (*DELAYED DEHISCENCE 1*) and *opr3* (*oxo-phytodienoic acid reductase 3*) mutants, in which *OPR3* is knocked out, are deficient in JA but not OPDA accumulation in response to wounding (Sanders et al., 2000; Stintzi and Browse, 2000). The *opr3/dde1* mutants also display a male sterile phenotype that can be restored by application of JA but not OPDA (Sanders et al., 2000; Stintzi and Browse, 2000).

OPR3 transcripts can be induced by JA (Müssig et al., 2000) and wounding (Strassner et al., 2002). In tomato, the wound induction kinetics of *OPR3* was found to

resemble that of the other forth-mentioned octadecanoid pathway genes (Strassner et al., 2002). But in contrast to these octadecanoid pathway genes, which all encode enzymes localized in the chloroplast, *OPR3* does not contain a chloroplast targeting sequence. Rather, OPR3 possesses a three-amino-acid carboxy-terminal extension (Ser-Arg-Leu) that constitutes a putative peroxisome-targeting signal (Olsen, 1998) that is absent from OPR1 and OPR2 (Stintzi and Browse, 2000). Indeed, both the OPR3 protein and activity were found exclusively in the peroxisome (Strassner et al., 2002).

Localization of OPR3 to the peroxisome provides strong support for the hypothesis that the later phase of JA formation occurs in the peroxisome (see section II.2). This result also indicated that in both tomato and Arabidopsis the biosynthesis of cyclopentanones (e.g. JA) and cyclopentenones (e.g. OPDA) is confined to the plastid and the peroxisome, respectively. This apparent spatial separation of cyclopentenones and cyclopentanones implies that transport processes might exist to shuttle OPDA from the chloroplast to the peroxisome (Strassner et al., 2002). Interestingly, most OPDA was found to be esterified to chloroplast galactolipids (Stelmach et al., 2001), suggesting that OPDA could be transferred between organellar membranes in lipid-bound form (Strassner et al., 2002).

Table 1.1. JA biosynthetic mutants in Arabidopsis and tomato.

Mutant	Plant	Description	References
fad3-2/ 7-1/8	Arabidopsis	Triple ω-3 fatty acid desaturase mutant; defective in the synthesis of trienoic fatty acids, male sterile, more susceptible to *Pythium irregulare* and *Bradysia impatiens*	McConn and Browse, 1996; McConn et al., 1997
dad1	Arabidopsis	Mutated in a phospholipase A1; male sterile	Ishiguro et al., 2001
dde1/opr3	Arabidopsis	Defective in OPDA reductase 3; male sterile, more susceptible to *Pythium irregulare* and *Bradysia impatiens*	Sanders et al., 2000; Stintzi and Browse, 2000; Stintzi et al., 2001
dde2	Arabidopsis	Mutated in the *AOS* gene; male sterile, defective in wound-induced JA accumulation	Park et al., 2002; von Malek et al., 2002
def1	Tomato	Defective in the octadecanoid pathway; deficient in wound-induced defense gene activation, more susceptible to arthropod herbivores, fertile.	Howe et al., 1996; Li et al., 2002a
spr2	Tomato	Mutated in an ω-3 fatty acid desaturase; suppressor of prosystemin-mediated responses, fertile, wound-induced JA-accumulation reduced to < 10% that of wild-type plants	Howe and Ryan, 1999; Li et al., 2002b

Abbreviations: *fad*, fatty acid desaturase; *dad*, defective in anther dehiscence; *dde*, delayed dehiscence; *def*, defenseless; *spr*, suppressor of prosystemin-mediated response; OPDA, 12-oxo-10,15(Z)-phytodienoic acid; AOS, allene oxide synthase.

I.6. β-Oxidation

Shortening of the OPC-8:0 side chain is achieved by three rounds of β-oxidation (Vick and Zimmerman, 1984). These reactions probably occur in the peroxisome, where enzymes for β-oxidation are known to be located in plants (Beevers, 1979; Gerhardt, 1983). There is little direct evidence for the subcellular localization of this part of the pathway for JA biosynthesis until recently. The specific β-oxidation enzymes involved in JA biosynthesis have yet to be identified. However, the demonstration of the peroxisomal localization of OPR3 (Strassner et al., 2002), which catalyzes the formation of OPC-8:0 as a substrate for subsequent β-oxidation, provides a strong support to the hypothesis that JA is finally formed in the peroxisome. Also noteworthy is that (Z)-jasmone, a common component of plant volatiles, is thought to be formed from JA by a further round of β-oxidation (Birkett et al., 2000).

I.7. Modification of Jasmonic Acid

JA can be metabolized to form its methyl ester MeJA and numerous conjugates and catabolites, many of which have biological activity (Hamberg and Gardner, 1992; Kramell et al., 1995). The methylation of JA to MeJA is catalyzed by an *S*-adenosyl-L-methionine:jasmonic acid carboxyl methyltransferase (JMT, Seo et al., 2001). High levels of *JMT* transcript were found in developing flowers and in wounded leaves. Transgenic Arabidopsis plants overexpressing *JMT* accumulated MeJA without altering JA content, stimulated the expression of jasmonate-responsive genes, and displayed enhanced

resistance to infection by *Botrytis cinerea* (Seo et al., 2001). However, it was not clear whether these phenotypes of the transgenic plants were caused by a general increase in jasmonate levels or by the specific accumulation of MeJA. Because MeJA is volatile, its production by JMT could conceivably mediate intracellular and intercellular signaling *in planta*, and could also function as an airborne signal mediating intra- and interplant communications to orchestrate defenses against insects (Farmer and Ryan, 1990; Karban et al., 2000; Seo et al., 2001).

The Arabidopsis *jar1* mutant exhibited reduced sensitivity to MeJA with regard to root growth inhibition and enhanced susceptibility to the soil fungus *Pythium irregulare* (Staswick et al., 1992; Staswick et al., 1998). However, even knockout alleles of *jar1* exhibited no obvious defects in stamen development (Staswick et al., 2002). *JAR1* was recently cloned and its predicted structure suggested that it belongs to the acyl adenylate-forming firefly luciferase superfamily of enzymes that activate the carboxyl groups of a variety of substrates for their subsequent biochemical modification (Staswick et al., 2002). The specificity of JAR1 for JA adenylation was demonstrated by an ATP-PPi isotope exchange assay (Staswick et al., 2002). These findings indicated that covalent modifications of JA are important for many (e.g. root growth inhibition and defense against soil pathogens) but not all (e.g. stamen development) jasmonate-mediated processes in plant (Staswick et al., 2002).

II. Regulation of JA Biosynthesis

The octadecanoid pathway leading to the formation of JA involves the coincident activation of at least five biosynthetic genes, the products of which are targeted to either the chloroplast or the peroxisome. The similar expression profile of the biosynthetic genes and the similar localization of the corresponding enzymes they encode suggest that the octadecanoid pathway is highly regulated and coordinated. This view is reinforced by the observations that transgenic overexpression of individual genes failed to increase JA levels (Laudert et al., 2000; Stenzel et al., 2003) or led to elevated JA levels that were functionally inactive (Harms et al., 1995).

II.1. Regulation of JA biosynthetic genes

Most if not all of the octadecanoid pathway genes are transcriptionally activated by treatment of plants with JA, wounding, or other stress conditions. Within 1 h of the treatment, induction of JA biosynthetic genes was documented in several plant species. These genes include *DAD1* (Ishiguro et al., 2001), *LOX* (Royo et al., 1996; Heitz et al., 1997), *AOS* (Laudert and Weiler, 1998; Howe et al., 2000; Maucher et al., 2000; Sivasankar et al., 2000), *AOC* (Stenzel et al., 2003); and *OPR3* (Müssig et al., 2000; Strassner et al., 2002). The promoter region of the Arabidopsis *AOS* was analyzed in detail (Kubigsteltig et al., 1999). The *AOS* promoter contains *cis*-elements such as CAAT and ACGT boxes similar to other known stress response elements (Goldsbrough et al., 1993). When the promoter was fused with the GUS reporter gene, GUS activity was found to be induced by wounding and JA treatment (Kubigsteltig et al., 1999). These data

were taken to be indicative of a positive feedback regulation in JA production (Laudert and Weiler, 1998; Sivasankar et al., 2000).

II.2. Cellular compartmentation of JA biosynthesis

The conversion of α-LA to OPDA occurs in the chloroplast, which contains an abundance of α-LA esterified in glycerolipids. At present, at least one isoform for each of the four octadecanoid enzymes (e.g. *DAD1*, *LOX2*, *AOS*, and *AOC*) has been shown to be localized in the chloroplast. These four enzymes contain a chloroplast transit peptide and their localization to the chloroplast was demonstrated by immunocytochemical analysis and by *in vitro* chloroplast import experiments. Furthermore, the corresponding enzymatic activities were found in the chloroplast (Bell et al., 1995; Maucher et al., 2000; Ziegler et al., 2000; Froehlich et al., 2001; Ishiguro et al., 2001). Co-localization of these enzymes in the chloroplast has been suggested to facilitate the metabolism of lipophilic or unstable intermediates of the JA branch of oxylipin metabolism (Froehlich et al., 2001).

The conversion of OPDA to JA, on the other hand, occurs in the peroxisome as indicated by the peroxisome-specific compartmentation of OPR3 and the β-oxidation enzymes (Beevers, 1979; Gerhardt, 1983; Strassner et al., 2002). It is interesting to note that more than 90% of the OPDA in Arabidopsis leaves is present as a novel lipid, sn1-O-(12-oxophytodienoyl)-sn2-O-(hexadecatrienoyl)-monogalactosyl diglyceride in chloroplast membranes (Stelmach et al., 2001). OPDA could be released from chloroplast membranes enzymatically by sn1-specific lipases, and this could account for the rapid transient increase in free OPDA and JA when leaves are wounded (Stelmach et al., 2001).

The spatial separation of OPDA and JA biosynthesis suggests that transfer of OPDA from chloroplast to peroxisome might be an important regulatory step in JA biosynthesis. The endogenous store of the lipid-bound OPDA also hints at its potential to rapidly supply OPDA as a signal molecule. However, a precursor-production relationship between lipid-bound OPDA and JA remains to be established.

II.3. Up-regulation of JA synthesis by the prosystemin/systemin pathway

A unique aspect of the wound signaling pathway of solanaceous plants is the involvement of the peptide signal systemin (Ryan and Pearce, 1998; Ryan, 2000). Systemin is an 18-amino-acid polypeptide originally isolated from leaves of tomato plants using a bioassay in which accumulation of PIs is measured in young excised tomato plants after a compound is supplied through their transpiration stream. Systemin was found to be active at a concentration of fmol/plant in the bioassay, which ranks it among the most powerful plant gene-activating compounds known (Pearce et al., 1991). Systemin is derived from the C-terminal end of a 200-amino-acid precursor called prosystemin (McGurl et al., 1992), whose cDNA has been found in potato, nightshade and bell pepper (Constabel et al., 1998). Most recently, two degenerate systemin-like peptides that can activate PI synthesis were isolated from suspension-cultured tobacco cells (Pearce et al., 2001). Molecular manipulation of the prosystemin cDNA has provided convincing evidence that prosystemin plays a critical role in the transduction of systemic wound signals. Antisense suppression of prosystemin expression in tomato plants compromised the systemic wound response (McGurl et al., 1992), whereas overexpression of the prosystemin cDNA driven by the CaMV 35S promoter (a genotype

designated as *35S::prosys*) resulted in constitutive activation of wound response genes in unwounded plants (McGurl et al., 1994).

The requirement of the octadecanoid pathway in mediating systemin signaling was first suggested by Farmer and Ryan (1992) and has been confirmed by several lines of evidence. When fed through the cut stem of tomato plants, systemin rapidly elevates JA levels (Doares et al., 1995; Howe et al., 1996). Conversely, inhibitors of the octadecanoid pathway, such as diethyldithiocarbamate (which converts 13-HPOT into a hydroxyl derivative) and SA (which represses AOS activity), prevent systemin-mediated activation of defense genes (Farmer et al., 1994; Doares et al., 1995). The most convincing evidence has come from the analysis of a tomato mutant called *def1* (Table 1.1), which is impaired in JA accumulation upon wounding and systemin feeding, and is deficient in the activation of defense genes (Howe et al., 1996). In keeping with this finding, a genetic screen in the *35S::prosys* background for mutants suppressed in the constitutive activation of defense genes has identified genes required for both JA synthesis (e.g. *spr2*, Table 1.1) and perception (Howe and Ryan, 1999; Li et al., 2001; 2002b).

Radioactively labeled systemin, when placed on fresh wounds of tomato leaves, was shown to move from wound sites to the petiole phloem, supporting its possible role as a mobile wound signal (Pearce et al., 1991; Narváez-Vásquez et al., 1995). However, the exact mode of action for systemin is still obscure. Recent models propose that systemin is the mobile systemic wound signal that is released from prosystemin upon wounding or herbivore attack and is transported throughout the plant where it interacts with its specific receptor at the surface membrane of target cells. As a consequence of

this interaction, α-LA is released and converted along the octadecanoid pathway to JA (Ryan and Pearce, 1998; Ryan, 2000). A systemin-binding activity was identified that displayed characteristics of a functional systemin receptor (Meindl et al., 1998; Scheer and Ryan, 1999). This putative receptor was purified to homogeneity from plasma membrane fractions of suspension cultured cells of *L. peruvianum*. It was found to be a 160-kD protein belonging to the leucine rich repeat (LRR) receptor kinase family that is most similar to the brassinolide receptor kinase BRI1 (Scheer and Ryan, 2002).

Interaction of systemin with its receptor is associated with several rapid biochemical events including cytosolic calcium influx, membrane depolarization, inhibition of a plasma membrane proton ATPase, and activation of a MAP kinase activity (Felix and Boller, 1995; Moyen and Johannes, 1996; Stratmann and Ryan, 1997; Moyen et al., 1998; Schaller and Oecking, 1999). These early signaling events in the systemin and wound response pathways are believed to culminate in the activation of a phospholipase A_2 (PLA2) activity that presumably releases fatty acid from membrane lipids (Narváez-Vásquez et al., 1999; Ryan, 2000). The PLA_2 activity, as measured by the accumulation of ^{14}C-lysophosphatidylcholine, was rapidly induced by wounding and systemin, but not by MeJA treatment (Narváez-Vásquez et al., 1999). The unresponsiveness of PLA_2 to MeJA contrasts other JA biosynthetic enzymes, indicating that this PLA_2 activity is specific to the systemin pathway. However, a causal role for this PLA_2 activity in wound- and systemin-mediated JA biosynthesis remains to be established.

III. The Jasmonate Signal Transduction Pathway

In the past decade jasmonates have gone from little-regarded secondary metabolites to the well-recognized central regulator of many plant defensive and developmental processes. Like other plant hormones, jasmonates have a profound impact on the biochemistry, metabolism and mRNA population of plant cells that can perceive and transduce the hormonal signal (Creelman and Mullet, 1997). The following section discusses our current understanding of the jasmonate signaling pathway.

III.1. Perception of jasmonates

By analogy to other plant hormones (Kende and Zeevaart, 1997), the jasmonate signal is thought to be transduced by the activation of a receptor that binds the hormone (Creelman and Mullet, 1997). Lack of high-specific-activity JA and the lipophilic or volatile nature of jasmonates made JA binding experiments, and thus the direct analysis of JA receptors, difficult (Creelman and Mullet, 1997). Another approach to identify the jasmonate receptor has been through analysis of jasmonate response mutants. In Arabidopsis, exhaustive mutant screens for response mutants to MeJA or its structural analog coronatine (Figure 1.1) have identified a number of loci important for jasmonate signaling (Table 1.2). Cloning and biochemical analysis indicated that *COI1* (Xie et al., 1998), *CEV1* (Ellis et al., 2002), and *JAR1* (Staswick et al., 2002) are not JA receptors. Evidence regarding the molecular basis of other mutations is still lacking.

Table 1.2. Jasmonate response mutants in Arabidopsis.

Mutant	Phenotype	References
jin1	Reduced root growth inhibition by MeJA; not yet cloned	Berger et al., 1996
jin4	Reduced root growth inhibition by MeJA; allelism to *jar1* not determined	Berger et al., 1996
jar1	Reduced root growth inhibition by MeJA, enhanced susceptibility to *Pythium irregulare*; JAR1 belongs to the acyl adenylate-forming luciferase family and displays adenylation activity toward JA	Staswick et al., 1992;1998; 2002
coi1	Reduced root growth inhibition by coronatine; enhanced susceptibility to *Pythium irregulare* and *Alternaria brassicicola*; male sterility; COI1 is an LRR-containing F-box protein.	Feys et al., 1994 Xie et al., 1998
cex1	Constitutive expression of several JA-inducible genes; not yet cloned	Xu et al., 2001
cev1	Constitutive expression of several JA-inducible genes; accumulates high level of JA; CEV1 encodes the cellulose synthase CeSA3	Ellis and Turner, 2001 Ellis et al., 2002
cet	Constitutive expression of the JA-inducible gene *THI 2-1*; not yet cloned	Hilpert et al., 2001

Abbreviations: *cet*, constitutive expression of the thionin gene; cev, constitutive expression of the vegetative storage protein 1 gene; *cex*, constitutive expression of JA-inducible genes; *coi*, coronatine insensitive; *jar*, jasmonic acid resistance; *jin*, jasmonic acid insensitive; LRR, leucine rich repeat.

Jasmonate perception is further complicated by the fact that OPDA acts as a signaling molecule in its own right. For instance, OPDA has been shown to be more potent than JA in inducing tendril coiling in *Bryonia dioica* (Weiler et al., 1993). The assessment of the relative contributions of JA and OPDA to the various physiological responses in Arabidopsis was made possible by the isolation of the *dde1/opr3* mutants (Sanders et al., 2000; Stintzi and Browse, 2000). The male sterility defects of the mutants (see section I.5) could be alleviated by application of JA but not by OPDA, providing clear evidence for a role of JA rather than OPDA in male developmental processes (Sanders et al., 2000; Stintzi and Browse, 2000). Surprisingly, *dde1/opr3* plants were found to be fully resistant to the dipteran insect *Bradysia impatiens* and the nectrotrophic fungus *Alternaria brassicicola*, demonstrating that OPDA can substitute for JA in mediating plant resistance (Stintzi et al., 2001). This suggests that, in Arabidopsis, OPDA and JA have overlapping as well as distinct functions. Accordingly, there may exist at least two pathways to transduce the OPDA and JA signals, one for recognition of either OPDA or JA for defense responses, and one for recognition of JA, but not OPDA, for stamen development.

III.2. The ubiquitin-mediated proteolysis pathway in jasmonate signal transduction

Selective proteolysis performed by the ubiquitin-proteasome pathway plays a key regulatory role in numerous cellular processes in both animals and plants, in which ubiquitin serves as a reusable tag to target proteins for degradation by the 26S proteasome (Voges et al., 1999). Covalent attachment of ubiquitin to substrate proteins involves a

cascade of three protein complexes called the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2) and the ubiquitin ligase (E3). Whereas eukaryotic organisms contain only a few E1s and dozens of E2s, the specificity and timing of substrate ubiquitination are primarily controlled by a large number of E3 complexes (Deshaies, 1998; Voges et al., 1999).

In plants, ubiquitin monomers are encoded by gene fusions that contain the polyubiquitin and the ubiquitin extension protein (UbEP) genes. The polyubiquitin gene consists of several tandem repeats of the ubiquitin coding unit whereas the UbEP gene encodes a single ubiquitin unit fused in frame with either of two ribosomal proteins (Finley et al., 1989). Both the polyubiquitin and the UbEP gene have been shown to be up-regulated by wounding or MeJA (Garbarino et al., 1992; Garbarino et al., 1995). Similarly, wound-induced accumulation of mRNA encoding the α proteasome subunit was described in Arabidopsis (Genschik et al., 1992). These observations suggest the involvement of the ubiquitin/proteasome pathway in the wound and the jasmonate signaling pathways, presumably through selective degradation of regulatory proteins.

In keeping with these earlier results, analysis of the Arabidopsis *coi1* mutants provided a clear link between ubiquitin-mediated proteolysis and jasmonate signaling. The *coi1* mutants were first isolated in a screen for plants insensitive to growth inhibition by the bacterial toxin coronatine (Figure 1.1; Table 1.2; Feys et al., 1994). Subsequently, it was found that *coi1* plants fail to express jasmonate-regulated genes, and also are unresponsive to growth inhibition by MeJA, male sterile, and susceptible to insect herbivores and to pathogens (Benedetti et al., 1995; McConn et al., 1997; Thomma et al.,

1998). The *COI1* gene encodes a 66-kD protein containing an F-box motif and an LRR domain (Xie et al., 1998). The F-box motif is a hallmark of proteins that associate with cullin and Skp1 proteins to form an E3 ubiquitin ligase known as the SCF complex (Bai et al., 1996; Deshaies, 1998). LRRs are short sequence motifs that mediate protein-protein interaction (Kobe and Deisenhofer, 1994). Therefore, F-box proteins are believed to function as receptors that recruit regulatory proteins as substrates for ubiquitin-mediated degradation (Bai et al., 1996; Deshaies, 1998).

The involvement of an F-box protein in jasmonate signaling suggests that jasmonate-inducible genes are transcriptionally activated by selective recruitment of a repressor of jasmonate responses for proteolysis (Figure 1.3). It has been postulated that upon binding with its receptor, a jasmonate signal activates a protein kinase cascade (Rojo et al., 1998), which leads to the phosphorylation of the repressor of jasmonate responses (Creelman, 1998). The phosphorylated repressor can then be recognized by the COI1-containing complex, polyubiquitinated and subsequently degraded by the 26S proteasome (Deshaies, 1998). Removal of the repressor then allows jasmonate-responsive genes to be transcribed (Creelman, 1998). The structure of SCF^{Skp2} (superscript denotes the F-box protein) has been recently determined. The complex adopts an elongated shape, with Cul1 forming the scaffold and Rbx1 and the Skp1/Skp2 complex segregated to opposite ends (Zheng et al., 2001). Physical association of COI1 with other SCF components has been demonstrated by immunoprecipitation experiments (Devoto et al., 2002; Xu et al., 2002), confirming that COI1 participates in the formation a functional E3-type ubiquitin ligase, SCF^{COI1}, in plants.

Figure 1.3. Hypothetical model showing presumed function of COI1 in the jasmonate signaling pathway. In this model, an elevated level of jasmonate is perceived as a signal resulting in the activation of a kinase catalyzing the phosphorylation of a regulatory protein (R). The phosphorylated protein R binds to COI1 in the SCF^{COI1} complex via the LRR domain. Following transfer of ubiquitin (Ub) from E1 to E2, the SCF^{COI1} complex (composed of AtCUL1, AtRbx1, either of the Arabidopsis Skp1-like proteins ASK1 or ASK2, and COI1) acts as a ubiquitin ligase complex to transfer ubiquitin to R. Following polyubiquitination, R is targeted to the 26S proteasome for degradation. Removal of R then allows the transcription of jasmonate-responsive genes. Composition of the SCF^{COI1} complex was recently determined by immunoprecipitation experiments (Devoto et al., 2002; Xu et al., 2002). Images in this figure are presented in color.

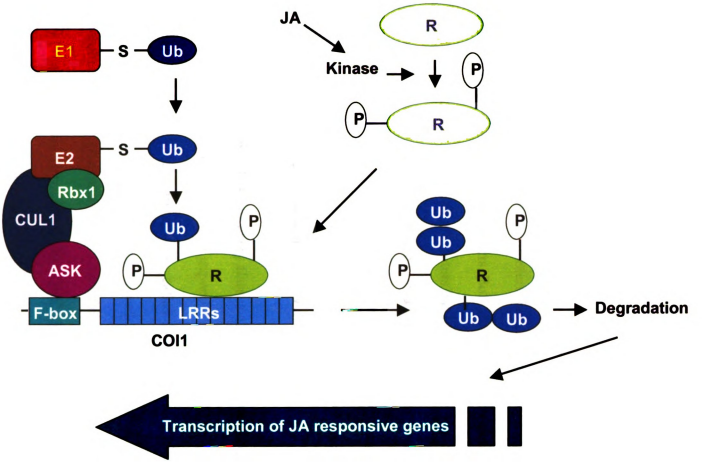

JA
R
Kinase
P
P
R
E1
S
Ub
E2
S
Ub
Rbx1
CUL1
ASK
Ub
P
P
R
F-box
LRRs
COI1
Ub
Ub
P
P
R
Ub
Ub
Degradation
Transcription of JA responsive genes

Figure 1.3

III.3. Transcriptional regulation of jasmonate responses

Microarray analysis showed that exogenous MeJA affects the mRNA level of numerous genes (Schenk et al., 2000). However, our knowledge of the *cis* acting elements of jasmonate responsive genes and the transcriptional factors involved in recognizing these elements is incomplete. The promoters of two jasmonate-inducible genes, *Pin2* and *VspB*, have been studied in some detail (Kim et al., 1992; Mason et al., 1993). A 50-bp domain was identified in both promoters that conferred MeJA responsiveness on truncated reporter gene constructs. Both domains contain a motif (CACGTG) called the G-box, which in other promoters has been shown to bind bZIP transcription factors (Williams et al., 1992). But the relevance of this G-box in conferring jasmonate responsiveness is still unsettled because it is found in numerous promoters that are not responsive to jasmonates. Further, mutation of the G-box in the *Pin2* promoter did not prevent MeJA induction of the gene (Lorbeth et al., 1992).

Jasmonates induce the biosynthesis of many classes of secondary metabolites in different plant species. Using the biosynthesis of a terpenoid indole alkaloid in *Catharanthus roseus* as a biochemical marker, a gene called *ORCA3* was identified. *ORCA3* mRNA was rapidly induced by MeJA treatment and its overexpression in untreated cells resulted in enhanced expression of several biosynthetic genes of the alkaloid pathway (van der Fits and Memelink, 2000). ORCA3 was shown to be a MeJA-responsive APETALA2 (AP2)-domain transcription factor that specifically activates transcription via a JA- and elicitor-responsive element (JERE) in the promoters of jasmonate-response genes, including the alkaloid biosynthetic gene *strictosidine synthase* (van der Fits and Memelink, 2001). OCRA3 has similarity to the ethylene response

factors (ERFs), which were originally isolated as GCC box binding proteins from tobacco (Ohmetakagi and Shinshi, 1995). Arabidopsis ERF genes (*AtERF1* to *AtERF5*) are differentially regulated by different stress conditions (Fujimoto et al., 2000). Particularly relevant to jasmonate signaling, *AtERF1* was found to be JA-inducible and required for induction of other jasmonate-responsive genes (Lorenzo et al., 2003). Cycloheximide, which inhibits protein synthesis, induces marked accumulation of *AtERF* mRNAs, suggesting that *ERF* transcription can be stimulated by protein turnover (Fujimoto et al., 2000). Thus, it seems possible that jasmonate responses in Arabidopsis require *ERF*-like transcription factors, whose activation is dependent on SCF^{COI1}-mediated proteolysis (Lorenzo et al., 2003).

III.4. Integration of jasmonate signaling with other defense signaling pathways

The jasmonate pathway is implicated in regulating responses to many abiotic stresses, defenses against insect herbivores and necrotrophic as well as biotrophic pathogens (Walling 2000; Ellis and Turner, 2001; Berger, 2002; Gatehouse 2002). Jasmonates also appear to regulate a broad spectrum of developmental processes (Creelman and Mullet, 1997), many of which also involve other plant signaling pathways. For instance, microarray analysis of gene expression in wild-type and *coi1* Arabidopsis plants that were subjected to wounding, insect attack, or water stress revealed a large overlap of *COI1*-dependent genes regulated by wounding and by water stress (Reymond et al., 2000). This result suggests crosstalk between the jasmonate and the abscisic acid (ABA) pathway, which is involved in water stress responses (Kende and Zeevaart, 1997).

In tomato, however, ABA was found not to be the primary signal for defense gene activation (Birkenmeier and Ryan, 1998).

The jasmonate signal pathway also interacts with the ethylene signal pathway in controlling some developmental and defensive responses. This is best exemplified in the expression of the Arabidopsis defensin gene *PDF1.2*, which encodes a 5-kD protein possessing antifungal properties. *PDF1.2* could be induced by challenge of pathogen, MeJA or ethylene but not SA treatment (Penninckx et al., 1996). Further analysis demonstrated the synergistic effect of JA and ethylene on the induction of *PDF1.2* and that activation of *PDF1.2* by pathogen depended on both the jasmonate and ethylene signal transduction pathway (Penninckx et al., 1998). It has been shown that *ERF1*, an ethylene response factor that binds to ethylene response elements, could be induced by both ethylene and JA treatments. Furthermore, overexpression of *ERF1* in transgenic plants could rescue the defense defects of both *coi1* and *ethylene insensitive2* plants (Lorenzo et al., 2003). These results suggest that the ethylene and jasmonate pathways converge at the transcriptional activation of *ERF1*.

The interaction between the SA and jasmonate pathways is often antagonistic (Niki et al., 1998). The inhibitory effect of SA on JA-inducible genes has been previously reported for the wound-induction of *PIs* in tomato (Doares et al., 1995). Conversely, JA-responsive genes were hyper-induced in transgenic plants expressing a bacterial salicylate hydroxylase gene (*NahG*) that fail to accumulate SA during pathogenesis (Reymond and Farmer, 1998). Phenylalanine ammonia lyase (PAL) is a key enzyme in SA biosynthesis. Silencing the expression of PAL in tobacco was found to reduce SA-mediated systemic

acquired resistance (SAR) to tobacco mosaic virus but enhanced herbivore-induced systemic resistance to the insect *Heliothis virescens.* Overexpression of PAL, on the other hand, enhanced SAR but reduced resistance to the insect (Felton et al., 1999). This inverse relationship has been observed in other plants species as well. In Arabidopsis, the *coi1* mutant exhibited robust resistance to the bacterial pathogen *Pseudomonas syringae* strain DC3000, which was correlated with elevated accumulation of SA and hyperactivation of the *pathogenesis related* (PR) genes following infection. These experiments suggest that the SA-mediated defense response pathway is sensitized in *coi1* plants (Kloek et al., 2001). Further, the *enhanced disease susceptibility 4* (*eds4*) mutation caused reduced accumulation of SA and enhanced susceptibility to infection by *P. syringae.* Not surprisingly, the *eds4* mutation also caused heightened jasmonate responses (Gupta et al., 2000).

In apparent contradiction to the evidence above, other studies suggest that the jasmonate and ethylene signaling pathways might be required for SA responses. The Arabidopsis mutant *nonexpression of PR1* (*npr1*) is insensitive to SA, fails to express SA-induced *PR* genes, and has reduced SAR (Cao et al., 1994). A screen for suppressor mutations of *npr1* yielded a dominant mutation named *ssi1* for *suppressor of SA insensitivity1.* The *ssi1* mutant has elevated levels of SA, constitutive expression of *PR* genes and restored resistance to *P. syringae.* A striking novel phenotype of *ssi1* plants was the constitutive expression of *PDF1.2*, which is normally induced by JA and ethylene. When SA accumulation in *ssi1* plants was prevented by expressing the *NahG* gene, all of the *ssi1* phenotypes were also suppressed, including the expression of *PDF1.2* (Shah et al., 1999). The results indicate that *SSI1* is a negative regulator of the

SA pathway and that in *ssi1* plants, elevated SA does not antagonize but rather enhances the expression of the JA and ethylene responsive *PDF1.2*.

Investigation of the Arabidopsis *cev1* mutant revealed interaction of the jasmonate signaling pathway with cell wall metabolism. The *cev1* mutant displayed constitutive expression of stress response genes and enhanced resistance to fungal pathogens (Ellis and Turner, 2001). These phenotypes were in keeping with the finding that *cev1* plants have increase production of JA and ethylene (Ellis et al., 2002). Cloning the *CEV1* gene showed that it encodes the cellulose synthase *CeSA3*, which is expressed preferentially in the roots. Consequently, *cev1* roots contained less cellulose than that in wild-type roots (Ellis et al., 2002). Significantly, the *cev1* mutant can be phenocopied by treating wild-type plants with inhibitors of cellulose synthesis. Other cellulose synthesis mutants, such as *rsw1*, also exhibited constitutive activation of JA-inducible genes (Ellis et al., 2002). These experiments established a link between cell wall metabolism and jasmonate signaling in plants.

IV. Physiological Roles of Jasmonates

Jasmonates play a dual role in regulating plant development and responses to numerous stresses. This conclusion was first evident by the development- and stress-regulated accumulation of JA (Creelman and Mullet, 1997). Levels of endogenous JA are highest in young growing tissue (Creelman and Mullet, 1995) and increase after treatment of cell cultures with elicitors or after subjecting plants to wounding, UV light, water deficit, pathogens and ozone (Conconi et al., 1996; Creelman and Mullet, 1997; Rao et al., 2000). Application of jasmonates induces the expression of a large number of genes that are also responsive to other stresses such as wounding and pathogen infection (Reymond et al., 2000; Schenk et al., 2000). The identification and analysis of mutants that are impaired in jasmonate biosynthesis, perception or signaling, and the analysis of transgenic plants with altered expression of JA biosynthetic genes or jasmonate signaling factors has begun to offer new insights into the function of jasmonates in plant.

IV.1. Jasmonates play a direct role in resistance to herbivores and pathogens

Several remarkable discoveries in the 1990s' triggered an awakening to the importance of jasmonates in plant defense. Farmer and Ryan (1990) observed that volatile MeJA produced by sagebrush could evoke defense gene expression in adjacent tomato plants. Zenk and colleagues subsequently found that many plant species tested as suspension cultured cells could accumulate defensive secondary metabolites in response to MeJA (Gundlach et al., 1992; Mueller et al., 1993). In tomato, several complementary pieces of evidence show that jasmonates play a crucial role in the defensive response to

herbivores. Plant defense against herbivore attacks was first reported by the wound-induced accumulation of *PI*s that inhibit digestive proteases of herbivores and reduce the nutritional quality of the ingested tissues (Green and Ryan, 1972). Further studies in tomato revealed that wounding causes a systemic reprogramming of leaf cells that results in the synthesis of over 20 defense-related proteins (Bergey et al., 1996; Ryan, 2000). Treatment of plants with JA or MeJA induced the same set of genes (Farmer and Ryan 1992; Howe et al., 1996; Howe et al., 2000), suggesting that jasmonates are the main regulator for the activation of defense genes. Finally, characterization of the tomato *def1* and *spr2* mutants (Table 1.1) showed that the mutant plants accumulate significantly less JA after wounding, herbivore predation and systemin treatment. The mutant plants also produced negligible levels of PIs and several other defensive proteins in response to, and were more susceptible to, lepidopteran insect attacks (Howe et al., 1996; Li et al., 2002a). In Arabidopsis, mutants that are defective in jasmonate biosynthesis or perception also exhibited impaired defense responses to insect herbivores (McConn et al., 1997; Stintiz et al., 2001).

In conifer trees, the production of terpenoid-based resins has long been studied for its role in defense against herbivores and pathogens (Phillips and Croteau, 1999; Trapp and Croteau, 2001). For example, in the genus *Picea*, stem resin is stored in axial resin canals in the cortex and in axial traumatic resin ducts (TDs). The resin appears after the tree has been subjected to mechanical wounding, insect predation, or fungal elicitation (Marin et al., 2002). Recent studies in the Norway spruce showed the induced formation of TDs by insect and pathogen attack, wounding and MeJA treatment (Franceschi et al., 2000; Martin et al., 2002). The complex defense responses induced by MeJA, including

de novo formation of TDs, terpenoid accumulation, and activation of enzymatic activities for terpene synthesis (Martin et al., 2002) provided new avenues to evaluate the role of jasmonates in plant defense.

Several observations in Arabidopsis implicated jasmonates in pathogen defense. JA levels in plants increase after treatment with the necrotrophic fungus *Alternaria brassicicola* (Penninckx et al. 1996). It was also shown that Arabidopsis mutants affected in jasmonate synthesis or signaling are more sensitive to attack by the necrotrophic pathogens *Pythium*, *A. brassicicola*, and *Erwinia carotovora* (Staswick et al.; 1998; Thomma et al.; 1998; Vijayan et al., 1998; Norman-Setterblad et al., 2000). In addition, transgenic plants overexpressing *JMT* are more resistant to the necrotrophic pathogen *Botrytis cinerea* (Seo et al., 2001). In contrast, *coi1* plants show higher resistance to the biotrophic bacterial pathogen *Pseudomonas syringae* (Kloek et al., 2001). Taking these results together, the jasmonate pathway seems to positively regulate the resistance of Arabidopsis plants to necrotrophic pathogens while negatively regulating resistance to biotrophic pathogens (Thomma et al., 1998). However, in disagreement with this scenario, the *cev1* mutant with elevated levels of JA is more resistant to the biotrophic fungal pathogen powdery mildew (Ellis and Turner, 2001). The jasmonate signaling pathway is also involved in induced systemic resistance (ISR) in which growth of Arabidopsis plants in soil containing the rhizobacterium *Pseudomonas fluorescens* results in enhanced resistance to subsequent pathogen infection (Pieterse and van Loon, 1998). Thus, the mode of action of jasmonates in regulating pathogen responses in Arabidopsis is determined by both the type of pathogen and the type of pathogenicity.

IV.2. Role of jasmonates in emission of plant volatiles and tritrophic interactions

The emission of specific volatile blends from plant tissue has long been recognized as an important component in the interaction between plants and insects, both in the attraction of pollinators and the deterrence of predators (Harborne, 1988; Gatehouse, 2002; Pichersky and Gershenzon, 2002). Often times, the synthesis and release of volatiles as part of the wounding response occurs both locally and systemically in plants (Rose et al., 1996). Activation of volatile synthesis and release by jasmonates has been reported (Thaler, 1999; Rodriguez-Saona et al., 2001).

Plant volatiles contribute directly to defense either through their toxicity or by inducing defensive genes. One such an example is the C6 aldehydes, alcohols and esters referred to as the "green leaf volatiles" (Pare and Tumlinson, 1999). These volatiles are formed by a branch of the octadecanoid pathway involving the action of hydroperoxide lyase (Hatanaka, 1993; Howe et al., 2000) and were found to be able to induce defense-related genes (Bate and Rothstein, 1998). Antisense suppression of the *hydroperoxide lyase* gene in transgenic potato plants led to lower levels of the volatile compounds and improved performance of the aphid *Myzus persicae* (Vancanneyt et al., 2001), demonstrating the importance of the green leaf volatiles in plant defense. As a further example, the fragrant compound MeJA has been studied as a volatile for long distance intraplant and plant-to-plant signaling. MeJA volatized from sagebrush was found to evoke defense gene expression in adjacent tomato plants (Farmer and Ryan, 1990). Genetic manipulation of MeJA production was achieved by overexpressing the Arabidopsis *JMT* gene. The *JMT* transgenic plants contained three times more MeJA

compared with wild-type plants and exhibited enhanced resistance to fungal pathogens (Seo, et al., 2001). In field experiments, tobacco plants grown adjacent to sagebrush were found to experience reduced natural herbivore damage when the sagebrush was clipped to release MeJA (Karban et al., 2000).

Plant volatiles also play a critical role in indirect defense strategies employed by plants, such as the tritrophic interaction in which the plant-derived compounds signal directly to the natural enemy of the herbivore infesting the host plant. Volatiles produced by corn, cotton, and *Brassica* plants during herbivory have been found to attract parasitic wasps to lepidopteran larvae preying on the plants (Geervliet et al., 1994; Turlings et al., 1995; Pare and Tumlinson, 1999). Volicitin, a conjugate of LA and L-glutamine purified from beet armyworm oral secretion, was identified as a powerful elicitor of volatile release (Alborn et al., 1997). The LA moiety is apparently plant-derived, whereas the glutamine is provided by the insect, which also performs the chemical reactions required to produce volicitin (Pare et al., 1998). Volicitin has been shown to up-regulate the expression of genes involved in biosynthesis of both indole (Frey et al., 2000) and terpene (Shen et al., 2000) volatiles in maize. Similar fatty acid conjugates have been isolated in oral secretions of other lepidopteran larvae (Halitschke et al., 2001). Identification of volicitin linked insect feeding damage to plant recruitment of natural enemies of the pest. Future experiments aimed at determining the requirement of the jasmonate biosynthetic and signaling pathways in volicitin-mediated volatile production should be helpful to elucidate the molecular control over the diverse responses elicited by herbivores.

IV.3. Role of jasmonates in plant growth and development

Reports suggesting that JA might be involved in regulating plant growth, e.g. to promote senescence, first appeared in 1980 (Ueda and Kato, 1980). Later, a wide variety of physiological responses to applied jasmonates were reported (Sembdner and Parthier, 1993; Creelman and Mullet, 1997). Besides their active role in plant defense, jasmonates are also implicated in responses to abiotic stimuli including salt and drought stress (Creelman and Mullet, 1995), UV irradiation (Conconi et al., 1996) and ozone exposure (Rao et al., 2000;). In addition, jasmonates appear to play a role in regulating diverse plant developmental processes including seedling growth (Staswick et al., 1992), tendril coiling (Weiler et al., 1993), tuberization (Pelacho and Mingo-Castel, 1991), fruit ripening (Czapski and Saniewski, 1992), pollen maturation and anther dehiscence (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi et al., 2000; Ishiguro et al., 2001), and senescence (Ueda and Kato, 1980; He et al., 2002).

Implication of jasmonates in many developmental processes has largely been based on observations of plant responses to applied jasmonates. While possibly informative, these observations do not necessarily indicate the endogenous activity of the hormone. In fact, evidence from analyzing jasmonate signaling and biosynthetic mutants suggests that jasmonates may be disposable for many aspects of plant growth and development, and that jasmonates may exert different effects on a particular plant process in different species. For instance, the reported effects of exogenous JA on seed germination are contradictory. Inhibition was noted in *Brassica napus*, flax (Wilen et al., 1991), and sunflower (Corbineau et al., 1988), whereas stimulation was reported in apple

(Ranjan and Lewak, 1992) and no effect was evident in Arabidopsis (Staswick et al., 1992). These findings suggest that a role for endogenous JA in seed germination, if any, is at least variable among species. The possible link between JA and senescence is also questionable because highest endogenous levels of JA generally occur in young growing tissues and not in senescing tissues (Creelman and Mullet, 1997). Furthermore, tomato and Arabidopsis mutants affecting JA synthesis or action all appear to senesce normally (Feys et al., 1994; Howe et al., 1996; McConn and Browse, 1996).

The best example for the involvement of jasmonates in development is their role in anther and pollen development in Arabidopsis. Initial evidence for this conclusion came from the observation that the Arabidopsis *fad3-2/fad7-2/fad8* mutant fails to produce viable pollen. Subsequently, mutants defective in other JA biosynthesis genes (Table 1.1) as well as the JA perception mutant *coi1* were all found to be male sterile. Significantly, all mutants exhibited identical characteristics in the male-sterile phenotype (McConn and Browse, 1996; Xie, et al., 1998; Sanders et al., 2000; Stintzi et al., 2000; Ishiguro et al., 2001). Floral organs of the mutants develop normally within closed buds, but the anther filaments do not elongate sufficiently to position the locules above the stigma at anthesis, and the anthers lack the proper dehiscence of the stomium at the time of flower opening. Although the tapetum is correctly broken down, pollen cannot be released from locules. The mutant plants produce mature tricellular pollens albeit in much smaller amounts as compared with wild-type plants. However, the mutant pollen does not germinate properly (McConn and Browse, 1996). The sterile phenotype of the triple-*fad* mutant can be restored by application of α-LA, OPDA, JA, and MeJA whereas the *opr3/dde1* mutants require JA/MeJA for recovery. Therefore, three distinct steps in

Arabidopsis male reproductive development can be distinguished in which JA/MeJA may function as a signal: elongation of anther filaments, maturation of pollen, and timing of anther dehiscence. It has been postulated, based upon the expression pattern of the *DAD1* gene, that temporal and spatial up-regulation of JA synthesis in filaments promotes water uptake from the locules and subsequent transport to the filaments, resulting in the elongation of filaments, maturation of pollen grains and dehiscence of anthers (Ishiguro et al., 2001).

It is clear from the above discussion that our knowledge of the function of jasmonates at the molecular and genetic levels is largely limited to studies conducted with Arabidopsis. Therefore, a comprehensive understanding of the action and function of jasmonates requires molecular genetic studies in diverse plant species. In the current dissertation, efforts to utilize cultivated tomato (*L. esculentem*) as an experimental model to dissect the role of jasmonates in the systemic wound response and the defense against herbivores, and in several developmental processes will be described. The first part of this dissertation research concerns the regulation of the tomato *prosystemin* gene. This work is presented in Chapter 2. The remainder of the dissertation (Chapter 3-6) focuses on the role of jasmonates in tomato defense and development using JA-insensitive mutants developed in this thesis research. Chapter 7 summarizes the major results of this dissertation research and suggests a few experiments to further elucidate the jasmonate pathway in tomato. The finding for a role of jasmonates in tomato glandular trichome development is presented in the Appendix.

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CHAPTER 2

Alternative Splicing of Prosystemin Pre-mRNA Produces Two Isoforms That Are Active as Signals in the Wound Response Pathway

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Abstract

Systemin and its precursor protein, prosystemin, play an essential role in the systemic wound response pathway of tomato plants. We report here the isolation from tomato of a novel prosystemin cDNA (*prosysB*) that differs from the reported cDNA sequence (*prosysA*) by the addition of a CAG trinucleotide. Inspection of the prosystemin genomic sequence, which was mapped to the central region of chromosome 5, indicated that *prosysA* and *prosysB* transcripts are generated by an alternative splicing event that utilizes different 3′ splice sites within intron 3. Quantitative RT-PCR analysis showed that *prosysB* transcripts accumulated to approximately twice the level of *prosysA* in all tissues that express the prosystemin gene. The relative abundance of the two mRNAs was unaffected by wounding or methyl jasmonate treatment, conditions that increase the level of total *prosys* mRNA. These findings indicate that alternative splicing of *prosys* pre-mRNA is a constitutive process. The amino acid sequence of prosysB is predicted to differ from that of prosysA by replacement of Arg_{57} with Thr-Gly in the non-systemin portion of the protein. Overexpression of the *prosysB* cDNA in transgenic tomato plants conferred constitutive expression of defense genes that are regulated by wounding and systemin. We conclude that *prosysB* is the major prosystemin-encoding transcript in tomato, and that this isoform is active as a signal in the wound response pathway.

Introduction

Systemin and its precursor protein, prosystemin, play an essential role in the regulation of systemic wound responses in tomato plants. An increasing body of evidence indicates that systemin regulates a complex signaling pathway that culminates in the systemic expression of proteinase inhibitor and other defense-related genes (reviewed by Bowles, 1998; Ryan and Pearce, 1998; Schaller, 1999; Ryan, 2000). Systemin is proposed to activate the signaling cascade upon binding to a 160-kD cell surface protein that displays characteristics of a functional systemin receptor (Meindl et al., 1998; Scheer and Ryan, 1999). Interaction of systemin with its receptor is associated with several rapid biochemical events including increased cytosolic Ca^{2+} levels, membrane depolarization, inhibition of a plasma membrane proton ATPase, and activation of a MAP kinase activity (Felix and Boller, 1995; Moyen and Johannes, 1996; Stratmann and Ryan, 1997; Moyen et al., 1998; Schaller and Oecking, 1999). These early signaling events in the systemin and wound response pathways culminate in the activation of a phospholipase A_2 activity that releases fatty acid precursors of jasmonic acid (JA) from membrane lipids (Narváez-Vásquez et al., 1999). Current models indicate that JA, together with wound-induced ethylene, is required as downstream signals for the activation of wound-responsive genes (Farmer and Ryan, 1992; O'Donnell et al., 1996; Ryan, 2000).

Systemin is derived from the C-terminal end of a 200-amino-acid precursor called prosystemin. Molecular cloning and analysis of a cDNA encoding the precursor has provided evidence that prosystemin plays a critical role in the transduction of systemic wound signals. First, anti-sense expression of the prosystemin cDNA in transgenic

tomato plants dramatically reduced the systemic wound response (McGurl et al., 1992). Second, overexpression of prosystemin from the CaMV 35S promoter in transgenic plants resulted in constitutive expression of wound response genes in unwounded plants (McGurl et al., 1994). Third, mutations that disrupt prosystemin-mediated signaling also impair wound-induced expression of downstream target genes (Howe and Ryan, 1999). Finally, recombinant prosystemin, like synthetic systemin, is a potent inducer of wound-responsive genes when supplied to tomato seedlings through the transpiration stream. Structure-function analysis of recombinant prosystemin further indicates that the bioactivity of prosystemin resides exclusively in the systemin domain of the molecule (Dombrowski et al., 1999). These results suggest that prosystemin itself might be active as a signaling component for wound-induced gene expression.

Despite the evidence that (pro) systemin is a required component of the systemic wound response pathway, the precise mechanism by which this polypeptide participates in the transduction of systemic signals remains to be elucidated. At least two hypotheses have been proposed. One model holds that systemin is transported through the plant following its proteolytic release from the C-terminal end of prosystemin (Pearce et al., 1991; Ryan and Pearce, 1998; Ryan, 2000). Proteolytic activities that digest prosystemin to smaller systemin-containing polypeptides have been identified in the apoplastic fluid from tomato leaves (Dombrowski et al., 1999). Characterization of these proteases and study of their role in systemic signaling is likely to provide insight into the proposed link between prosystemin processing and the production of a mobile peptide signal. An alternative hypothesis is that prosystemin functions locally to facilitate or amplify a very rapid systemic signal, such as electrical signals that are produced in response to

wounding (Wildon et al., 1992; Bowles, 1998). Both hypotheses appear to be consistent with the observation that prosystemin expression is restricted to vascular tissues, through which systemic signals are likely to travel (Jacinto et al., 1997). The observed increase in vascular-specific expression of prosystemin in response to wounding and other elicitors of the wound response, such as JA, may reflect a mechanism to amplify (pro) systemin-mediated signals (McGurl et al., 1992; Jacinto et al., 1997).

Here we describe a novel prosystemin cDNA that differs from the reported sequence by including a CAG trinucleotide located at the boundary of intron 3 and exon 4. Our results indicate that two isoforms of prosystemin are produced by an alternative splicing event that utilizes two 3′ splice sites within intron 3 of the prosystemin gene. Analysis of transgenic tomato plants that overexpress either of the two cDNAs indicates that both isoforms of prosystemin are active signaling components of the wound response pathway.

Materials and Methods

Plant material and treatments

Lycopersicon esculentum cv Micro-Tom was used for all experiments except where otherwise indicated. The 35S::*prosysA* transgene present in cv Castlemart was introduced into the Micro-Tom genetic background by repeated backcrossing (three times) using Micro-Tom as the pistillate parent, with selection for the semi-dwarf and constitutive wound response phenotypes in each generation. Seeds of *L. pennellii* (LA0716), *L. pimpinellifolium* (LA2184), *L. chmielewskii* (LA1306), *L. chilense* (LA1963), *L. parviflorum* (LA1326), *L. hirsutum* (LA1223), and the introgression lines used for RFLP mapping were obtained from the Tomato Genetics Resource Center (Davis, CA). Plants were grown in Jiffy peat pots (Hummert International) and maintained in growth chambers as described previously (Howe et al., 2000). Methyl jasmonate (MeJA) treatments were accomplished by placing 40 three-week-old plants in a closed Lucite box (31×27×14 cm). Two µl of MeJA (Bedoukian Research Inc.) was diluted into 50 µl ethanol and the resulting solution was applied to nine cotton wicks evenly distributed within the box. For each time point after MeJA treatment, five plants were removed from the box for extraction of RNA from leaf tissues. Plants were challenged with tobacco hornworm larvae as previously described (Howe et al., 2000). Proteinase inhibitor and polyphenol oxidase levels were measured as previously described (Howe and Ryan, 1999).

Cloning of prosys cDNAs and intron sequences

Five μg of total RNA isolated from tomato leaf tissue was reverse transcribed using the SuperScript Preamplification System (Gibco-BRL) and an oligo(dT)$_{12-18}$ primer as recommended by the manufacturer. cDNA products of the reaction were used as template for a PCR reaction that employed the primers PS1 (5′- GCG AAT TCG ATG AGT ATA TAA AGC TCA GC) and PS2 (5′- GCG GAT CCG AAG TTA CTT TTC TAA CGG GAG AC). The resulting 0.76-kb PCR products were digested with *Bam*HI and *Eco*RI, gel purified, and ligated into the corresponding sites of pBluescript SK(-) to generate plasmid pPS-B. The *prosysB* cDNA insert was sequenced in its entirety using T7 and T3 primers. The *prosysB* cDNA contained the entire open reading frame for prosysB as well as 57 bp of the 5′ UTR and 100 bp of the 3′ UTR.

For DNA sequencing of prosystemin intron 3 from wild tomato species, genomic DNA prepared from these plants was PCR-amplified using primers PSD1 (5′ - GCG GAT CCA TCT CTT CAT ATG T) and PSD2 (5′ - GCG AAT TCC CTC CTC ATG TTC CAT). The resulting PCR products were cloned into a pGEM-T Easy vector (Promega, Madison, WI) and sequenced in using a T7 primer. Alignment of intron 3 sequences from various tomato species was performed using the Clustal method in the Megalign program (DNAStar, Madison, WI).

Northern and Southern blot analyses

Total RNA was isolated from tomato tissue and analyzed by RNA blot hybridization as previously described (Howe *et al.*, 2000). ^{32}P-labeled DNA probes were prepared using a T7 Quickprime Kit (Pharmacia Biotech) according to the manufacturer's instructions. A 760-bp *Eco*RI-*Bam*HI fragment containing the entire *prosysA* cDNA was used as a probe for detection of prosystemin transcripts. Hybridization signals were visualized by autoradiography using Kodak XAR-5 film, or were measured using a Phosphor-Imager (Molecular Dynamics). Hybridization signals were normalized to those obtained using a probe for translation initiation factor eIF4A mRNA as previously described (Howe et al., 2000).

Tomato genomic DNA was purified from young leaf tissue as previously described (McCouch et al., 1988). Five μg aliquots of DNA were digested with *Hind*III, fractionated on a 0.8% agarose gel, and blotted by capillary action to Hybond^{TM}-N^{+} membrane (Amersham). Probes were prepared as described above. Pre-hybridization and hybridization were performed in a solution containing 5×SSPE, 5×Denhardt's reagent, 0.5 % SDS (w/v), and 50 μg/ml denatured salmon testes DNA. Blots were washed at 65°C in a solution of 2×SSC and 0.1 % SDS (w/v), followed by repeated washing at 65°C in 1×SSC and 0.1% SDS. Probes were stripped from hybridized blots by boiling the membrane in 0.5% SDS (w/v) for 2 min.

Quantitative PCR

Five μg of total RNA was reverse transcribed in a reaction volume of 20 μl using the SuperScript Preamplification kit as described above. One μl of this reaction served as DNA template for a 25-μl PCR reaction containing 2.5 μl 10X reaction buffer supplied by the manufacturer (Gibco-BRL), 5 pmol each of primers PSD1 and PSD2, 5 nmol dNTPs, and 1.25 U *Taq* DNA polymerase. Primer PSD2 was end-labeled with ^{32}P using T4 polynucleotide kinase (Gibco-BRL), as recommended by the manufacturer. PCR reactions were denatured at 94°C for 3.5 min, and subjected to 20 cycles of amplification (94°C, 1 min; 56°C, 1 min; 72°C, 35 sec). Two μl PCR products were mixed with 4 μl formamide loading buffer (Sambrook et al., 1989), heated at 70°C for 5 min, and chilled on ice. The samples were loaded on an 8% denaturing (7.6 M urea) polyacrylamide gel and electrophoresed at 1,500 volts for 2 hrs. The gel was pre-electrophoresed at 1500 volts for 0.5 hr prior to running the samples. Gels were washed in 5% methanol and acetic acid solution for 30 min and dried in gel-dryer (model 583, Bio-Rad). The signals were visualized by autoradiography and quantified with a Phosphor-Imager.

S1 nuclease experiments

S1 nuclease (Gibco-BRL) was used to probe PCR products for regions of heteroduplex DNA as described by Eckhart et al. (1999). Reactions (30 μl) contained approximately 100 ng template DNA, 33 mM sodium acetate, 50 mM NaCl, 0.03 mM $ZnSO_4$, pH 4.5. S1 nuclease was added to a concentration of 20U/μg template DNA, and

reactions were incubated at 37°C for 1 hr. Approximately one ng of reaction products was fractionated on a 2% agarose gel and blotted on Hybond^{TM}-N^{+} membrane. Blots were hybridized to a radiolabeled *prosysA* cDNA probe, as described above, for detection of S1 cleavage products.

Transformation

The pPS-B plasmid harboring the *prosysB* cDNA was digested with *Eco*RI and *Bam*HI. The resulting 766-bp fragment containing the cDNA was gel-purified and blunt-ended with Klenow fragment (Gibco-BRL). This fragment was cloned into *Sma*I and *Sst*I sites, blunt-ended with Klenow fragment, of the binary vector pBI121 (Clontech). The resulting construct was transformed into *Agrobacterium tumefaciens* strain LBA4404. Transformation of cotyledon explants (cv Micro-Tom) was performed as previously described (McCormick, 1991). Eleven independent primary transformants (T_1) were regenerated on kanamycin-containing medium. Introduction of the transgene was confirmed by PCR using a primer set of PS3 (5'- GCG GAT CCG TGG AGA TGA CAA AGA GAC TCC) and PS2 (see above). These primers were designed to amplify 1030-bp and 290-bp products corresponding to the endogenous prosystemin gene and the *35S::prosysB* transgene, respectively. Regenerated plants were transferred to the greenhouse for collection of T_2 seeds.

Results

ProsysA and prosysB are generated by alternative splicing of intron 3

During the sequencing of prosystemin cDNAs from *L. esculentum*, we isolated several clones that contained a CAG insertion between nucleotides 273 and 274 of the cDNA sequence (accession number M84801) reported by McGurl et al. (1992). For clarity, we have designated the novel CAG-containing cDNA as *prosysB* and the original sequence reported by McGurl et al. as *prosysA*. Of a total of 24 independent cDNAs that were isolated, sequence analysis showed that 15 of them (62.5%) corresponded to *prosysB* and the remainder corresponded to *prosysA*. To rule out the possibility that the two transcripts are encoded by different genes, we used Southern blot and RFLP analysis to confirm the single copy nature of the gene and to map its chromosomal position. In agreement with previous results (McGurl et al., 1992), extensive Southern blot analysis failed to provide evidence for more than one copy of the prosystemin gene (data not shown). A set of introgression lines that harbor defined segments of *L. pennellii* DNA (Eshed and Zamir, 1994) was screened for the presence of a *Hind* III-generated RFLP that distinguishes the prosystemin gene in *L. pennellii* from that in *L. esculentum*. Only one line (IL5-3; LA3497) displayed the *L. pennellii* RFLP pattern, indicating that the prosystemin gene is located within the central region of chromosome 5 (Figure 2.1a). Hybridization of blots to a RFLP marker (CT118A) known to map to the introgressed DNA segment contained in LA3497 confirmed this conclusion (Figure 2.1b). These results indicate that that *prosysA* and *prosysB* are not derived from two different genes.

Figure 2.1. Mapping of the prosystemin gene by Southern blot analysis of tomato introgression lines. (a) Genomic DNA from *L. esculentum* (L.e.), *L. pennellii* (L.p.), an F_1 derived from a *L. pennellii* × *L. esculentum* cross (L.e. x L.p.), and introgression lines (IL5-2, IL5-3, IL5-4 and IL3-5) carrying different segmental substitutions of *L. pennellii* DNA were digested with *Hind* III and analyzed by Southern blotting with a *prosysA* cDNA probe. The position of migration of molecular weight standards (kb) is shown. (b) Hybridization of the blot shown in (a) to CT118A, an RFLP marker known to map to the introgressed DNA segment present in IL5-3.

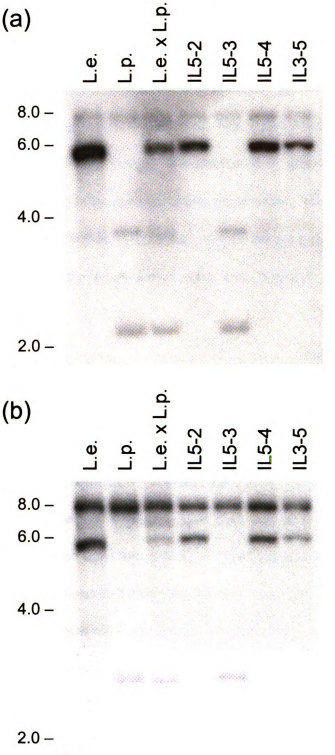
(a)
L.e.
L.p.
L.e. x L.p.
IL5-2
IL5-3
IL5-4
IL3-5
8.0 –
6.0 –
4.0 –
2.0 –
(b)
L.e.
L.p.
L.e. x L.p.
IL5-2
IL5-3
IL5-4
IL3-5
8.0 –
6.0 –
4.0 –
2.0 –

Figure 2.1

Comparison of the *prosys* cDNA and genomic sequences revealed that the CAG insertion within *prosysB* coincided precisely with the location of intron 3 (Figure 2.2). That the CAG polymorphism resulted from alternative splicing was suggested by the location of a second 3′ splice site (site B) three nucleotides upstream from the AG 3′ splice site (site A) used for generation of *prosysA*. The A and B splice sites are 71% and 57% identical to the UGY**AG**|GU consensus sequence for 3' splice sites of plant introns (Lorković et al., 2000), with both sites containing the invariant AG dinucleotide. These observations strongly suggest that *prosysA* and *prosysB* are the products of an alternative splicing event that utilizes two juxtaposed 3′ splice sites (A and B, respectively) in intron 3 (Figure 2.2).

To determine whether the two juxtaposed 3′ splice sites are conserved in different genetic backgrounds of tomato, we used primers PSD1 and PDS2 (Figure 2.2a) to PCR-amplify and clone prosystemin intron 3 from *L. esculentum* and six wild species of tomato including *L. pimpinellifolium*, *L. chmielewskii*, *L. chilense*, *L. pennellii*, *L. parviflorum*, and *L. hirsutum*. DNA sequencing showed that intron 3 of *L. esculentum* is 109 bp in length, whereas the same intron in the six wild species ranged between 97 bp (*L. pennellii*) to 118 bp (*L. chmielewskii*) (data not shown). Sequence comparisons indicated that *L. esculentum* intron 3 was most similar (95.3% identical) to that of *L. pimpinellifolium*, and most dissimilar to that of *L. hirsutum* (62.6% identity). Despite the various levels of polymorphism between *L. esculentum* and the wild species, the juxtaposed 3' splice site (CAGCAG|GA) was perfectly conserved in all wild species examined. This result suggests that the alternative event splicing observed in *L. esculentum* also occurs in distantly related tomato species.

Figure 2.2. Proposed model for alternative splicing of prosystemin pre-mRNA. (a) Schematic diagram of the tomato prosystemin gene organized into 11 exons (vertical bars) and 10 introns (horizontal lines). Systemin is encoded within exon 11 (black bar). The vertical arrow indicates the location of the alternative splicing at the 3′ end of intron 3. Also shown are the annealing sites for the primers used in this study (horizontal arrows). (b) Proposed scheme for alternative splicing of prosystemin intron 3. Exon-intron boundaries are indicated by "|". The extreme 3′ end of intron 3 contains two "ag" dinucleotides (underlined) that compete as splice acceptor sites. Splicing at the upstream ag (site B) results in production of *prosysB*, whereas splicing at the downstream ag (site A) generates *prosysA*. At the protein level, the deduced effect of alternative splicing is substitution of Arg_{57} in prosysA with a Thr-Gly sequence in prosysB.

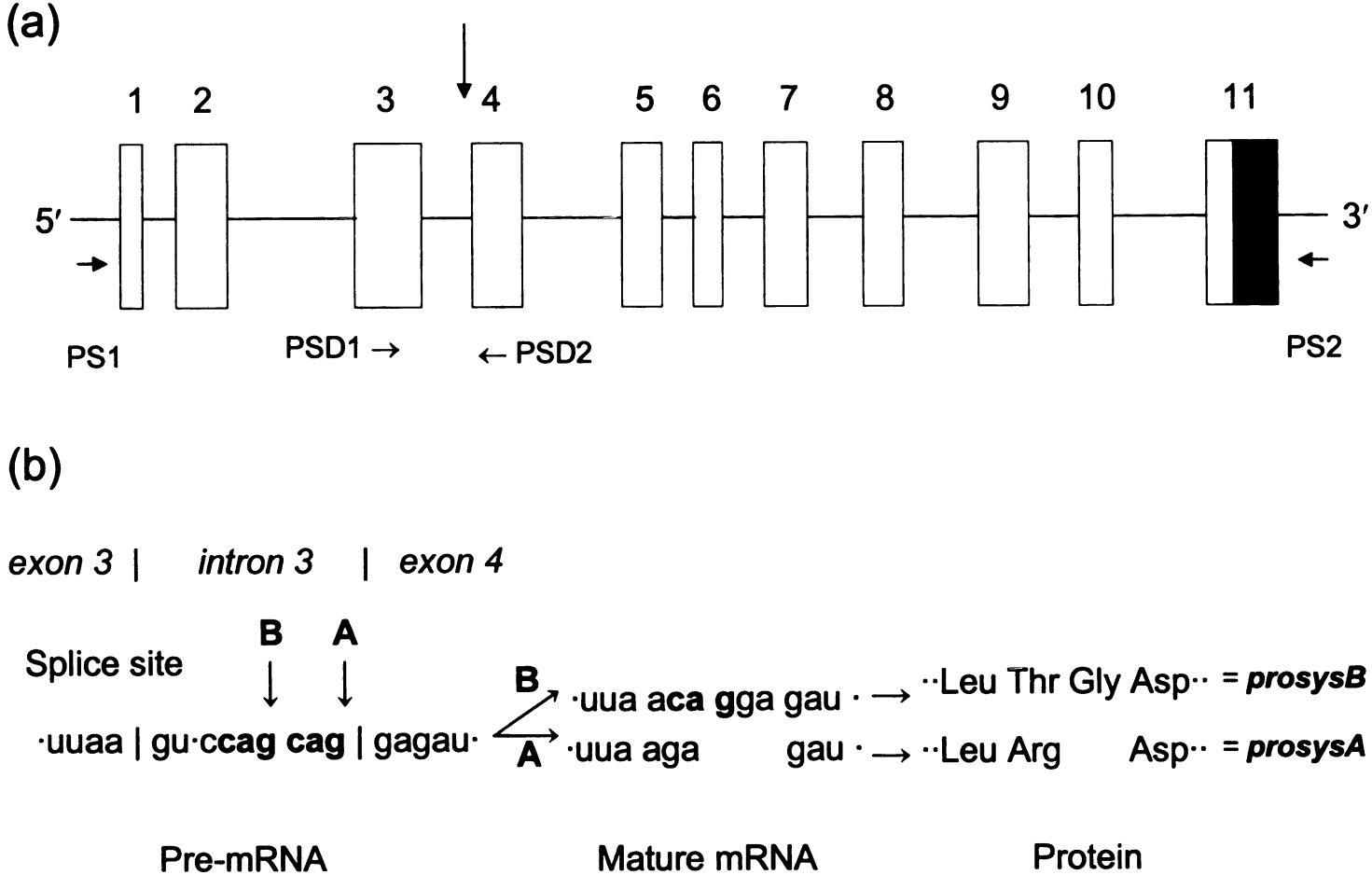
(a)
1 2 3 4 5 6 7 8 9 10 11
5′
3′
PS1
PSD1 →
← PSD2
PS2
(b)
exon 3 | intron 3 | exon 4
Splice site
B A
·uuaa | gu·ccag cag | gagau·
B
A
·uua aca gga gau · → ··Leu Thr Gly Asp·· = prosysB
·uua aga gau · → ··Leu Arg Asp·· = prosysA
Pre-mRNA
Mature mRNA
Protein

Figure 2.2

An S1 nuclease assay was used to obtain additional evidence for the accumulation of *prosysA* and *B* transcripts in samples of total RNA isolated from *L. esculentum*. This assay, which takes advantage of the ability of S1 nuclease to cleave heteroduplex DNA in regions of nucleotide mispairing, has been used to detect small insertion/deletion DNA polymorphisms (Shenk et al., 1975; Eckhart et al., 1999). Control experiments were performed with plasmids pPS-A and pPS-B that harbor full-length cDNAs for *prosysA* and *prosysB*, respectively. Primers (PS1 and PS2; Figure 2.2a) corresponding to the 5′- and 3′-UTRs of the cDNAs were used to PCR-amplify the prosystemin open reading frame. PCR products were treated with S1 nuclease and then analyzed by Southern blotting (Figure 2.3). PCR products derived from a single plasmid template (pPS-A or pPS-B alone) did not produce specific cleavage products when treated with S1 (Figure 2.3, lanes 1-4). However, S1 treatment of PCR products generated from a mixture of pPS-A and pPS-B generated cleavage products of approximately 530 and 220 bp (Figure 2.3, lanes 5 and 6). The sizes of these fragments are consistent with that expected for cleavage at the site of the CAG polymorphism. To test for the presence of *prosysA* and *prosysB* transcripts in tomato leaf tissue, total RNA was reverse transcribed (RT) and PCR-amplified using the same primer set. Analysis of the S1 cleavage products revealed the 535-bp band corresponding to the DNA located 3′ to the polymorphism (Figure 2.3, lane 8). Longer exposures of the autoradiograph also revealed the smaller 220-bp cleavage product (not shown), which was often difficult to detect owing to its diffusion from the gel. These results demonstrate the co-existence of *prosysA* and *prosysB* transcripts in RNA isolated from tomato. The absence of additional S1-generated

Figure 2.3. S1 nuclease cleavage assay for detection of heteroduplex DNA formed by prosystemin cDNAs. PCR products were generated by amplification of various *prosys* cDNA templates with primers PS1 and PS2 (Figure 2.2a). For control reactions, cloned *prosys* cDNAs contained in plasmids pPS-A and pPS-B were used as PCR templates as follows: p*rosysA* alone (A; lanes 1-2), *prosysB* alone (B; lanes 3-4), or an equal mixture of *prosysA* and *prosysB* cDNAs (A+B; lanes 5-6). Alternatively, *prosys* cDNAs were generated by reverse transcription of total leaf RNA (RT; lanes 7-8). PCR products were incubated in the presence (+) or absence (-) of S1 nuclease and a portion (approximately one ng) of the reaction was separated by gel electrophoresis, blotted to Hybond membrane, and hybridized to a *prosysA* cDNA probe. Hybridization signals were visualized by autoradiography. The band indicated by the asterisk (*) denotes the larger of the two DNA fragments produced by S1 cleavage of the *prosysA/prosysB* heteroduplex.

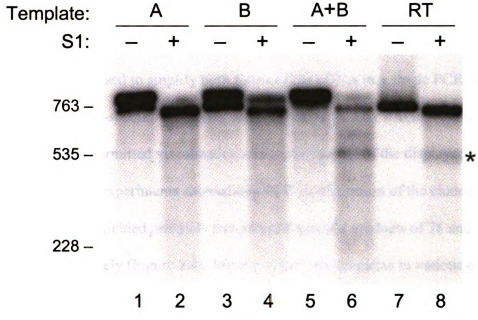

Figure 2.3

fragments (Figure 2.3, lane 8) further suggests that *prosysA* and *B* comprise the two most abundant prosystemin transcripts in tomato leaves.

Expression pattern of prosysA and prosysB

Alternative splicing of pre-mRNA is often regulated by specific factors expressed in response to developmental or environmental cues. To address this question in relation to prosystemin pre-mRNA splicing, we developed a quantitative RT-PCR assay to estimate the relative abundance of the *prosysA* and *prosysB* transcripts in total RNA samples. Primers (PSD1 and PSD2; Figure 2.2a) that span the site of the CAG polymorphism were used to amplify both forms of the cDNA in a single PCR reaction. The resulting isoform-specific fragments were then separated by PAGE. Radiolabeling of one primer (PSD2) permitted visualization and quantification of the displayed products after PAGE. Control experiments showed that PCR amplification of the cloned *prosysA* and *prosysB* cDNAs yielded *prosysA*- and *prosysB*-specific products of 78 and 81 nucleotides, respectively (Figure 2.4). Mixing of the two templates in various proportions prior to PCR yielded an excellent correlation (R value = 0.99) between the ratio of template DNA in the reaction and the relative abundance of the two isoform-specific products obtained (Figure 2.4). These results indicate that the PCR assay is quantitative with respect to the relative abundance of the two templates in the reaction.

To examine the possibility that the abundance of *prosysA* and *B* is regulated during development, total RNA prepared from different tomato tissues was reverse-

Figure 2.4. Quantitative PCR amplification of *prosysA* and *prosysB* cDNAs in a single reaction. (a) P*rosysA* and *prosysB* cDNA fragments contained in plasmids pPS-A and pPS-B, respectively, were gel-purified and mixed in various ratios such that the proportion of *prosysA* in the mixture was as follows: 100% (lane 1), 90% (lane 2), 80% (lane 3), 70% (lane 4), 60% (lane 5), 50% (lane 6), 40% (lane 7), 30% (lane 8), 20% (lane 9), 10% (lane 10), and 0% (lane 11). The total amount of *prosysA* + *prosysB* DNA in each mixture was 30 fg. These samples served as templates for a PCR reaction using primers PSD1 and PSD2, where the latter primer was end-labeled with ^{32}P. PCR products obtained from 20 cycles of amplification were separated by denaturing PAGE and visualized by autoradiography. The PCR products derived from *prosysA* and *prosysB* were 78 and 81 bp in size, respectively. (b) The products shown in (a) were quantified using a Phosphor-Imager. The relative proportion of the two products in each reaction (expressed as % *prosysA*) was calculated and plotted against the proportion of *prosysA* present in the template DNA.

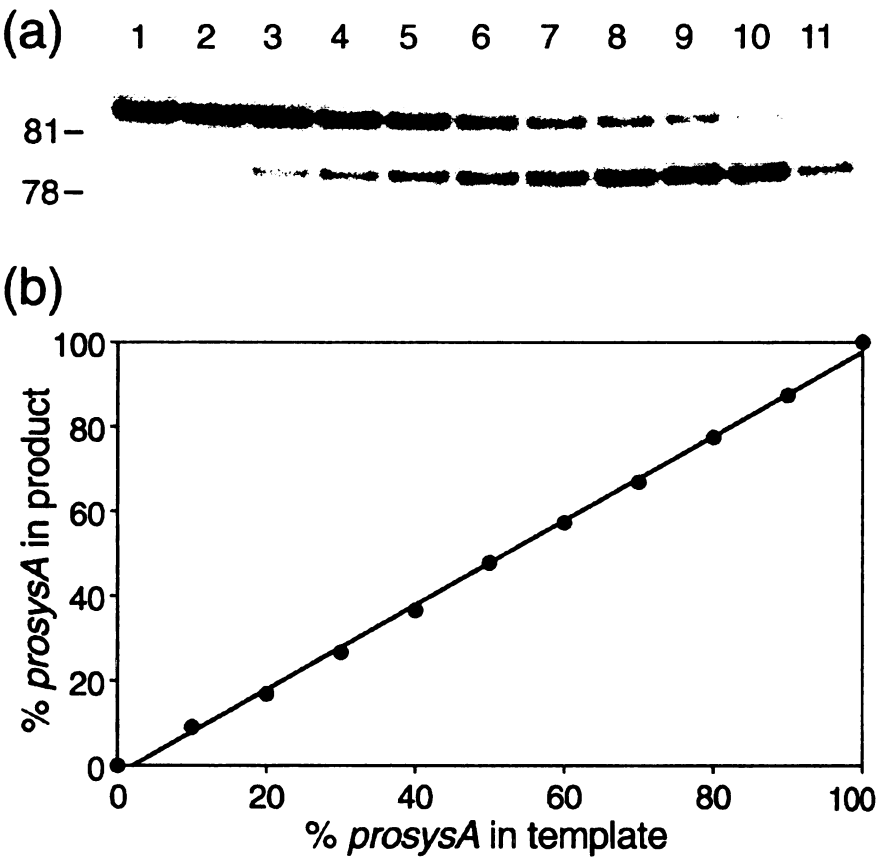
(a)
1 2 3 4 5 6 7 8 9 10 11
81–
78–
(b)
100
80
60
40
20
0
% prosysA in product
0 20 40 60 80 100
% prosysA in template

Figure 2.4

transcribed and subjected to the quantitative PCR analysis described above. Display of the radiolabeled products on PAGE revealed the presence of *prosysA*- and *prosysB*-specific bands in all tissues in which *prosys* mRNA is known to be expressed (Figure 2.5a). Furthermore, the absolute abundance of *prosys* mRNA (*prosysA* + *B*) observed in different organs was consistent with that previously observed by RNA blot analysis (McGurl et al., 1992). The ratio of *prosysA* to *prosysB* was approximately 1:2 in RNA prepared from all tissues examined (Figure 2.5b). This finding indicates that *prosysB* is the most abundant form of *prosys* mRNA.

Previous studies have shown that *prosys* mRNA accumulation is up-regulated by wounding or by treatment of plants with methyl jasmonate (MeJA), a potent inducer of wound response genes (Jacinto et al., 1997; McGurl et al., 1992). To determine whether this effect reflects increased accumulation of one or both forms of the mRNA, plants were exposed to MeJA and total leaf RNA was prepared at different times thereafter for analysis by RT-PCR. The effectiveness of the MeJA treatment was evident by a several hundred-fold increase in accumulation of proteinase inhibitor II (PI-II) mRNA during the time course (Figure 2.6a). Although the effect of MeJA on prosystemin mRNA accumulation was considerably less, we did observe an increase of at least 5-fold. Analysis of *prosysA* and *prosysB* transcripts in these RNA samples showed that the level of both forms increased in parallel during the time course, such that the relative abundance of the two transcripts remained constant (Figure 2.6b and c). Similar results were obtained using RNA prepared from tomato leaves that were subjected to wounding by tobacco hornworm (*Manducta sexta*) larvae (data not shown). Taken together, these results indicate that alternative splicing of prosystemin pre-mRNA within intron 3 is

Figure 2.5. Relative abundance of *prosysA* and *prosysB* transcripts in different tissues of tomato. (a) Total RNA isolated from cotyledon (lane 1), stem (lane 2), petiole (lane 3), leaf (4), flower bud (5), or open flower (6) was reverse transcribed and subjected to the quantitative PCR assay described in Figure 2.4. "A" and "B" denote the isoform-specific products derived from *prosysA* and *prosysB*, respectively. (b) Products in (a) were quantified by Phosphor-Image analysis. The gray and black portions of each bar represent the relative abundance of p*rosysA* and *prosysB*, respectively.

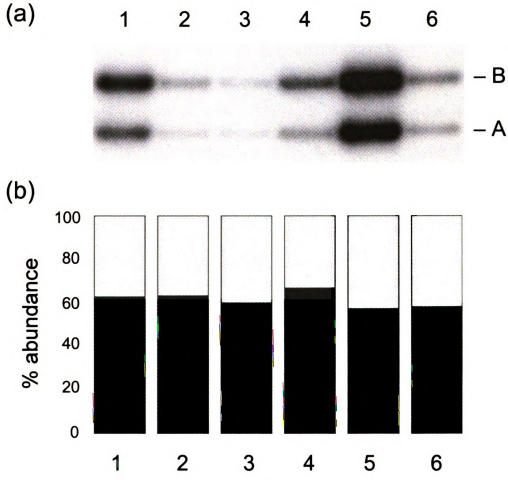

Figure 2.5

Figure 2.6. Relative abundance of *prosysA* and *prosysB* transcripts in response to methyl jasmonate treatment. (a) Three-week-old Micro-Tom plants were exposed to MeJA vapor and leaf tissue was harvested for RNA isolated at various times (h) thereafter. Five μg aliquots of total RNA were subjected to Northern blot analysis using cDNA probes for *prosysA* (top panel) and *proteinase inhibitor II* (PI-II; middle panel). *eIF4A* (bottom panel) was the loading control. (b) The RNA samples analyzed in (a) were reverse transcribed and subjected to the quantitative PCR assay described in Figure 2.4. (c) The relative proportion of the isoform-specific products shown in (b) was quantified. The gray and black portions of each bar represent the relative abundance of p*rosysA* and *prosysB*, respectively, in each reaction.

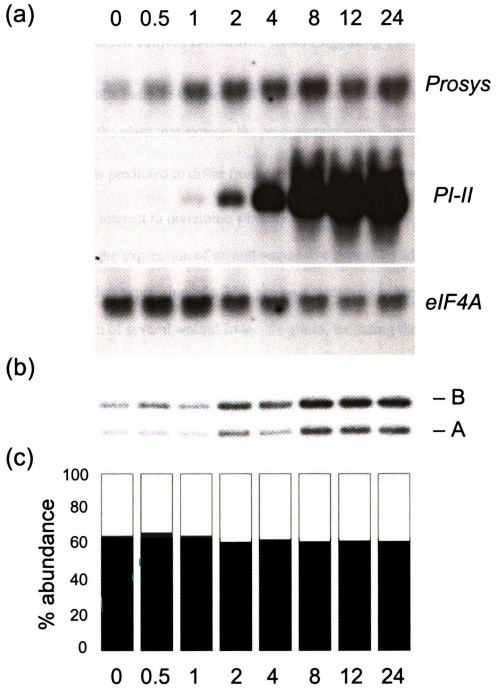
(a)
0 0.5 1 2 4 8 12 24
Prosys
PI-II
eIF4A
(b)
– B
– A
(c)
% abundance
100
80
60
40
20
0
0 0.5 1 2 4 8 12 24

Figure 2.6

constitutive, and is not regulated by developmental or environmental conditions that regulate the expression of the prosystemin gene.

Overexpression of prosysB confers constitutive wound signaling

The above results indicate that *prosysB* is the predominant species of prosystemin mRNA in all parts of the plant that express the prosystemin gene. Because the amino acid sequence of prosysB is predicted to differ from prosysA by an Arg_{57}→Thr-Gly substitution, it was of interest to determine whether this change affects the ability of prosystemin to signal the expression of wound responsive genes. To address this question we relied on the fact that expression of a *35S::prosysA* transgene in tomato activates the constitutive expression of several wound-inducible genes, including those encoding PI-I, PI-II and polyphenol oxidase (PPO) (McGurl et al., 1994; Constabel et al., 1995; Bergey et al., 1996). Genetic analysis has shown that this phenotype results from constitutive activation of the endogenous wound response pathway (Howe and Ryan, 1999). To determine whether overexpression of *prosysB* confers such a constitutive signaling phenotype, *Agrobacterium*-mediated transformation was used to generate transgenic tomato plants (cv Micro-Tom) that harbor a *35S::prosysB* transgene. The presence of the transgene in regenerated plants was confirmed by PCR and Southern blot analysis (data not shown). As a control for these experiments, the *35S::prosysA* transgene originally introduced into the Better Boy cultivar (McGurl *et al.*, 1994) was backcrossed into the Micro-Tom genetic background (see Experimental procedures). Northern blot analysis showed that leaf tissue from the *35S::prosysA* and *35S::prosysB* lines accumulated significantly greater levels of *prosys* mRNA than were observed in untransformed control

plants (Figure 2.7a, upper panel). RT-PCR analysis of the same RNA samples showed that the *prosysB* transcript accounted for 96% of the total *prosys* mRNA detected in *35S::prosysB* plants, whereas the *prosysA* transcript accounted for 90% of the total *prosys* mRNA detected in *35S::prosysA* plants (Figure 2.7a, lower panel). This result established that the two transgenic lines have dramatically different isoform-specific expression patterns. The RT-PCR assay did not distinguish the endogenous *prosysA* transcript from *35S::prosysA*-derived transcripts, or endogenous *prosysB* transcripts from those derived from *35S::prosysB*. However, this experiment did indicate that overexpression of one isoform does not increase the accumulation of the other isoform derived from the endogenous prosystemin gene. This finding suggests that ectopic expression of prosystemin from a *35S::prosys* transgene does not positively regulate the expression of the endogenous prosystemin gene.

We next compared the different genotypes with respect to the level of PI-II and PPO produced in leaf tissue from unwounded plants (Figure 2.7b). The level of PI-II in both *35S::prosysA* and *35S::prosysB* plants was extraordinarily high, and represented an approximate 30-fold increase over the level observed in untransformed plants. Similarly, the constitutive level of PPO activity in both transgenic lines was significantly elevated (>10-fold) over that of control plants. The PI-II and PPO levels observed in *35S::prosysB* plants were comparable to those in *35S::prosysA* plants. These results demonstrate that alteration of the ratio of prosysA to prosysB *in planta* does not affect the constitutive signaling phenotype. We therefore conclude that *prosysB*, like *prosysA*, encodes a form of prosystemin that activates the expression of wound-response genes.

Figure 2.7. Overexpression of either *prosysA* or *prosysB* in transgenic tomato results in a constitutive wound response phenotype. (a) Five μg total RNA prepared from leaf tissue of untransformed control plants (lane 1), *35S::prosysB* plants (lane 2), or *35S::prosysA* plants (lane 3) was subjected to Northern blot analysis for determination of *prosys* mRNA levels (top panel). A second blot was hybridized to an *eIF4A* probe as a loading control (middle panel). The relative proportions of *prosysA* and *prosysB* in the same samples were determined using the RT-PCR assay described in Figure 2.4 (lower panel). (b) Proteinase inhibitor II (PI-II; black bars) and polyphenol oxidase (PPO; gray bars) levels in leaves of five-week-old unwounded plants (1, untransformed; 2, *35S::prosysB*; and 3, *35S::prosysA*). Values represent the mean ± sd of six plants of each genotype.

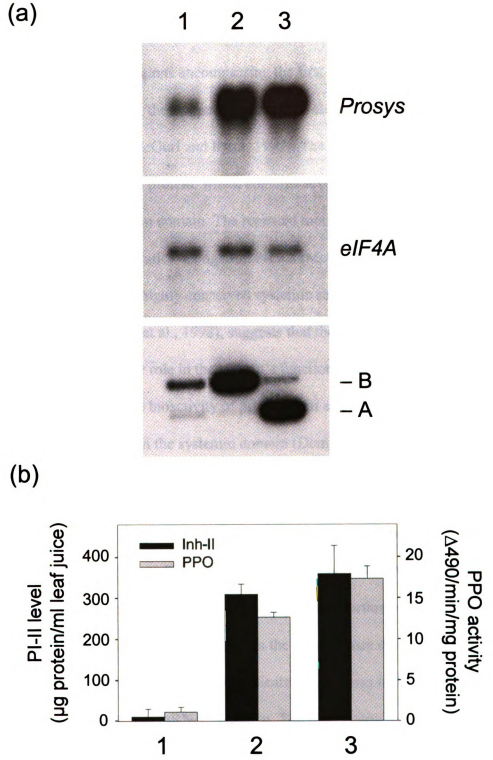
(a)
1 2 3
Prosys
eIF4A
– B
– A
(b)
PI-II level
(μg protein/ml leaf juice)
Inh-II
PPO
400
300
200
100
0
20
15
10
5
0
PPO activity
(Δ490/min/mg protein)
1 2 3

Figure 2.7

Discussion

The prosystemin gene of tomato consists of 10 introns and 11 exons that are organized into five repeated regions encompassing the first ten intron-exon pairs (McGurl and Ryan, 1992). Interestingly, these repeats exclude the eleventh exon in which the systemin sequence is found (McGurl and Ryan, 1992). The repeat structure of the gene is also evident in the prosystemin protein, which contains several repeated motifs that also exclude the C-terminal systemin domain. The repeated motifs are thought to have arisen through successive gene duplication-elongation events (McGurl and Ryan, 1992). This observation, together with the highly conserved systemin region found in other solanaceous plants (Constabel et al., 1998), suggests that the non-systemin portion of the polypeptide plays only a minor role in the protein's function. This view is consistent with a recent study showing that the bioactivity of prosystemin as a signal for wound responses resides exclusively in the systemin domain (Dombrowski et al., 1999).

In the present study, we show that different forms of prosystemin are produced by alternative splicing. Initial insight into this phenomenon came from the identification of a novel prosystemin cDNA (*prosysB*) that differs from the reported cDNA sequence (*prosysA*) by the addition of a CAG within the non-systemin portion of the open reading frame. An S1 nuclease assay was used to confirm the co-existence of *prosysA* and *prosysB* transcripts in total RNA, and further indicated that *prosysA* and *B* comprise the two major forms of prosystemin mRNA in tomato. The single copy nature of the prosystemin gene, which we mapped to the central region of chromosome 5, excludes the possibility that the two transcripts are derived from different genes. Rather, our results

indicate that *prosysA* and *B* are generated by an alternative splicing event that uses one of two juxtaposed 3′ splice sites in intron 3. At the protein level, the predicted outcome of alternative splicing is an Arg_{57}→Thr-Gly substitution. Based on comparisons of deduced prosystemins isolated from various solanaceous plants (Constabel et al., 1998), it is apparent that this polymorphism is located in one of the most variable regions of prosystemin. Constabel et al. (1998) suggested that amino acid deletions found in bell pepper prosystemin and one isoform of potato prosystemin may result from a shift in intron-exon boundaries in this region. Our results suggest that alternative splicing may also be responsible for generating sequence variability in this region.

ProsysA and *prosysB* transcripts accumulate at a relatively fixed ratio in all tissues that express the prosystemin gene. The relative proportion of the two transcripts was not significantly altered under conditions (e.g., wounding or MeJA treatment) that increase the accumulation of total *prosys* mRNA. These results indicate that alternative splicing of prosystemin intron 3 is constitutive, and is not likely to be regulated by developmental or environmental cues. Quantitative PCR experiments showed that the level of *prosysB* transcripts was approximately twice that of *prosysA* transcripts. This value is consistent with the higher proportion (62%) of *prosysB* cDNAs revealed by direct sequencing of cloned prosystemin cDNAs. In further support of this, we found that all the prosystemin cDNAs sequenced to date as part of the tomato EST project (http://www.tigr.org/tdb/lgi/) correspond to the *prosysB* isoform. Taken together, we conclude that *prosysB* is the most abundant prosystemin transcript in cells that express the gene. Assuming that both transcripts are subject to similar post-transcriptional regulation, we predict that prosysB is

the major protein isoform in cell-types that express prosystemin. It may be possible to test this hypothesis using chromatography techniques to separate the two protein isoforms.

The prevalence of *prosysB* over *prosysA* is consistent with the spliceosome scanning model for recognition and selection of 3′ splice sites (Smith et al., 1993). This model proposes that 3′ splice site selection is determined by spatial relationships between the branch point, the polypyrimidine tract downstream of the branch point, and the 3′ splice site. The spliceosome scans in a 5′-to-3′ direction from the splice branch point and selects the first AG encountered. In the event that an additional AG lies downstream of the proximal AG, the most competitive splice site is selected. Determinants of splice site competition include not only the proximity of the AG to the branch point, but also the sequence context surrounding the splice site. In the case of prosystemin, it is interesting to note that the downstream splice site (site A) conforms to the 3′ splice site consensus better than does the upstream site (site B). However, our data indicate that the upstream AG is the preferred splice site *in vivo*. This result lends direct support to the scanning model, and in particular to the notion that proximity of the splice site to the branch point is an important determinant in splice site competition.

The functional significance of alternative splicing of prosystemin pre-mRNA remains to be determined. However, strict conservation of the two juxtaposed 3' splice sites in all wild tomato species examined suggests that alternative splicing of this intron serves an important role. For example, it is possible that the efficiency of splicing is enhanced by the close juxtaposition of two 3' splice sites. In support of this idea is the fact that several other genes have been shown to utilize an AGNAG| motif at the 3′ end

of an intron to affect alternative splicing (Maurer et al., 1981; Shelness and Williams, 1984; Cook et al., 1985; Sun and Baltimore, 1991; Manrow and Berger, 1993; Vogan et al., 1996). Given the close juxtaposition of the two AG splice sites, and their separation by three nucleotides, such a mechanism to increase splicing efficiency is likely to minimize alteration to the function of the protein. The constitutive signaling phenotypes observed in both *35S::prosysA* and *35S::prosysB* plants support this view. The functional equivalence of prosysA and prosysB in the transgenic assay is also consistent with the recent finding that the systemin portion of prosystemin is necessary and sufficient for the biological activity of prosystemin (Dombrowski et al., 1999). Of course, our results do not exclude the possibility that prosystemin isoforms differ in functional attributes that cannot be discerned from the overexpression phenotype. For example, prosystemin isoforms could differ in their intracellular location within the specific cell types in which prosystemin is normally expressed, interaction with other components of the wound signaling pathway, or susceptibility to proteolysis. Additional experiments aimed at determining the precise role of prosystemin in the transduction of systemic wound signals should provide further insight into these possibilities.

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CHAPTER 3

The Tomato Mutant jai1-1 Is Insensitive to Jasmonates and Defective in Defense against Herbivores

The work presented in Figure 3.2 and Table 3.1 was done in collaboration with Bonnie McCaig and Youfu Zhao, respectively.

Abstract

Jasmonates are endogenous signaling compounds that regulate a wide variety of developmental and defense-related processes in plants. Here we report the characterization of a fast neutron-induced recessive mutant (called *jasmonic acid-insensitive1-1*) of tomato that is deficient in jasmonate signaling in many, if not all, tissues. *jai1-1* plants exhibited normal vegetative growth but produced fruit that lacked viable seed. Although *jai1* pollen showed reduced germination and viability, sterility of the mutant resulted from a defect in female reproductive development. Expression profiling of ~ 500 tomato genes identified 40 transcripts whose levels were up-regulated by exogenous methyl jasmonate in wild-type but not *jai1-1* leaves. Floral tissues of the mutant were also deficient in the expression of jasmonate-regulated defensive *proteinase inhibitor* (*PI*) genes that are constitutively expressed in wild-type flowers. Wound-induced local and systemic expression of *PI* genes and other 'late' wound response genes was abolished in *jai1-1* leaves, as was *PI* expression in response to the PI-inducing compounds systemin, 12-oxo-phytodienoic acid, bestatin, and hydrogen peroxide. Expression of 'early' wound response genes in *jai1-1* plants was either reduced or not affected, indicating the existence of multiple wound signaling pathways that differ in their requirement for *Jai1*. Failure of *jai1-1* plants to express jasmonate-regulated genes was correlated with increased susceptibility to herbivores. These findings demonstrate that *Jai1* is an essential component of the jasmonate signaling pathway in tomato.

Introduction

Jasmonic acid (JA) and its cyclic precursors and derivatives, collectively called jasmonates, comprise a family of oxylipins that are synthesized from linolenic acid via the octadecanoid pathway (Schaller, 2001; Turner et al., 2002; Weber, 2002). Jasmonates act either alone or in combination with other phytohormones to promote a wide range of biological activities (Creelman and Mullet, 1997; Wasternack and Hause, 2002). Jasmonates are perhaps best known for their ability to regulate plant defense responses to attack by herbivores and some microbial pathogens (Walling, 2000; Weber, 2002). For instance, JA synthesized in response to herbivory activates the expression of genes involved in the production of phytochemicals that have direct toxic or anti-nutritive effects on the herbivore, or that act indirectly to attract natural predators to the herbivore (Creelman and Mullet, 1997; Walling, 2000; Memelink et al., 2001; Kessler and Baldwin, 2002). Jasmonates are also implicated in responses to abiotic stimuli including salt and drought stress (Creelman and Mullet, 1995), UV irradiation (Conconi et al., 1996) and ozone exposure (Rao et al., 2000). In addition, jasmonates play a role in regulating a number of plant developmental processes including tendril coiling (Weiler et al., 1993), pollen and anther development (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001), and senescence (He et al., 2002).

Regulation of gene expression by endogenous jasmonates requires numerous cellular activities to integrate the biosynthesis and perception of the hormone in specific tissues at specific times. The octadecanoid pathway for JA biosynthesis from linolenic acid is initiated by lipoxygenase in the chloroplast, with the terminal steps of

cyclopentenone reduction and oxidation taking place in peroxisomes (Schaller, 2001; Strassner et al., 2002). Genes encoding all of the JA biosynthetic enzymes have been identified and studied in detail (Schaller, 2001; Turner et al., 2002; Feussner and Wasternack, 2002). In contrast to knowledge about JA synthesis, much less is known about the process by which jasmonates regulate changes in gene expression. The isolation and characterization of jasmonate response mutants of Arabidopsis has begun to yield valuable insight into this question (Berger, 2002; Turner et al., 2002). For instance, the JA-insensitive *coi1* mutant defines a gene encoding an F-box protein that is essential for many stress-induced and developmental responses (Feys et al., 1994; Xie et al., 1998). The COI1 protein is involved in the formation of an ubiquitin-E3-ligase complex that presumably targets transcriptional repressors of jasmonate responsive genes for degradation by the 26S proteasome (Devoto et al., 2002; Xu et al., 2002). Forward genetic analysis also led to the identification of *JAR1*, which encodes an enzyme involved in the covalent modification (e.g., adenylation) of JA (Staswick et al., 2002). Despite these significant advances, the mechanism by which JA and its derivatives are perceived in the cell remains to be elucidated.

A complete understanding of the jasmonate signaling pathway and its role in the plant life cycle may be facilitated by the identification of jasmonate response mutants in plants other than Arabidopsis. In our efforts to characterize the systemic wound response pathway in tomato (*Lycopersicon esculentum*), we conducted various genetic screens that led to the identification of mutants that are deficient in jasmonate perception (Li et al., 2001; Howe et al., 2002). Reciprocal grafting experiments performed with these mutants showed that jasmonate perception is essential for the recognition of the transmissible

wound signal in undamaged responding leaves, whereas JA synthesis is essential for the production of that signal in damaged leaves (Li et al., 2002b). In addition to providing a powerful tool for studying long-distance wound signaling, jasmonate perception mutants of tomato may prove useful for studying other processes for which tomato has been used as a model system, including fruit development (Giovannoni, 2001), peptide signaling (e.g., systemin; Ryan, 2000), and plant interactions with herbivores, pathogens, and nematodes (Berger, 2002; Kessler and Baldwin, 2002; Li et al., 2002a; Kennedy, 2003). Here, we report the identification and characterization of a fast-neutron-induced mutant, called *jai1-1*, that is defective in JA signaling in many, if not all, tissues of the plant. Our results indicate that the signaling pathway defined by *jai1* is essential for the expression of a subset of wound responsive genes in leaves, as well as the constitutive expression of several defense-related genes in flowers. Our results also provide strong evidence for a role of jasmonate in female reproductive development.

Materials and Methods

Plant material and treatments

Tomato seedlings (*Lycopersicon esculentum* Mill cv Micro-Tom and cv Castlemart) were grown under 17 h days at 27 °C with light at 200 μmol m^{-2} sec^{-1} and 7 h at 16 °C in darkness. The original mutant 406A was backcrossed twice using wild-type plants (cv Micro-Tom and cv Castlemart) as the recurrent pistillate parent. All experiments involving *jai1-1* were performed with homozygous (*jai1-1/jai1-1*) lines. For selection of mutant plants in F_2 populations, resistance to inhibition of root growth and anthocyanin accumulation by MeJA was assayed as follows. Seeds were placed on a piece of water-saturated filter paper in a shallow Tupperware box and allowed to germinate in the dark at ambient temperature for 4-5 days until the emerging radicals were 1 cm in length. The filter paper was then re-saturated with a solution of 1 mM MeJA, which was prepared by mixing 2 μl pure MeJA (Bedoukian Research, Danbury, CT) with 75 μl ethanol. This mixture was then diluted into 10 ml distilled water. Following growth in the dark for approximately 24 to 36 h, phenotypes of the seedlings were scored for the presence of *jai1-1/jai1-1* homozygotes using two criteria: root growth and anthocyanin accumulation. MeJA treatment of wild-type seedlings causes root growth inhibition and anthocyanin accumulation in the hypocotyls. In contrast, *jai1-1/jai1-1* roots grow in the presence of MeJA, and hypocotyls do not accumulate anthocyanin (see Figure 3.1c).

MeJA treatment of adult plants was perfomed by incubating three-week-old plants (cv Micro-Tom) in a sealed Lucite box (60 x 32 x 17 cm) in which 1 µl pure MeJA was distributed to several evenly spaced cotton wicks. For each time point of sampling, five plants were removed from the box for extraction of RNA from leaf tissues (Li and Howe, 2001). Tobacco hornworm (*Manduca sexta*) feeding trials were performed as described previously (Howe et al., 1996).

Isolation of jai1-1

Fast neutron irradiation of tomato (*Lycopersicon esculentum* cv Micro-Tom) seed was performed at the International Atomic Energy Agency (Seibersdorf, Austria), using calibrated doses in the range of 12.7 and 17.9 Gy. M_2 seed was collected separately from each M_1 plant. The screen for MeJA-insensitive mutants was conducted as follows. Approximately 120 eighteen-day-old M_2 seedlings were enclosed in the Lucite box containing 5 µl MeJA applied to cotton wicks. Plants were exposed to MeJA vapor for 24 h, followed by an additional 24 h of incubation in the absence of MeJA. A small piece of leaf tissue was then sampled from each individual plant and assayed for PPO activity as previously described (Howe and Ryan, 1999). Plants showing reduced PPO activity were re-tested for PI-II accumulation using a radial immunodifusion assay (Howe and Ryan, 1999).

Pollen germination

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 percent germination from 10 arbitrarily selected microscopic fields.

cDNA microarray and RNA gel blot analysis

Tomato EST (expressed sequence tags) clones were obtained from the Clemson Genomics University Genomics Institute (Clemson, SC). cDNA inserts were amplified by polymerase chain reaction (PCR) in a 100-µl reaction volume using pBluescript KS(-) primers T3 and T7, or amplified using gene specific primers. PCR products were precipitated with ethanol and resuspended in 25 µl of 3 x SSC (1x SSC 0.15 M NaCl and 0.015 M sodium citrate). One µl of PCR product was run on 1% agarose gel for quality control before arraying. DNA was printed onto amine-coated glass slides (Telechem, Sunnydale, CA) using an Omnigridder robot (Gene Machines, San Carlos, CA) equipped with ArrayIt chipmaker 4 pins (Telechem). Each DNA sample was printed in triplicate on each slide. Slides were blocked according to the recommended protocol from Telechem.

Total RNA (100 µg) isolated from tomato leaves was used to synthesize the cDNA probes for microarray analysis. The RNA was purified according to the RNAeasy

kit cleanup protocol (Qiagen, Valencia, CA) and was labeled by direct incorporation of Cy3- or Cy5-conjugated deoxy UTP (Amersham Pharmacia Biotech, Piscataway, NJ) during reverse transcription. Briefly, RNA was mixed with 6 μg oligo-dT23V (Invitrogen) in a total volume of 16.5 μl and incubated at 70 °C for 10 min. The mixture was then chilled on ice for 5 min and 2 μl of FluoroLink Cy3- or Cy5-dUTP, 3 μl of 0.1 M DTT, 6 μl of 5X first-strand buffer, 0.5 μl of 50X dNTPs mix (25 mM dATP, dCTP, dGTP, 9 mM dTTP), and 2 μl of Superscript II (Invitrogen) were added to the mixture. Reactions were incubated at 42 °C for 120 min. RNA was hydrolyzed by adding 0.5 μl of RNase A (10 mg/ml) and 0.25 μl of RNase H (Invitrogen) at 37 °C for 30 min. The labeled cDNA probe was first purified on a Microcon YM-30 filter (Millipore, Bedford, MA) and then by a PCR purification kit (Qiagen). The purified, Cy3- and Cy5-labeled probes were then combined, concentrated, and resuspended in 4 μl 10 mM EDTA, pH8.0. The labeled probes were denatured at 95 °C for 10 min and 30 μl SlideHyb buffer 1 (Ambion, Austin, TX) was added to the denatured probes. The mixture was then hybridized to slides at 54 °C in a hybridization chamber for 16 to 20 h. After hybridization, slides were washed twice in 2X SSC, 0.5% SDS for 5 min at 65 °C, in 0.1X SSC, 0.2% SDS for 5 min, and in 0.1X SSC for 5 min at room temperature. After washing, slides were dried by centrifugation and scanned by Affymetrix 428 Array Scanner (Affymetrix, MA). Spot intensities were quantified using Axon GenePix Pro 3 image analysis software (Axon, Foster City, CA). Ratio data were extracted and normalized as previously described (Schaffer et al. 2001).

For RNA gel blot analysis, total RNA isolation and gel blot hybridization were carried out as previously described (Li and Howe, 2001). Gels were also stained with ethidium bromide to check for equal loading. A cDNA for tomato translation initiation factor eIF4A (cLED1D24) was used as the loading control.

Results

Isolation of jasmonate-insensitive1-1

Polyphenol oxidase (PPO) and proteinase inhibitor II (PI-II) are defense-related proteins that accumulate in tomato leaves in response to wounding, systemin, and exogenous JAs (Farmer and Ryan, 1992; Constabel et al., 1995). To identify tomato mutants affected in the jasmonate signaling pathway, we used a rapid PPO activity assay to screen a fast-neutron-mutagenized population of Micro-Tom plants for individuals that fail to accumulate PPO in response to exogenous MeJA (Figure 1.1a; Howe and Ryan, 1999; Howe et al., 2002). Out of a total of 24,077 M_2 plants tested, eighteen putative mutants were identified that accumulated reduced levels of both PPO and PI-II in response to MeJA. One plant (406A) showing normal vegetative growth (Figure 3.1b) and no detectable expression of either PPO or PI-II in response to MeJA was chosen for further analysis. F_1 plants derived from a cross between 406A pollen and a wild-type pistillate parent accumulated normal levels of leaf PPO and PI-II in response to MeJA, and thus were sensitive to the hormone. Analysis of 687 F_2 plants showed that the ratio of MeJA-sensitive to -insensitive plants was 519:168 (Figure 3.1a; $\chi^2 = 0.089$ for 3:1 hypothesis). These results indicate that the deficiency in MeJA-induced accumulation of PPO and PI-II in line 406A is caused by a single recessive mutation, which we designated *jasmonic acid-insensitive1-1* (*jai1-1*).

Figure 3.1. *jai1-1* plants exhibit normal vegetative growth and insensitivity to exogenous MeJA. (a) MeJA-induced PPO activity in tomato leaves. Fourteen-day-old plants were treated with MeJA and assayed for PPO activity as described in the Material and Methods. Wild-type (WT) plants were treated with MeJA (+) or ethanol (-) as a control. All F_2 plants, which were derived from a cross between *jai1-1* and WT (cv Castlemart), were treated with MeJA. (b) Four-week-old WT (left) and *jai1-1* (right) plants. (c) Response of germinating seedlings to MeJA. WT (cv Micro-Tom), *jai1-1*, and F_1 seeds were germinated and exposed to MeJA (+) for 2 days. A WT seedling grown in the absence of MeJA (-) is shown as a control. Images in this figure are presented in color.

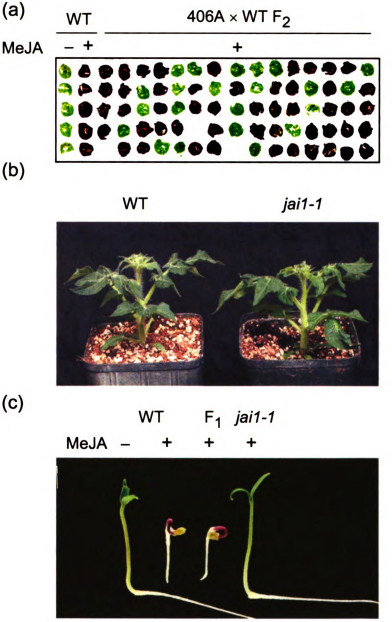

Figure 3.1

Jasmonate-induced phenotypes of seedlings

Exogenous jasmonates have several well-documented effects on seedling growth and development (Corbineau et al., 1988; Staswick et al., 1992), including inhibition of root growth in tomato (Tung et al., 1996). Treatment of four-day-old wild-type seedlings with 1 mM MeJA resulted in inhibition of root and hypocotyl growth, and accumulation of anthocyanin in the hypocotyl (Figure 3.1c). To determine whether these responses are suppressed by *jai1-1*, an F_2 population segregating for the mutation was exposed to MeJA. Approximately one-quarter of the seedlings appeared to be identical to untreated wild-type seedlings (Figure 3.1c), indicating that they were MeJA-insensitive. Re-testing of these MeJA-insensitive seedlings, after growth for three weeks in soil, showed that they also lacked MeJA-induced PPO/PI-II accumulation in leaves. These results demonstrate that MeJA-induced responses in both seedlings (i.e., root growth, hypocotyl elongation, and anthocyanin production) and mature plants (PPO and PI-II accumulation in leaves) require *Jai1*.

Reproductive phenotypes of jai1-1 plants

The gross morphology and timing of development of *jai1-1* flowers was similar to that of wild-type flowers (Figure 3.2a, b). One exception to this was that stigma of mutant flowers protruded from the anther cone during later stages of flower development (Figure 3.2a, b). Although stigma exertion can reduce the efficiency of self-pollination and fruit set, the number of fruit produced by self-fertilized *jai1-1* plants was comparable to that of wild-type. The size of immature (green) *jai1-1* fruit was also similar to wild-type (Figure

Figure 3.2. Reproductive development of wild-type and *jai1-1* plants. (a) Flowers of wild-type (WT) plants. (b) *jai1-1* flowers. Arrowheads indicate the protruded stigma. (c) Developing fruits of WT plants. (d) Developing fruits of *jai1-1* plants. (e) Mature WT (top) and *jai1-1* (bottom) fruits. (f) Enlargement of the *jai1-1* fruit showing the small, undeveloped seeds. (g and h) WT and *jai1-1* pollen, respectively, after germination. Images in this figure are presented in color.

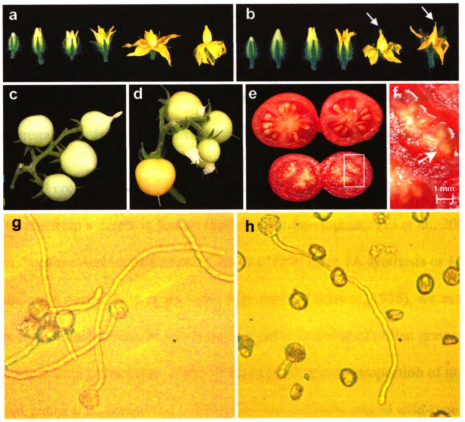

Figure 3.2

3.2c, d), as was the general timing of fruit ripening. The size of ripe *jai1-1* fruit, however, was significantly less than ripe fruit from wild-type plants (Figure 3.2e). The average weight of ripe fruit from wild-type and mutant plants was 5.4 ± 1.1 g and 2.9 ± 0.4 g, respectively (mean ± SD; n = 100 fruit/genotype; P<0.0001). Wild-type fruit yielded approximately 30 seed, each having a dry weight of approximately 2.5 mg. Ripened *jai1-1* fruit contained numerous undeveloped seeds having a dry weight < 0.7 mg (Figure 3.2f). Based on these observations, we estimated that the yield of mature seed from *jai1-1* fruit was <0.01% of that from wild-type fruit. Examination of several hundred fruit from *jai1-1/jai1-1* plants obtained from three successive backcrosses showed that the sterility strictly co-segregated with the MeJA-insensitive phenotype of seedlings and leaves.

Reciprocal crosses between wild-type and *jai1-1* plants showed that sterility of the mutant results from a defect in female reproductive development (Li et al., 2001). Nevertheless, 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 (Alexander, 1969; 1980) showed that the proportion of non-aborted pollen grains (i.e., containing cytoplasm) from similarly-staged wild-type and mutant anthers was approximately 94% and 67%, respectively. Fluorescien diacetate/propidium iodine co-staining (Oparka and Read, 1994) further revealed a significant reduction in the viability of mutant pollen (28% viability) compared to that of wild-type pollen (82% viability, P<0.0001). 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* pollen ranged between 9 and

Figure 3.3. Accumulation of MeJA-responsive transcripts in wild-type and *jai1-1* leaves. Three-week-old plants (cv Micro-Tom) were exposed to MeJA vapor and leaf tissues harvested for RNA extraction at various times (h) thereafter. RNA was also prepared from untreated plants (0) for control. RNA gel blots were hybridized to cDNA probes indicated on the right. *eIF4A* was used as a loading control.

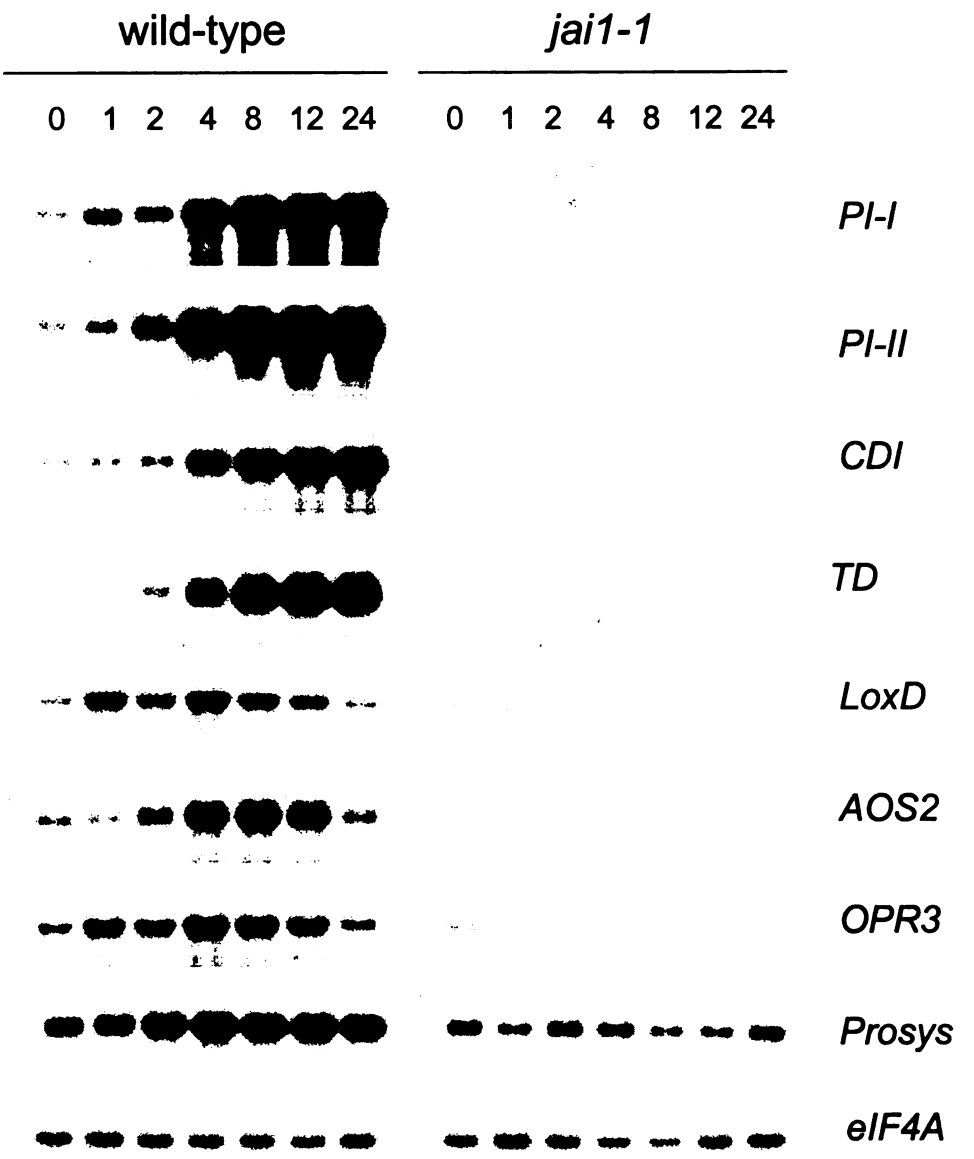
wild-type
jai1-1
0 1 2 4 8 12 24
0 1 2 4 8 12 24
PI-I
PI-II
CDI
TD
LoxD
AOS2
OPR3
Prosys
eIF4A

Figure 3.3

10%, compared to >55% germination of wild-type pollen from flowers at a similar stage. Despite this reduced germination, the overall morphology and tube length of germinated *jai1-1* pollen was comparable to that of germinated wild-type pollen (Figure 3.7g, h). These observations indicate that the defects in *jai1-1* pollen development, although significant, are not sufficient to cause male sterility.

Jasmonate-induced gene expression in jai1-1 plants

To investigate the effect of *jai1-1* on jasmonate-induced gene expression in leaves, RNA blot analysis was used to determine the steady state level of several transcripts whose expression is known to be wound- and JA-inducible in wild-type leaves (Figure 3.3). One set of genes, represented by *PI-I*, *PI-II*, *cathepsin D inhibitor* (*CDI*), and *threonine deaminase* (*TD*), was expressed at relatively low levels in untreated (0 h) wild-type plants. Upon treatment with MeJA, these mRNAs began to accumulate within 2 to 4 h, and reached maximum levels 12 h after treatment. *PI-I*, *PI-II*, *CDI*, and *TD* mRNA accumulation was undetectable in MeJA-treated *jai1-1* plants, with the exception that very weak expression (< 2% of wild-type) was observed at the 24 h time point. Exposure of wild-type plants to MeJA also stimulated moderate and transient accumulation of transcripts encoding the octadecanoid pathway enzymes lipoxygenase D (LoxD; Heintz et al., 1997), allene oxide synthase 2 (AOS2; Howe et al., 2000), 12-oxo-phytodienoic acid reductase 3 (OPR3; Strassner et al., 2002), as well as prosystemin (Prosys; Jacinto et al., 1997). In contrast to *PI* and *TD* genes, the steady state level of *LoxD*, *AOS2*, *OPR3*, and *Prosys* mRNA was reduced but not abolished in untreated (0 h) *jai1-1* plants.

Table 3.1. List of genes that are differentially regulated in wild-type and *jail-1* plants by exogenous MeJA.

GenBank accession no.	Description	Expression Ratio	
		wild-type	*jail-1*
AI485116	Threonine deaminase	41.88	ND
Q10712	Leucine aminopeptidase	32.98	1.33
AI485529	Unknown	25.89	0.66
AI486173	Similar to protein translation inhibitor	19.84	ND
AI897750	Miraculin;Kunitz-type protease inhibitor	14.83	0.76
AI490282	Unknown	14.32	0.91
AW037833	Metallocarboxypeptidase inhibitor	12.70	ND
AI487422	Pto-responsive gene 1	12.46	1.00
K03291	Proteinase Inhibitor II	12.41	0.75
AI488657	Cathepsin D inhibitor	11.18	ND
AW649919	Putative GDSL-motif lipase/acylhydrolase	10.95	1.28
AW624058	Lipoxygenase A-9	10.79	1.37
U09026	Allene oxide cyclase	9.23	0.84
Z12838	Polyphenol oxidase F	8.38	1.28
AI490318	Unknown	8.24	1.01
AW092579	Nucleoside diphosphate kinase	5.73	1.00
AI897184	Unknown	5.50	0.87
K03290	Proteinase Inhibitor I	5.46	0.84
AI486025	4-coumarate:coenzyme A ligase	5.17	1.11
AW040669	Thioredoxin M-type 3 chloroplast precursor	5.13	0.95
AI483889	NAC domain protein NAC2	4.77	0.87
AI486916	Proteinase inhibitor PID	4.53	1.08
AI486546	Wound-inducible carboxypeptidase	4.47	1.09
AI489221	WIZZ	4.32	1.03
AW032472	Unknown	4.20	0.65
Z21793	DAHP-synthase 2	4.18	1.17
AW034958	12-oxophytodienoate reductase 3	4.16	1.07
AI771886	RD2 protein	4.00	1.13
AI483527	Unknown	3.99	0.92
AW220064	Glutathione S-transferase	3.87	1.41
AF230371	Allene oxide synthase 2	3.76	0.99
AW648549	Indole-3-glycerol phosphate synthase	3.46	1.15
U37840	Lipoxygenase D	3.38	1.01

Table 3.1. cont'd.

GeneBank accession no.	Description	Expression Ratio	
		wild-type	*jai1-1*
AI895589	Allene oxide synthase 1	3.04	0.95
AI897620	Putative prephenate dehydratase	2.91	1.20
M84800	Prosystemin	2.91	1.18
AI483536	TMV response-related protein	2.67	0.86
BE459901	Caffeoyl-CoA O-methyltransferase	2.58	0.65
AW038929	Cathepsin B-like cysteine proteinase	2.48	1.12
AI485737	Glucosyl transferase	2.35	0.98
AI488332	Unknown	2.19	0.94
AI489097	Putative embryo-abundant protein	1.41	2.70
AI488782	Cold-inducible glucosyl transferase	1.30	6.47
AI484542	Eukaryotic initiation factor 4A-2	1.01	0.98

Three-week-old wild-type and *jai1-1* plants (cv Micro-Tom) were treated with MeJA or ethanol (mock) for 8 h. Leaf tissue was harvested for RNA isolation after the treatment. RNA from ethanol mock untreated plants was also prepared as controls. For microarray analysis, customer-made slides were hybridized simultaneously to probes derived from RNA isolated from mock and MeJA-treated plants. Numbers (average of two independent biological replicates) represent the expression ratio of MeJA and mock treated samples. Genes that were differentially regulated (up-regulated > 2-fold in response to MeJA in either wild-type or *jai1-1* plants) are listed and their GeneBank Accession numbers and annotation given. The cDNA for eukaryotic initiation factor 4A-2 (AI484542) was included in the array as a spiking control. ND, not detectable.

Moreover, a low level of *LoxD*, *AOS2*, *OPR3*, and *Prosys* mRNAs was maintained in *jai1-1* plants throughout the time course of MeJA treatment. These results suggest the existence of two classes of MeJA-responsive genes in tomato leaves: those genes whose induction by MeJA is completely dependent on *Jai1* (e.g., *PI-I*, *PI-II*, *CDI*, and *TD*), and those whose expression is only partially dependent in *Jai1* (e.g., *LoxD*, *AOS2*, *OPR3*, and *Prosys*).

DNA microarray analysis was used to further examine the spectrum of genes whose expression in response to MeJA is abrogated by *jai1-1*. For this purpose, we constructed a microarray slide containing 607 tomato cDNAs corresponding to approximately 500 unique genes involved in various aspects of herbivore and pathogen defense, signal transduction, lipid metabolism, and hormone synthesis. To identify jasmonate-responsive genes among this collection of sequences, the slide was hybridized simultaneously to probes derived from mRNA isolated from mock- and MeJA-treated (8 h) wild-type leaves. This time point was chosen because RNA blot analysis indicated that it maximizes the chance of detecting MeJA-responsive genes with different response kinetics (Figure 3.3). This experiment identified 40 genes that were up-regulated > 2-fold in response to MeJA (Table 3.1). Included among these were all eight of the genes analyzed by RNA blot analysis (Figure 3.3), as well as other previously identified JA-responsive genes including *leucine amino peptidase* (*LAP*; Chao et al., 1999), *polyphenol oxidase* (*PPO*; Constabel et al., 1995), *metallocarboxypeptidase inhibitor* (*MCPI*; Villanueva et al., 1998), *allene oxide cyclase* (*AOC*; Hause et al., 2000), *lipoxygenase A* (*LoxA*; Beaudoin and Rothstein, 1997), a wound-inducible *serine carboxypeptidase* (*CP*;

Figure 3.4. Developmental accumulation of transcripts encoding defense-related proteins. Tissues from wild-type (WT, cv Micro-Tom) and *jai1-1* plants were harvested for RNA extraction. R, roots; P, petioles; L, leaves; and F, young flower buds (< 10 mm). cDNA of several defense-related genes were used as probes. A blot was also probed with *eIF4A* as a loading control.

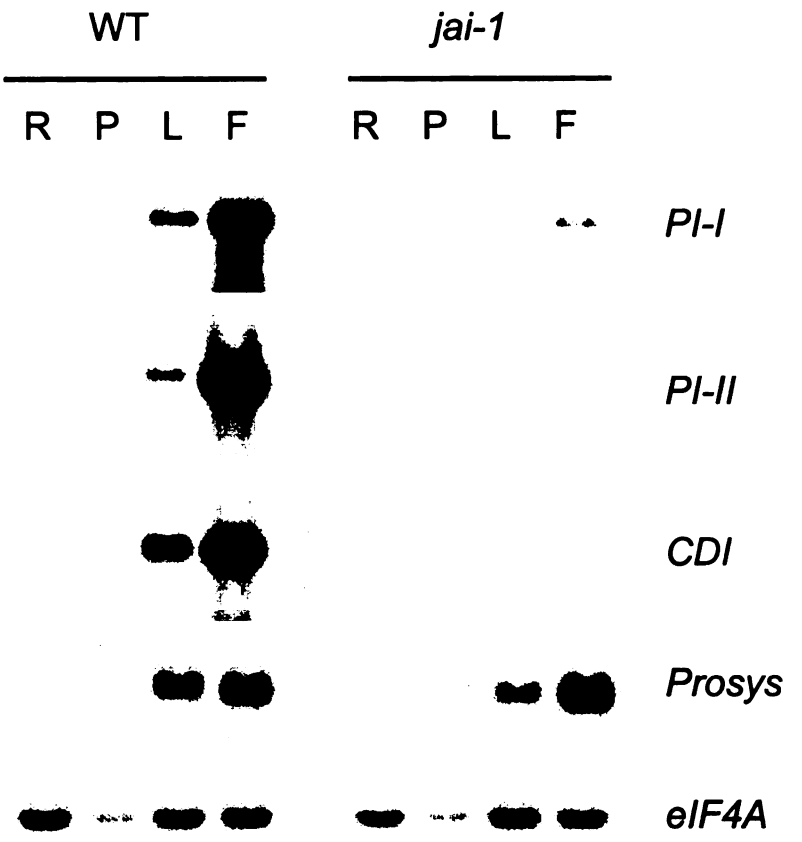

Figure 3.4

Moura et al., 2001), and *AOS1* (Sivisankar et al., 2000). To determine whether MeJA-induced expression of these genes involves *Jai1*, slides were hybridized to probes derived from mock- and MeJA-treated mutant plants. Of the 40 MeJA-responsive genes identified in wild-type, none of them were up-regulated by MeJA in *jai1-1* (Table 3.1). Interestingly, transcript levels for two genes encoding putative glucosyl transferases were up-regulated by MeJA in *jai1-1* plants but not in wild-type plants (Table 3.1).

Effects of jai1-1 on the developmental expression pattern of PI genes

Several genes that are induced by wounding and JAs in leaves have been shown to be constitutively expressed in reproductive organs (Wingate and Ryan, 1991; Peña-Cortés et al., 1991; Benedetti et al., 1995; Chao et al., 1999). The accumulation of high levels of JAs in flowers (Hause et al., 2000) and young fruit (Fan et al., 1998) of tomato suggested to us that constitutive expression of wound-responsive genes in reproductive organs is caused by constitutive activation of the JA response pathway in these tissues. To test this hypothesis, we compared the organ-specific expression of *PI* genes in wild-type and *jai1-1* plants (Figure 3.4). The results showed that *PI-I*, *PI-II*, and *CDI* mRNAs accumulated to high levels in wild-type flowers, whilst little or no expression of these genes was detected in *jai1-1* flowers. Consistent with this, the level of PI-II protein in wild-type flowers was 209 ± 42 μg/ml crude extract, but undetectable in mutant flowers. In contrast to *PI* genes, constitutive accumulation of *Prosys* mRNA in flowers was not significantly affected by *jai1-1*. This finding indicates that although *Prosys* expression in leaves is partially dependent on JA signaling (Figure 3.3; Jacinto et al., 1997), expression of prosystemin in floral tissues is not.

Figure 3.5. Response of wild-type and *jai1-1* plants to mechanical wounding.

Tomato seedlings (cv Castlemart) at the two-leaf stage were wounded with a hemostat on the lower leaves. Total RNA was isolated separately from the lower wounded (local) and the upper unwounded (systemic) leaves at various times after wounding. RNA was prepared from unwounded plants (0) as a control. cDNA probes representing different classes of wound responsive genes (see text for details) were used for hybridization as shown on the right. *eIF4A* was the loading control.

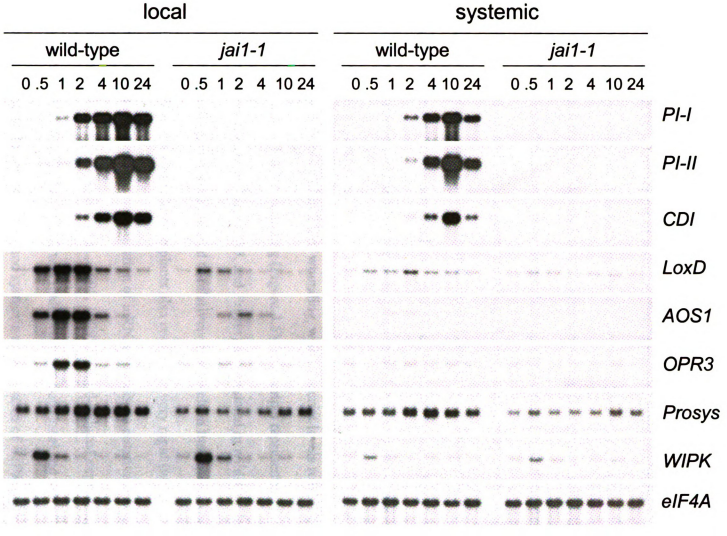
local
systemic
wild-type
jai1-1
wild-type
jai1-1
0 .5 1 2 4 10 24
0 .5 1 2 4 10 24
0 .5 1 2 4 10 24
0 .5 1 2 4 10 24
PI-I
PI-II
CDI
LoxD
AOS1
OPR3
Prosys
WIPK
eIF4A

Figure 3.5

Expression of wound response genes in jai1-1 plants

Recent studies indicate that wound responsive genes in tomato can be divided into at least two classes that differ with respect to their temporal and spatial pattern of induction (Ryan, 2000). Transcripts of so-called 'late' response genes, including defensive PIs, begin to accumulate both locally and systemically about 2 h after wounding, and reach maximal levels 8 to 12 h after wounding. By contrast, mRNAs transcribed from 'early' response genes accumulate rapidly (within 1 h) and transiently in response to wounding, and include genes encoding JA biosynthetic enzymes as well as other signaling components. To investigate the role of *Jai1* in wound-induced gene expression, we determined the temporal and spatial (i.e., local and systemic) expression pattern of representative early and late response genes in wild-type and *jai1-1* plants. Local and systemic expression of the late response genes (*PI-I*, *PI-II* and *CDI*) was detected in wild-type plants within 2 h of wounding, with transcript levels reaching maximal levels about 10 h after wounding (Figure 3.5). Transcripts representing four early response genes (*LoxD*, *AOS1*, *OPR3*, and *Prosys*) accumulated in wild-type leaves 2 to 4 h after wounding, and were induced to relatively low levels in the unwounded systemic leaves. Wounding of wild-type plants also resulted in very rapid (within 0.5 h) and transient local and systemic accumulation of mRNA encoding a putative mitogen-activated protein kinase (WIPK; Seo et al., 1995, 1999). Wound-induced expression of late response genes (*PI-I*, *PI-II* and *CDI*) was undetectable in both local and systemic leaves of *jai1-1* plants. In contrast, local and systemic accumulation of *WIPK* mRNA was not affected in the mutant, indicating that wound induction of this gene does not require JA signaling. Wound- induced expression of other early response transcripts (*LoxD*,

Table 3.2. Response of wild-type and *jai1-1* plants to PI-inducing factors.

Elicitors	PI-II (μg/ml juice)[a]	
	Wild-type	*jai1-1*
Buffer[b]	14 ± 10	ND
Systemin	124 ± 24	4 ± 11
OPDA	166 ± 21	ND
Bestatin	126 ± 14	ND
Glucose	74 ± 12	ND
Glucose + oxidase	145 ± 35	ND

a Fifteen-day-old wild-type and *jai1-1* seedlings were tested for the accumulation of PI-II in leaves using a radial immuno-diffusion assay. ND, not detectable. The detection limit of the assay is ~ 5μg/ml leaf juice.

b Seedlings were supplied through their cut stems with a buffer control (15 mM sodium phosphate, pH 7.0), or with elicitors dissolved in the buffer: systemin (5 pmol per plant), OPDA (10 nmol per plant), bestatin (50 nmol per plant), glucose (50 μM), and glucose (50 μM) plus glucose oxidase (0.01 U per plant). Data represent the mean ± SD of six plants.

AOS1, *OPR3*, and *Prosys*) was also evident in *jai1-1*, although transcript levels for these genes were reduced to approximately 20% of wild-type levels. These results indicate that the JA signaling pathway defined by *jai1* is essential for wound-induced expression of defensive *PI* and other late response genes. However, this pathway appears to function together with a JA-independent wound signaling pathway to control the expression of early wound response genes.

Responses of jai1-1 plants to PI-inducing compounds

Two general classes of PI-inducing compounds that act at different points in the jasmonate signaling pathway have been described. One class of elicitors functions either to activate the octadecanoid pathway leading to JA biosynthesis (e.g., systemin) or as metabolic precursors of JA (e.g., 12-OPDA). Because *jai1-1* plants are insensitive to JA, we hypothesized that these compounds would not induce PI expression in the mutant. Indeed, measurement of PI-II protein accumulation in response to exogenous systemin and 12-OPDA confirmed this prediction (Table 3.2). A second class of compounds induces *PI* expression by acting downstream of JA. Included within this group are the aminopeptidase inhibitor, bestatin, and the reactive oxygen species, H_2O_2 (Schaller et al., 1995; Orozco-Cárdenas et al., 2001). We found that neither bestatin nor a glucose/glucose oxidase mixture capable of generating H_2O_2 induced PI-II accumulation in *jai1-1* plants (Table 3.2). These results indicate *PI* expression in response to bestatin and H_2O_2 requires the action of *Jai1*.

jai1-1 plants are compromised in resistance to herbivores

Previous studies have established that the octadecanoid pathway for JA biosynthesis plays an important role in defense of cultivated tomato against a broad spectrum of arthropod herbivores (Howe et al., 1996; Li et al., 2002a). To determine whether JA signaling is required for anti-herbivore defense responses, wild-type and *jai1-1* plants were challenged with *Manducta sexta* (tobacco hornworm) larvae for five days. Hornworm feeding on wild-type plants resulted in PI-II accumulation in damaged (176 ± 17 μg/ml, n=9 leaves) and undamaged (205 ± 16 μg/ml, n=9 leaves) leaves. In contrast, very low levels (< 20 μg/ml) of PI-II were detected in damaged *jai1-1* leaves. RNA blot analysis of the same tissues showed that transcript levels for several defense-related genes were induced by hornworm attack both locally and systemically in wild-type but not in *jai1-1* plants (Figure 3.6). In addition to these effects on host gene expression, we also found that *jai1-1* plants were defoliated much faster than wild-type plants. The average weight of larvae fed on *jai1-1* plants (71.9 ± 25 mg, n=35 larvae) was approximately twice that of larvae fed on wild-type plants (44.5 ± 4 mg, n=30 larvae) for the same period of time. These results indicate that foliage from *jai1-1* plants is a better food source for hornworm larvae.

Figure 3.6. Accumulation of defense-related transcripts in response to tobacco hornworm attack. Newly hatched hornworm larvae were allowed to feed on wild-type (cv Micro-Tom) and *jai1-1* plants for 5 days. Total RNA was then isolated separately from hornworm damaged (L) and intact (S) leaves of the same plant. RNA from leaf tissue of the same plants immediately before the trial was also prepared as a control (C). cDNA probes used are indicated on the right. *eIF4A* was used as loading control.

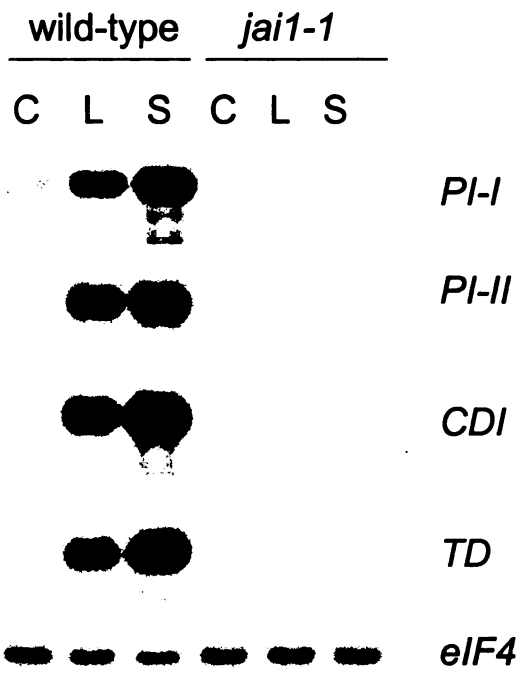

Figure 3.6

Discussion

Forward genetic analysis of the jasmonate signaling pathway

Forward genetic approaches have been instrumental in dissecting the jasmonate signaling pathway in Arabidopsis. Various genetic screens for jasmonate responsive mutants have revealed a number of loci that play important roles in the jasmonate signal transduction pathway (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1996; Ellis and Turner, 2001; Hilpert et al., 2001; Xu et al., 2001). Cloning the corresponding genes and subsequent biochemical analysis of the gene products have begun to yield valuable insight into the mechanism of jasmonate signal transduction and the interaction of jasmonate signaling with other cellular processes (Xie et al., 1998; Ellis et al., 2002; Staswick et al., 2002).

Whereas additional genetic screens in Arabidopsis are likely to further our understanding of the jasmonate response pathway, forward genetics in tomato constitutes another powerful approach expand to our knowledge in this important field of research. The rational lies in the fact that tomato, the most advanced model for species bearing a fleshy berry type of fruit, has a well studied and distinctive wound repose pathway. The best characterized wound response genes in tomato, including PIs and PPO, are absent in Arabidopsis (van der Hoeven et al., 2002). The systemin pathway that is essential for the systemic wound responses in tomato can not be detected in Arabidopsis. Therefore, studies aimed at elucidating the jasmonate signaling pathway in tomato promise to identify new components of the pathway and to complement our knowledge of the functions of genes already identified.

Tomato is well suited for forward genetics thanks to recent technical and genomic advances. Adoption of the determinate miniature *L. esculentum* cultivar Micro-Tom provided further benefits for analysis of the wound responses. Meissner et al. (1997) reported that Micro-Tom has several attributes that make it ideal for genetic analysis. Included among these are a short life cycle, growth of populations at high density, and amenability to high frequency of transformation by *Agrobacterium*. We have found that Micro-Tom is well suited for the study of wound- and herbivore- induced responses. For example, brief (<10 min) feeding by a single tobacco hornworm on a lower leaf triggered a >50-fold increase in *PI-II* mRNA in upper undamaged leaves of the plant (Howe et al., 2000). In the current research, we report the isolation and characterization of a tomato loss of function mutant, *jai1-1*, from a fast-neutron mutagenized population of Micro-Tom plants.

Jai1 is a positive regulator of jasmonate responses

The *jai1-1* mutant was isolated in a screen for plants that fail to accumulate MeJA-induced PPO and PI-II in leaves (Figure 3.1.a). Further characterization of *jai1-1* plants indicated that the mutation defines a positive regulator of jasmonate signaling in most if not all tomato tissues. This conclusion was first evident from expression profiling of approximately 500 genes contained on a custom DNA microarray. In wild-type leaves, MeJA treatment increased the mRNA level of 40 genes (Table 3.1). MeJA induction of 40 genes was abolished by the *jai1-1* mutation, suggesting that expression of most if not all JA-responsive genes require *Jai1*. Furthermore, wound- and herbivore-induced defense gene activation was also abrogated in *jai1-1* leaves, which correlated with

enhanced susceptibility of *jai1-1* leaves to insect attack (Figure 3.5, 3.6). In germinating seedlings, *Jai1* is required for MeJA-mediated root growth and hypocotyl elongation inhibition, and anthocyanin accumulation (Figure 3.1c).

With respect to reproductive development, the timing of flower and fruit onset in *jai1-1* plants appears normal. Nevertheless, *jai1-1* plants were sterile and only produced nonviable seed with arrested embryos (Li et al., 2001; McCaig B and Howe GA, unpublished data). We found that pollen grains from *jai1-1* plants have reduced viability and *in vitro* germination. Nevertheless, a small portion of *jai1-1* pollen (~2% of total pollen) appears to germinate and grow normally (Figure 3.2g, h). This finding is consistent with the observation that pollen collected from *jai1-1* plants is capable of inducing normal fruit and seed set when manually applied to emasculated wild-type pistils (Li et al., 2001). These results indicate that while reduced pollen fitness might contribute to the sterility of *jai1-1* plants, the jasmonate signaling pathway is not strictly required for pollen development in tomato.

Reciprocal crosses between wild-type and *jai1-1* plants clearly showed that the sterility of the mutant lies in female reproductive development (Li et al., 2001). A proposed role for jasmonate signaling in female reproductive development in tomato contrasts the well-documented studies in Arabidopsis where JA biosynthesis and perception are essential for male, but not female, gametophyte development (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001). A role for jasmonate in female reproduction in tomato is consistent with several observations. In a number of plant species including tomato, pistil and ovary as well as developing embryo contain relatively high levels of jasmonates (Wilen et al.,

1991; Creelman and Mullet, 1997; Hause et al., 2000). Also, many JA biosynthetic genes have been found to be highly expressed in female flower organs (Hause et al., 2000; Sanders et al., 2000; Wasternack and Hause, 2002). In addition, a number of jasmonate-inducible genes in leaves are highly expressed in female reproductive tissues (Peña-Cortés et al., 1991; Kim et al., 1998; Chao et al., 1999). We found that the constitutive expression of defense genes in reproductive organs was abolished in *jai1-1* flowers (Figure 3.4). While it is conceivable that these proteins might play defensive roles, they might also serve as storage proteins or be involved in supply of nutrients from maternal tissues to the developing embryos. Further characterization of *jai1* mutants with regard to pistil- or ovary-specific gene expression will likely provide clues for how jasmonate signaling is coupled to female fertility in tomato.

Jai1-dependent and -independent wound-signaling pathways in tomato

Based on their genetic requirement for *Jai1*, the wound response genes in tomato can be classified into at least three groups, i.e. the late wound response genes that encode defensive proteins, the early response genes that include JA biosynthetic genes, and the third group of genes that rapidly respond to wounding in a jasmonate-independent manner (Figure 3.5). The late response genes were strongly induced by wounding both locally and systemically in wild-type plants, with maximal induction at about 10 h after wounding (Figure 3.5). The activation of the late response genes was completely abolished in *jai1-1* plants, indicating a requirement for JA signaling in the activation of these genes. Furthermore, the induction of late response genes by several chemical elicitors was also completely abolished by the *jai1-1* mutation (Table 3.2), indicating that

the signaling events mediated by these elicitors converge at *Jai1* to orchestrate induction of the late response genes. Expression of the early response genes was rapidly induced by wounding in wounded leaves of wild-type plants though systemic induction of these genes was rather weak (Figure 3.5). In *jai1-1* plants, wound induction of these genes was much reduced compared to wild-type plants (Figure 3.5), suggesting that the jasmonate pathway functions together with a JA-independent wound signaling pathway to control the expression of early wound response genes. This finding is in keeping with the previous observation that activation of early response genes was only partially dependent on JA synthesis (Howe et al., 2000). The third group of genes, (e.g. *WIPK*), was very rapidly (< 0.5 h) induced by mechanical wounding both locally and systemically in wild-type as well as *jai1-1* plants. The apparent jasmonate perception-independent expression of *WIPK* indicates the existence of yet another jasmonate-independent signaling pathway in mediating the wound responses in tomato.

Although jasmonates play a central role in the wound response, jasmonate-independent wound responses have previously been observed in plant species such as tomato (O'Donnell et al., 1998; Howe et al., 2000) and Arabidopsis (Rojo et al., 1998; León et al., 2001; LeBrasseur et al., 2002). Based on the differential induction kinetics of the wound responsive genes, our results support a scenario in which jasmonate-independent wound signaling pathways could be responsible for the induction of *WIPK* (Figure 3.5) and other genes, which might lead to increased jasmonate levels (Seo, et al., 1995; 1999). The jasmonate-independent pathways may function together with the *Jai1*-mediated jasmonate signaling pathway to activate the expression of early wound response genes, which might contribute to further mobilization of the jasmonate pools (Ryan,

2000). These early signaling events might ultimately lead to the induction of the late response genes whose expression requires jasmonate perception (Figure 3.5). A variety of signal molecules have been implicated in wound signaling. Included among them are physical signals such as electrical pulses (Wildon et al., 1992) and hydraulic waves (Malone and Alarcón, 1995), and chemical signals such as systemin (Pearce et al., 1991), ABA (Peña-Cortés et al., 1991), and ethylene (O'Donnell *et al.*, 1996). Although the signaling pathways mediated by these signals are important for optimal wound responses, none has been explicitly demonstrated as the primary wound signal. The interaction between these and the jasmonate signaling pathways is also need to be further elucidated. Isolation and characterization of the *jai1-1* mutant, therefore, offers an opportunity to gain greater insight into the genetic complexity of the wound response pathways and the mechanism by which different signaling pathways interact to regulate wound responsive genes in plants.

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CHAPTER 4

Genetic Analysis of Wound Signaling in Tomato: Evidence for a Dual Role of Jasmonic Acid in Defense and Female Fertility

The work presented in this chapter has been published:

Li L, Li CY, Howe GA (2001) Plant Physiol. 127, 1414–1417.

The experiment described in Table 4.1 was done in collaboration with Chuanyou Li.

Abstract

Tomato provides an attractive system in which to study jasmonate biosynthesis and signaling in regulating plant defense against herbivores and other processes. To genetically dissect the jasmonate pathway, several screens have been conducted in tomato for mutants that block wound-, prosystemin-, or MeJA-induced expression of downstream target genes (Lightner et al., 1993; Howe and Ryan, 1999; Chapter 3). Included among these mutants are *jai1-1* and 124A (*jai1-2*) that are defective in MeJA-induced responses. In the current chapter, we demonstrated that the two jasmonate signaling mutants are genetically allelic. Therefore, the *jai1* mutations define a single locus required for jasmonate signaling in tomato. Furthermore, reciprocal crosses to wild-type plants revealed that *jai1* plants are female sterile, indicating a role for jasmonate signaling in female reproductive development in tomato.

Introduction

Genetic analysis of the wound response pathway in tomato (*Lycopersicon esculentum*) indicates that prosystemin and systemin are upstream components of a defensive signaling cascade that involves complex regulation of jasmonic acid (JA) biosynthesis and the ability of cells to perceive and respond to JA. Recent identification of JA response mutants provides evidence for the hypothesis that the JA transduction pathway also plays an important role in female reproductive development.

Many plants respond to insect attack and wounding by synthesizing an array of phytochemicals that decrease the ability of herbivores to colonize, feed, or reproduce on the plant (Green and Ryan, 1972; Karban and Baldwin, 1997). Wound-inducible proteinase inhibitors (PIs) provide an attractive model system in which to study the signal transduction pathways that regulate this form of defense. In tomato, damage to a single leaflet by mechanical wounding or herbivory results in localized and systemic expression of two Ser PI-encoding genes (*PI-I* and *PI-II*) within about 2 h (Ryan, 2000; Howe et al., 2000). These proteins can accumulate to high levels in leaves of the damaged plant, where they play a defensive role by inhibiting digestive proteases of some lepidopteran insects. In their pioneering study of wound-inducible PIs 30 years ago, Green and Ryan (1972) proposed that chemical signals generated at the site of wounding traverse the vascular system to activate the systemic expression of PIs. Although many of the signals involved in this response have been identified, relatively little is known about the mechanisms by which they are produced and transported between cells.

A unique component of the wound response pathway in tomato is the peptide signal systemin and the precursor protein prosystemin, from which it is derived (Pearce et al., 1991; McGurl et al., 1992). Tomato prosystemin is encoded by a single gene whose primary transcript is alternatively spliced to generate two active forms of the protein (Li and Howe, 2001). Several lines of genetic evidence indicate that prosystemin is essential for wound-induced expression of *PI* and other defense-related genes. First, transgenic plants expressing an antisense prosystemin cDNA are deficient in wound-induced systemic expression of *PI* genes (McGurl et al., 1992). Second, overexpression of prosystemin from a *35S::prosys* transgene constitutively activates *PI* expression in unwounded plants (McGurl et al., 1994). Third, mutations that suppress *35S::prosys*-mediated signaling block wound induction of *PI*s (Howe and Ryan, 1999). It has been proposed that systemin functions as a mobile wound signal following its proteolytic release from prosystemin (McGurl et al., 1992). Expression of *PI* genes in tomato leaves in response to wounding and systemin is mediated by JA, a terminal product of the octadecanoid pathway (Farmer and Ryan, 1992; Creelman and Mullet, 1997). This model has been refined to reflect the fact that wound- and systemin-induced expression of PIs involves synergism between JA and ethylene (O'Donnell et al., 1996). Recent studies provide evidence that reactive oxygen species function downstream of JA to amplify wound- and systemin-induced responses (Orozco-Cárdenas et al., 2001). Due to space limitations, the reader is referred to recent reviews for a detailed discussion of the wound-signaling pathway (Bowles, 1998; Ryan, 2000; Walling, 2000; León et al., 2001).

Genetic Analysis of Wound Signaling

We are using tomato as a model system for genetic dissection of signaling pathways that regulate wound responses and, more broadly, defense against herbivores. To further define the function of prosystemin and systemin in the wound response, we conducted a screen for mutations that suppress *35S::prosys*-mediated expression of downstream target genes (Howe and Ryan, 1999). We identified 13 independent mutants, designated *spr* (*s*uppressed in *p*rosystemin-mediated *r*esponses). Eight mutants define four genetic complementation groups called *Spr1*, *2*, *3*, and *4*. Two mutants define new alleles of *def1*, a JA-deficient mutant that is compromised in wound-inducible *PI* expression and resistance to *Manduca sexta* larvae (Lightner et al., 1993; Howe et al., 1996). The three remaining mutants were sterile and thus were not further characterized in the initial study. Mutations in *Def1*, *Spr1*, and *Spr2*, in addition to suppressing the action of *35S::prosys*, impair wound- and systemin-induced *PI* expression. This finding provides strong genetic evidence that prosystemin is an essential upstream component in the wound response pathway. The ability of *def1*, *spr1*, and *spr2* plants to respond to exogenous JA suggests that these mutations affect processes required for JA biosynthesis or accumulation (Figure 4.1). Support for this interpretation comes from the finding that *def1* plants are deficient in JA accumulation in response to wounding and systemin (Howe et al., 1996).

Identification of mutants that are impaired in JA perception would provide a valuable tool to further elucidate the mechanism of wound signaling and its relationship

Figure 4.1. Proposed action of mutations in the wound response pathway. The signaling pathway depicted is consistent with the model proposed by Farmer and Ryan (1992). All mutants listed are deficient in wound-inducible systemic expression of *PI*s and also lack *PI* expression in response to systemin and *35S::prosystemin*. *def1*, *spr1*, *spr2*, and *spr5* plants are responsive to applied MeJA and JA, whereas *jai1* plants are insensitive to these signals. See text for details.

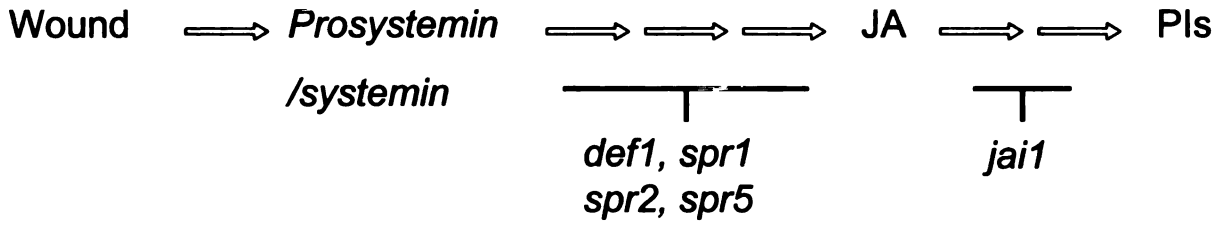

Figure 4.1

to induced defense. Toward this goal, we screened a fast-neutron-mutagenized population of tomato (cv Micro-Tom) for plants that fail to express polyphenol oxidase and PI-II upon exposure to gaseous methyl-JA (MeJA; Li et al., 2001). One mutant, designated *jasmonic acid-insensitive 1-1* (*jai1-1*), completely lacked these proteins upon treatment with MeJA or wounding. *jai1-1* plants displayed normal vegetative growth but produced fruit that lacked mature seed. In over 1,000 *jai1-1* fruit examined, only two viable seeds were recovered. Reciprocal crosses to wild-type showed that *jai1-1* is female-sterile; it failed to set seed following pollination with wild-type pollen but readily pollinated and fertilized wild-type pistils. F_1 plants derived from this cross were fully responsive to JA/MeJA and fertile. In a segregating population (108 F_2 plants), the JA-insensitive and sterile phenotypes always co-segregated as if conditioned by a single recessive mutation.

The female-sterile phenotype of *jai1-1* plants prompted us to investigate the sterile *spr* lines that were previously generated by ethyl methane sulfonate-mutagenesis (see above; Howe and Ryan, 1999). Attention was focused on two lines, 124A and 436G, that developed flowers but produced either no fruit or fruit containing no viable seed. Reciprocal backcrosses to wild-type indicated that both lines were female-sterile. Analysis of F_2 populations derived from these crosses showed that one-quarter of the progeny lacked wound-inducible PI-II expression both in the wounded leaf and the undamaged systemic leaf (Table 4.1). Wound-insensitive 436G plants accumulated normal levels of PI-II in response to exogenous MeJA (Table 4.1), similar to the phenotype of *def1*, *spr1*, and *spr2* plants (Figure 4.1). Complementation tests showed

Table 4.1. Proteinase inhibitor II accumulation in response to wounding and MeJA.

Values indicate the mean ± SD of PI-II levels (μg/ml leaf juice) in leaf tissue of wild-type and two mutant lines that are suppressed in *35S::prosys*-mediated signaling.

Genotype[a]	Unwounded	Local	Systemic	MeJA[b]
Wild-type	6 ± 5	77 ± 5	72 ± 4	140 ± 7
436G (*spr5*)	5 ± 5	3 ± 5	ND	135 ± 11
124A (*jai1-2*)	ND	ND	ND	ND

a F_2 populations segregating for *spr-5* (line 436G) or *jai1-2* (line 124A) were wounded with a hemostat on the lower leaf, and PI-II levels were measured 24 h later in both the wounded leaf (Local) and the distal undamaged leaf (Systemic). The ratio of wound-responsive to wound-insensitive plants was 59:21 for *spr5* and 402:118 for *jai1-2* (χ^2 = 0.067 and 1.477, respectively, for the 3:1 hypothesis). Data are shown for wound-insensitive F_2 plants and unwounded control plants grown in the same flat. ND, not detectable.

b *spr5* and *jai1-2* plants were selected by screening the corresponding segregating F_2 population for seedlings (11-d-old) that do not accumulate PI-II in wounded cotyledons. Eighteen-day-old selected plants were treated with MeJA as previously reported (Li and Howe, 2001), and PI-II levels were measured in leaves 24 h later.

Table 4.2. Genetic complementation tests between *jai1-1* and 124A.

Male Parent	Female Parent	JA sensitive[a]	JA insensitive[a]	χ^{2} [b]
124A	*Jai1-1/jai1-1*	26	29	0.16 (1:1)
jai1-1/jai1-1	WT × 124A (F_1)[c]	48	59	1.13 (1:1)
Jai1-1/jai1-1	WT × 124A (F_1)[c]	93	27	0.40 (3:1)

a Progeny from the indicated crosses were tested for responsiveness to applied MeJA as described in Table 4.1, using both PI-II and polyphenol oxidase as JA-responsive markers. The number of progeny displaying a JA-sensitive or JA-insensitive phenotype is shown.

b χ^{2} values were calculated for the expectation that *jai1-1* and the mutation in 124A are allelic, where the predicted segregation ratio is indicated in parenthesis (JA sensitive:JA insensitive). If *jai1-1* and the mutation in 124A were non-allelic, all progeny from each cross would be JA sensitive.

c A line heterozygous for the mutation in 124A was produced by crossing 124A as a staminate parent to wild-type (WT).

that the recessive mutation harbored by 436G defines a novel locus, designated *Spr5*. Backcrossed lines that are homozygous for *spr5* produced viable seed, albeit at reduced levels relative to wild-type. This finding indicates that the sterile phenotype of 436G can be attributed in part to a mutation other than *spr5*.

In contrast to *spr5*, MeJA-treated 124A plants expressed no detectable PI-II (Table 4.1) or polyphenol oxidase (data not shown). This indicates that 124A, like *jai1-1*, is blocked in JA perception or the ability to respond appropriately to the hormone. Complementation tests between 124A and *jai-1* were performed using heterozygous maternal parents, and the results showed that the two mutants define the same locus (Table 4.2). We henceforth refer to the fast-neutron allele of *jai1* as *jai1-1* and the EMS allele of 124A as *jai1-2*. Plants homozygous for *jai1-2* were also unresponsive to relatively high concentrations of JA (50 nmol/plant) and systemin (5 pmol/plant) supplied through the cut stem (Figure 4.2). Recovery of a JA-insensitive mutant in a screen for suppressors of *35S::prosys* is significant because it demonstrates that *35S::prosys*-mediated signaling requires a functional JA response pathway. The unresponsiveness of *jai1-1* plants to wounding and applied systemin likewise indicates that JA action is essential for wound- and systemin-induced *PI* expression (Figure 4.1).

Figure 4.2. Proteinase inhibitor II levels in wild-type and *jai1-2* plants in response to JA and systemin. Fifteen-day-old seedlings were supplied with buffer (B; 15 mM sodium phosphate, pH 7.0), JA (J; 50 nmol/plant), or systemin (S; 5 pmol/plant) through the cut stem. PI-II levels were measured in the leaves 24 h later. Plants homozygous for *jai1-2* were selected as described in Table 4.1. Values represent the mean ± SD of at least six plants.

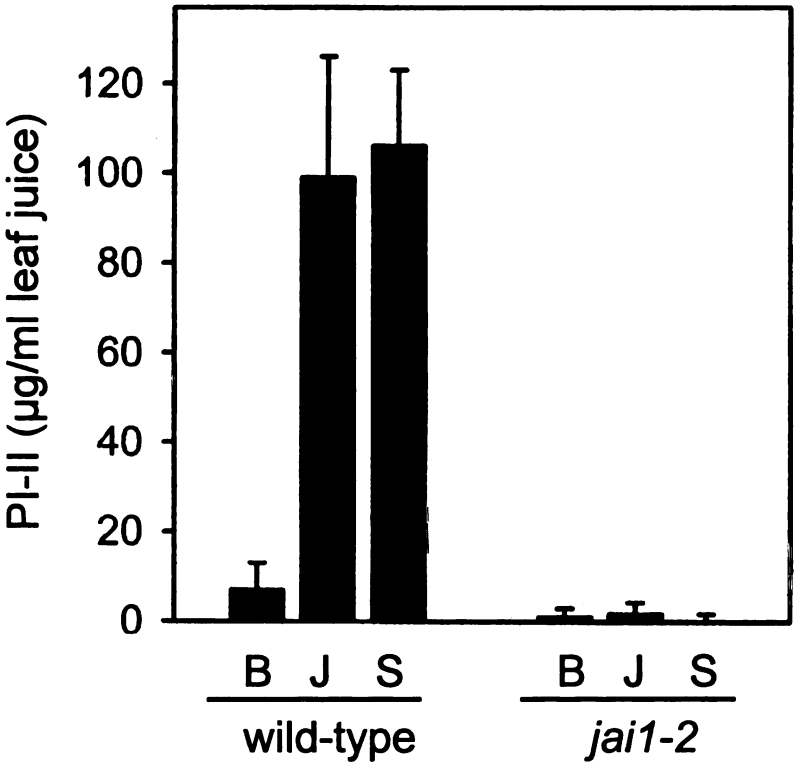
PI-II (µg/ml leaf juice)
120
100
80
60
40
20
0
B J S
B J S
wild-type
jai1-2

Figure 4.2

A Role for JA in Female Reproductive Development?

The JA insensitivity and female sterility of independent mutants (*jai1-1* and *jai1-2*) argues against the possibility that the two phenotypes result from mutations in different genes. Rather, our results support the hypothesis that a single gene, *Jai1*, is required both for wound-induced expression of defensive genes and female reproductive development. Conclusive proof will require cloning of *Jai1* and functional complementation of the mutant. The sterility of *jai1-1* plants could result from a defect in ovule development, embryogenesis, or another maternal process required for seed production. A dysfunction in embryogenesis would be consistent with previous studies implicating JA as an endogenous regulator of embryo development in oilseeds (Wilen et al., 1991). A similar situation could exist in tomato, where JA, its precursor 12-oxo-phytodienoic acid, and various amino acid conjugates of JA are abundant in female organs of the flower (Hause et al., 2000).

A proposed role for JA in female reproductive development in tomato stands in contrast to well-documented studies in Arabidopsis where JA biosynthesis and perception are essential for male, but not female, gametophyte development (McConn and Browse, 1996; Feys et al., 1994; Sanders et al., 2000; Stintzi and Browse, 2000). Although we cannot exclude a role for JA in male gametophyte development in tomato, the ability of *jai1-1* pollen to induce normal seed set when crossed to a wild-type pistillate parent indicates that JA perception and downstream signaling events are not essential for the production of viable pollen. How might such species-specific differences in jasmonate function be explained? One possibility is that oxylipins such as JA first evolved as low-

abundance signaling molecules for the regulation of stress responses (e.g. defense) in vegetative tissues and, subsequently, these compounds were recruited to perform other physiological functions (e.g. reproduction) in specific plant lineages. This scenario was previously suggested to account for species-specific differences in the requirement for flavonoids in male fertility (Burbulis et al., 1996). The range of physiological processes controlled by JA may ultimately reflect the function of specific genes whose expression is regulated by the hormone in a tissue- or cell type-specific manner. The wound response mutants described herein should provide useful tools to investigate the molecular mechanisms by which jasmonates regulate diverse physiological processes.

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CHAPTER 5

Distinct Roles for Jasmonate Synthesis and Action in the Systemic Wound Response of Tomato

The work presented in this chapter has been published:

Li L, Li C, Lee GI, Howe GA (2002) Proc. Natl. Acad. Sci. USA 99, 6416–6421.

Quantification of jasmonates was performed by Gyu In Lee. Experiements presented in Figure 5.2 were done with collaboration with Chuanyou Li.

Abstract

Plant defense responses to wounding and herbivore attack are regulated by signal transduction pathways that operate both at the site of wounding and in undamaged distal leaves. Genetic analysis in tomato indicates that systemin and its precursor protein, prosystemin, are upstream components of a wound-induced, intercellular signaling pathway that involves both the biosynthesis and action of jasmonic acid (JA). To examine the role of JA in systemic signaling, reciprocal grafting experiments were used to analyze wound-induced expression of the *proteinase inhibitor II* gene in a JA biosynthetic mutant (*spr2*) and a JA response mutant (*jai1*). The results showed that *spr2* plants are defective in the production, but not recognition, of a graft-transmissible wound signal. Conversely, *jai1* plants are compromised in the recognition of this signal, but not its production. It was also determined that a graft-transmissible signal produced in response to *35S::prosys* expression in rootstocks was recognized by *spr2* but not by *jai1* scions. Taken together, the results indicate that (pro)systemin-mediated activation of the jasmonate biosynthetic pathway in wounded leaves is required for the production of a long-distance signal whose recognition in distal leaves requires jasmonate signaling. These findings provide strong support for the hypothesis that jasmonate is an essential component of the transmissible wound signal.

Introduction

Higher plants respond to insect attack and wounding by activating the expression of genes involved in herbivore deterrence, wound healing, and other defense-related processes (Karban and Baldwin, 1997). An important aspect of many induced defense responses is their occurrence in undamaged leaves located distal to the site of attack (Green and Ryan, 1972). Wound-inducible proteinase inhibitors (PIs) in tomato (*Lycopersicon esculentum*), which are expressed within about 2 h after mechanical wounding or herbivory (Howe et al., 2000; Ryan, 2000), represent one of the best examples of this phenomenon. In their landmark study of wound-inducible PIs, Green and Ryan (1972) proposed that specific signals generated at the wound site travel through the plant and activate PI expression in undamaged responding leaves. Although several chemical and physical signals have since been implicated in the systemic wound response (reviewed in Ryan, 1992; Bowles, 1993; Malone, 1996; Ryan, 2000; Walling, 2000; León et al., 2001), very little is known about how these signals interact with one another to effect cell-to-cell communication over long distances.

Among the proposed intercellular signals for wound-induced *PI* gene expression are systemin, an 18-amino-acid peptide derived from proteolytic cleavage of a larger precursor protein called prosystemin (Pearce et al., 1991; McGurl et al., 1992), and jasmonate signals such as jasmonic acid (JA) and its methyl ester, methyl-JA (MeJA; Farmer and Ryan, 1990; Farmer et al., 1992). According to a recent model of wound signaling in tomato (Ryan, 2000), systemin is transported through the plant as a mobile signal following its proteolytic release from prosystemin. Interaction of systemin with a plasma membrane-bound receptor (Meindl et al., 1998; Scheer and Ryan, 1998) then

triggers a signaling cascade leading to activation of a lipase that releases linolenic acid from membrane lipids (Narváez-Vásquez et al., 1999; Ishiguro et al., 2001). Jasmonates are synthesized from linolenic acid via the octadecanoid pathway, and are considered to be key regulators for stress-induced gene expression in virtually all plants (Reymond et al., 2000; Schaller, 2001). Recent studies have shown that 12-oxo-phytodienoic acid (OPDA), a cyclopentenone precursor of JA/MeJA, is a signal for defense gene expression without its prior conversion to JA (Stintzi et al., 2001). Activation of *PI* expression in response to wounding, systemin, and jasmonates involves the coordinate biosynthesis and action of ethylene (Felix and Boller, 1995; O'Donnel et al., 1996), and is also associated with the production of reactive oxygen species that act downstream of JA (Orozco-Cárdenas et al., 2001).

Of relevance to the mechanism of wound-induced intercellular signaling is the observation that genes encoding prosystemin and some JA biosynthetic enzymes are expressed in vascular bundle cells, whereas defensive *PI* genes are expressed in adjacent palisade and spongy mesophyll cells (Shumway et al., 1970; Jacinto et al., 1997; Kubigsteltig et al., 1999; Hause et al., 2000). The cell-type-specific expression pattern of these signaling components has led to the hypothesis that wound-induced release of systemin into the vascular system activates JA biosynthesis in surrounding vascular tissues in which JA biosynthetic enzymes are located (Ryan, 2000). Active transfer or diffusion of a jasmonate signal from its site of synthesis could, in turn, induce *PI* expression in neighboring mesophyll cells. A role for jasmonates in intercellular signaling is supported by the fact that application of JA/MeJA to one leaf induces *PI* expression in distal untreated leaves (Farmer et al., 1992), and that exogenous JA is

readily transported in the phloem (Zhang and Baldwin, 1997). In addition, it has been demonstrated that cultured plant cells secrete JA into the medium (Parchmann et al., 1997). Recent studies suggest that the conversion of JA to MeJA by a specific JA carboxyl methyltransferase is an important regulatory step in jasmonate-mediated intercellular signaling (Seo et al., 2001).

We are using tomato as a model system for genetic analysis of systemic wound signaling and its role in plant defense. Toward this goal, plant genotypes defective in wound-induced systemic expression of *PI* and other defense-related genes have been identified in various genetic screens (Lightner et al., 1993; Howe and Ryan, 1999; Li et al., 2001). These mutants can be classified into two phenotypic groups: jasmonate biosynthesis mutants that are insensitive to systemin but responsive to JA/MeJA, and jasmonate response mutants that are insensitive to both systemin and JA/MeJA (Li et al., 2001). Here we report the use of grafting experiments to determine whether these mutants are defective in the production of a long-distance wound signal, or the recognition of that signal in distal undamaged leaves. The results reveal distinct roles for jasmonate biosynthesis and signaling in the generation and recognition, respectively, of a long-distance wound signal for activation of defense gene expression.

Materials and Methods

Plant material and treatments

Lycopersicon esculentum cv Castlemart was used as the wild-type variety for all experiments. Plants were grown and maintained as described previously (Howe et al., 2000). The original *spr2* and *jai1-2* mutants (Howe et al., 2000; Li et al., 2001) were backcrossed to wild-type, and homozygous mutants lacking the *35S::prosys* transgene were selected from the resulting F_2 population. Seed for the *spr2* mutant was collected from a *spr2/spr2* homozygote that had been back-crossed three times to wild-type. Because of the reduced fertility of *jai1-2* plants (Li et al., 2001), plants homozygous for *jai1-2* were obtained from a segregating F_2 population using the screening procedure described by Li *et al.* (2001). Seed for the *35S::prosys* transgenic line was collected from a *35S::prosys/35S::prosys* homozygote that had been back-crossed five times to wild-type. Wounding was performed as described in the figure legends. Treatment of plants with MeJA (Bedoukian Research) was performed as described previously (Li and Howe, 2001).

Grafting experiments

Four-week-old plants were grafted using a modification of the procedure described by McGurl *et al.* (1994). A longitudinal incision of approximately 1.5 cm was made in the middle of the rootstock stem. The scion stem was trimmed to the shape of a

wedge, and then tightly fastened to the cortex flaps of the rootstock using water-soaked raffia. All but one leaf immediately beneath the apical meristem were excised from the scion. Scions were enclosed in a plastic bag that was fastened at the graft junction. One week after grafting, the plastic bag and raffia were removed. Grafted plants contained two or three leaves on the rootstock and two newly emerging leaves on the scion. Four days after removal of the bag, plants were subjected to mechanical wounding as follows. All leaflets (approximately 10) on leaves of the stock were crushed with a hemostat across the midvein. This procedure was repeated 2 h later, such that the second wound was parallel to the first wound and proximal to the petiole. Eight hours after the second wound, wounded leaflets from the stock and undamaged leaflets of the scion were harvested separately for RNA isolation. Equal amounts of leaf tissue from three plants of the same graft combination were pooled prior to RNA isolation. RNA blot analysis of *PI-II* mRNA levels was performed as previously described (Li and Howe, 2001), using an *eIF4A* cDNA probe as a loading control. PI-II protein levels in grafted plants were measured by radial immunodiffusion assay (Ryan, 1967).

Quantification of jasmonic acid

Plants containing two fully expanded leaves and an emerging third leaf were wounded with a hemostat on each leaflet of the two expanded leaves. Leaves (10 g FW) were harvested for extraction and quantification of JA using a modification of the procedure described by Weber *et al.* (1997). Harvested leaves were frozen in liquid nitrogen and ground to a fine powder using a chilled mortar and pestle. The tissue was dissolved in 28 ml methanol containing 500 ng dihydrojasmonic acid (DHJA) as an

internal standard, and then homogenized with a Polytron for 1 min at 4°C. The homogenate was incubated for 2 h at 4°C with shaking, diluted with 12 ml ice-cold water, and then centrifuged at 3500 x *g*. The resulting supernatant was recovered and the pH adjusted to 8.0 with NH_4OH. This solution was passed through a tC_{18}-SepPak cartridge (Waters) preconditioned with 70% (v/v) methanol and collected in a new tube. The cartridge was washed with 7 ml 75% (v/v) methanol. Eluates from both the sample and the wash steps were combined and adjusted to pH 4.0 with 10% (v/v) formic acid. This solution was diluted with 160 ml ice-cold water and then loaded on a tC_{18}-SepPak column that was prewashed sequentially with methanol, diethylether, methanol, and water. After washing the column with 7 ml 15% (v/v) ethanol and 7 ml water, the JA fraction was eluted with 10 ml diethylether. The eluate was partially dried over anhydrous $MgSO_4$ and then dried completely under a stream of nitrogen gas. The dried paste was dissolved in 0.5 ml methanol and subjected to methylation by the addition of diazomethane in 0.5 ml diethylether. This mixture was dried under nitrogen gas and resuspended in 20 µl hexane.

The amount of JA/MeJA in leaf extracts was quantified by GC-MS using a Hewlett-Packard GC 5890 equipped with a Hewlett-Packard 5970 mass detector. The GC was fitted with a DB-5 column and run at 250 °C in the isothermal mode. GC-MS analysis was performed in the SIM mode with monitoring of ions specific for MeJA (m/z = 224) and MeDHJA (m/z = 226). For quantification of JA/MeJA, a standard curve was generated from samples in which MeJA and MeDHJA were mixed in known ratios. Because peaks corresponding to both the 3R,7S and 3R,7R isomers of endogenous

JA/MeJA were detected, the areas of the two peaks were combined. JA levels reported in the Results section represent the mean ± standard error of at least three independent experiments. DHJA was prepared by PtO_2-cataylzed hydrogenation of (±)-JA (Sigma) as previously described (Weber et al., 1997). The authenticity of the standard, as well as the absence of endogenous DHJA/MeDHJA in tomato leaf extracts, was verified by GC-MS.

Results

Mutations affecting either JA biosynthesis or JA signaling abolish wound-induced systemic expression of PI genes

Wound response mutants that are defective either in JA biosynthesis or JA responsiveness were used to study the role of JA in systemic wound signaling. The *spr2* and *jai1-2* mutations were previously identified as suppressors of defense-related responses that are constitutively activated in transgenic tomato plants that overexpress prosystemin from the *35S::prosys* transgene (Howe et al., 2000; Li et al., 2001). *spr2* plants lack wound-induced systemic expression of the well-characterized *PI-II* gene, but nevertheless respond normally to applied MeJA (Figure 5.1). This phenotype is very similar to that conditioned by *def1*, a non-allelic mutation that reduces wound-induced JA accumulation to about 30% of wild-type levels (Howe et al., 1996). To determine whether *spr2* plants are defective in JA synthesis, JA was extracted from wounded and control (unwounded) leaves of wild-type and *spr2* plants, and quantified using gas chromatography-mass spectrometry. The results showed that undamaged wild-type leaves contained 12 ± 1 pmol JA/g FW. In response to mechanical wounding, JA levels increased to 262 ± 41 and 151 ± 26 pmol JA/g FW 1 h and 3 h, respectively, after wounding. The JA level in unwounded *spr2* leaves was 3 ± 1 pmol JA/g FW, which rose to 22 ± 9 and 7 ± 1 pmol JA/g FW 1 h and 3 h, respectively, after wounding. This finding indicates that the wound response phenotype of *spr2* plants results from a defect in jasmonate biosynthesis.

Figure 5.1. Induction of the *proteinase inhibitor II* gene in response to wounding and MeJA. Two-leaf stage wild-type (WT) and mutant (*spr2* and *jai1-2*) tomato plants were wounded once with a hemostat across the midvein of the lower leaf. Eight hours later, leaf tissue was harvested separately from the wounded leaf (L; Local response) and the upper undamaged leaf (S; systemic response) for RNA isolation. RNA was also isolated from a set of plants treated for 12 h with MeJA (J), and a set of untreated control plants (C). Five-μg aliquots of total RNA were analyzed by RNA blot analysis for *PI-II* mRNA levels. As a loading control, a duplicate blot was probed with a cDNA encoding *eIF4A*.

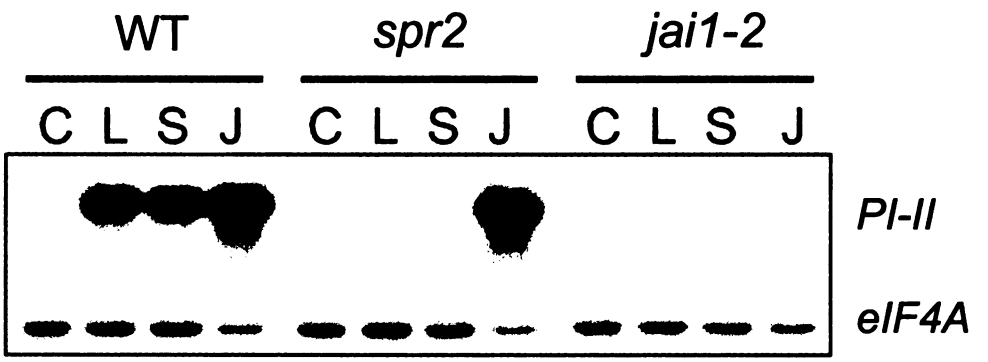

Figure 5.1

Wounded *jai1-2* plants accumulate *PI-II* mRNA to < 5% of the levels observed in wild-type (Figure 5.1). The residual wound-induced expression of *PI-II* in this mutant likely results from partial activity of the *jai1-2* allele, as we have observed that plants homozygous for a deletion allele of *jai1* (*jai1-1*) accumulate no detectable *PI-II* transcripts in response to wounding (Figure 3.5). In contrast to JA biosynthetic mutants, *jai1-2* plants fail to accumulate *PI-II* transcripts in response to exogenous JA/MeJA (Figure 5.1; Li et al., 2001). This phenotype is indicative of a defect in jasmonate perception or subsequent signaling events that are necessary for *PI* activation.

Jasmonate signaling is required for functional recognition of a long-distance wound signal

To examine the role of jasmonate action in the systemic wound response, we analyzed wound-induced *PI-II* expression in grafts between wild-type and *jai1-2* plants. Four-week-old plants were grafted such that both the rootstock (stock) and the scion contained at least two healthy leaves (Figure 5.2). Following sufficient time for healing of the graft junction, stock leaves were wounded and *PI-II* mRNA levels were measured 11 h later in both the damaged stock leaves (local response) and the undamaged scion leaves (systemic response). Control experiments showed that the grafting procedure itself induced some *PI-II* expression in wild-type stock and scion leaves (Figure 5.3a, lane 1; data not shown). However, subsequent wounding of stock leaves induced local and systemic *PI-II* expression well above this background level (Figure 5.3a, lane 2). This experiment demonstrates that wounding of wild-type stock leaves leads to the production

Figure 5.2. Photograph of a typical grafted tomato plant. The arrow indicates the position of the graft junction between the stock and scion. Systemic *PI-II* expression was measured in undamaged scion leaves 11 hr after wounding of the stock leaves. The distance between wounded leaflets on the stock and undamaged leaflets on the scion is approximately 20 cm. Images in this figure are presented in color.

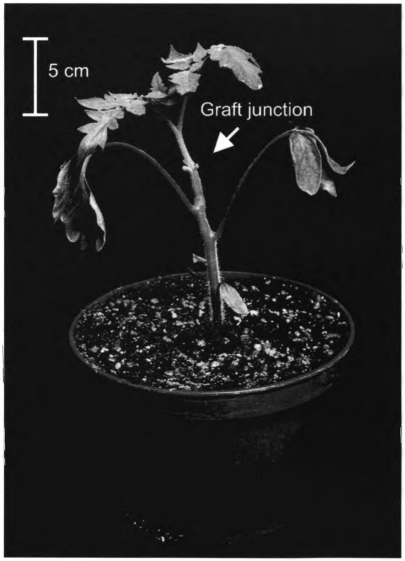

Figure 5.2

of a graft-transmissible signal that is recognized in undamaged scion tissues. Consistent with the wound response observed in *jai1-2* seedlings (Figure 5.1), wound-induced expression of *PI-II* in both stock and scion leaves of grafted *jai1* plants was < 5% of that in wild-type plants (Figure 5.3a, lanes 3-4). Analysis of *jai-1*/wild-type hybrid grafts showed that wounding of *jai1* stock leaves resulted in full activation of *PI-II* expression in wild-type scion leaves (Figure 5.3a, lanes 5-6). In the reciprocal combination, however, undamaged mutant scion leaves failed to express *PI-II* in response to wounding of the wild-type stock (Figure 5.3a, lanes 7-8). These results demonstrate that *jai1* does not affect the production of the graft-transmissible systemic signal at the site of wounding, but rather disrupts the recognition or proper interpretation of that signal in distal undamaged leaves.

Jasmonate biosynthesis is required for generation of a long-distance wound signal

Wound-induced *PI-II* expression in graft combinations between wild-type and *spr2* plants was examined to investigate the role of jasmonate biosynthesis in the systemic wound response. In contrast to the results obtained with *jai1* plants, wild-type scions responded very weakly to wounding of *spr2* stock leaves (Figure 5.3b, lanes 5-6). Moreover, *spr2* scions exhibited a strong response to a signal emanating from wounded leaves of WT stock (Figure 5.3b, lanes 7-8). Analysis of grafts between wild-type plants and the *def1* mutant, which is also deficient in JA biosynthesis (Howe et al., 1996), gave results that were similar to those for *spr2*/wild-type hybrid grafts (data not shown). These results indicate that JA, or a

Figure 5.3. Wound-inducible *PI-II* expression in grafts between wild-type plants and mutants defective in jasmonate signaling (*jai1*) or jasmonate biosynthesis (*spr2*). Wild-type (WT) and *jai1-2* plants (a) or WT and *spr2* plants (b) were grafted in the four combinations indicated. The genotypes listed above and below the horizontal line correspond to the scion and stock, respectively. For each graft combination, plants were divided into a control (C) and experimental (W) group consisting of three grafted plants per group. For the experimental group, each leaflet on the stock was wounded as described in the Experimental procedures. Eleven hours after wounding, leaf tissue was harvested separately from wounded stock leaves (stock) and undamaged scion leaves (scion) for RNA extraction. The control set of plants received no wounding, other than that inflicted by the grafting procedure itself. Levels of *PI-II* mRNA were analyzed by RNA blot analysis, using an *eIF4A* cDNA probe as a loading control.

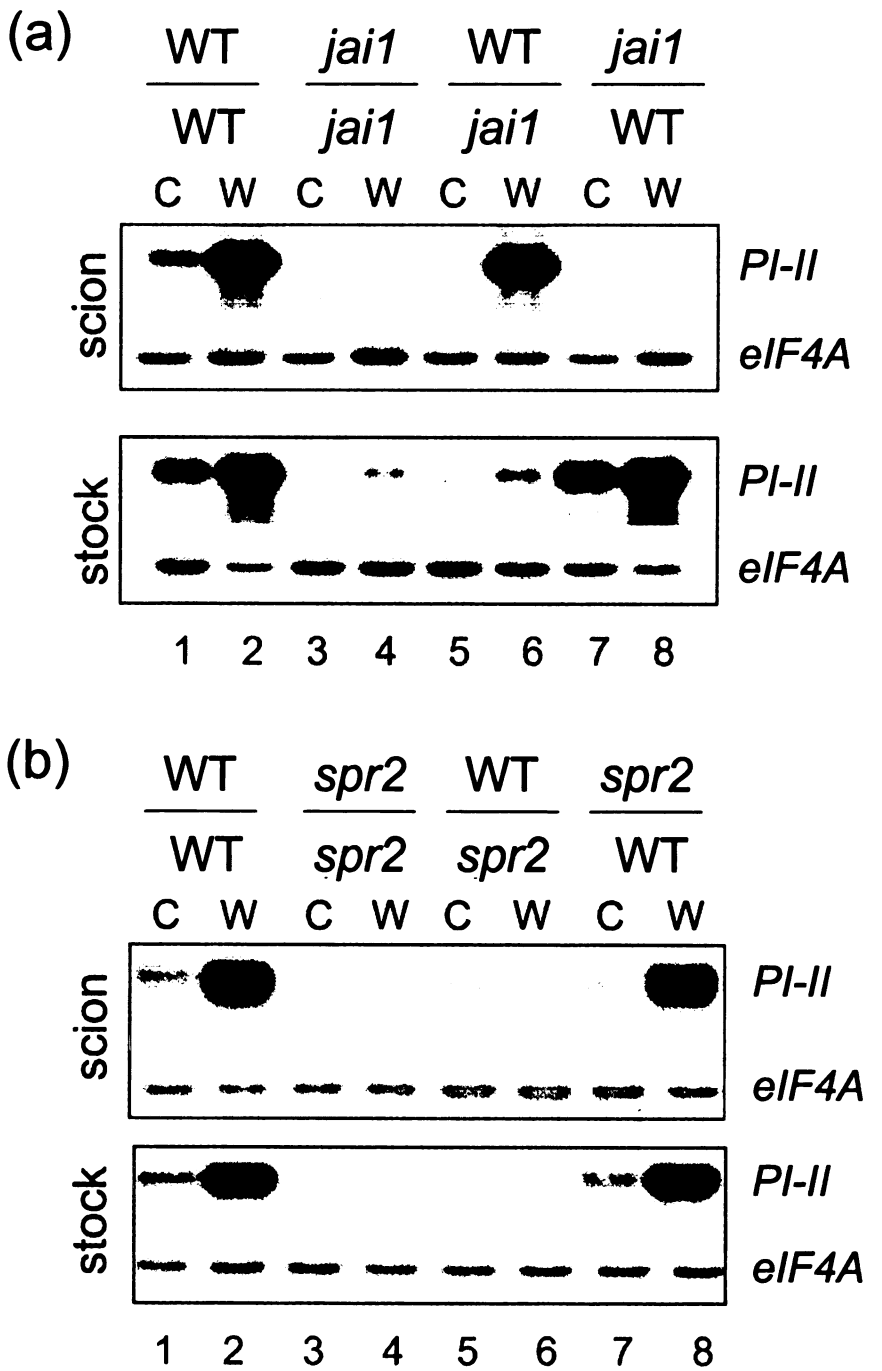
(a)
WT
jai1
WT
jai1
WT
jai1
jai1
WT
C W C W C W C W
scion
PI-II
eIF4A
stock
PI-II
eIF4A
1 2 3 4 5 6 7 8
(b)
WT
spr2
WT
spr2
WT
spr2
spr2
WT
C W C W C W C W
scion
PI-II
eIF4A
stock
PI-II
eIF4A
1 2 3 4 5 6 7 8

Figure 5.3

Figure 5.4. Wound-induced systemic *PI-II* expression in grafts between *jai1* and *spr2* plants. Wound-induced systemic expression of *PI-II* was assessed in the various graft combinations indicated, as described in Figure 5.3. WT, wild-type. *PI-II* and *eIF4A* (loading control) mRNA levels in the undamaged scion leaves of unwounded control (C) plants, and plants wounded on the stock leaves (W), are shown.

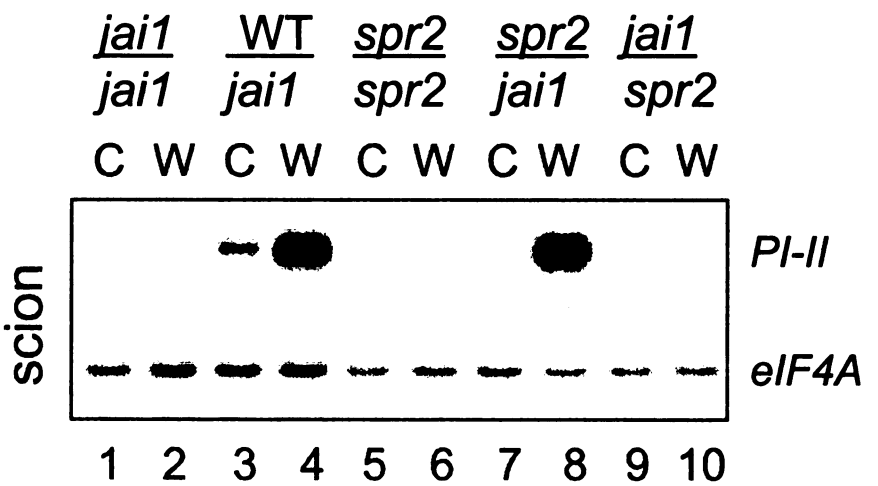
jai1 WT spr2 spr2 jai1
jai1 jai1 spr2 jai1 spr2
C W C W C W C W C W
scion
PI-II
eIF4A
1 2 3 4 5 6 7 8 9 10

Figure 5.4

related octadecanoid pathway-derived compound, is an essential component of the graft-transmissible wound signal. The results shown in Figure 5.3b (lanes 7–8) further suggested that recognition of the long-distance signal and subsequent *PI* expression in undamaged *spr2* scions does not require JA biosynthesis in these tissues. However, an alternative explanation was that grafting of *spr2* scions to wild-type stock simply restored the ability of the mutant scion to synthesize JA in response to a signal produced in wounded wild-type stock leaves. To examine this possibility, *spr2* scion leaves that had been grafted to wild-type stock were wounded and then tested for PI-II protein accumulation. Control experiments showed that wild-type scion leaves grafted to either wild-type or *spr2* stock were responsive to wounding (Table 5.1). *spr2* scion leaves grafted to *spr2* stock failed to accumulate PI-II in response to wounding, and this deficiency was not relieved by grafting to wild-type stock. This finding supports the interpretation that *PI-II* activation in *spr2* scions (Figure 5.3b, lanes 7-8) does not involve *de novo* JA biosynthesis in these leaves, but rather requires a functional octadecanoid biosynthetic pathway in the wild-type stock.

Despite the fact that both *spr2* and *jai1* abrogate systemic wound signaling, the reciprocal nature of the grafting phenotypes conditioned by each mutation (Figure 5.3) predicted that grafted plants lacking both jasmonate responsiveness in stock leaves and jasmonate biosynthesis in scion leaves would be capable of systemic signaling. To test this idea, we examined *PI-II* expression in *spr2* scion leaves in response to wounding of *jai1* stock leaves (Figure 5.4). The results showed that wounded *jai1* stock leaves produce a graft-transmissible signal that activates *PI-II* expression in *spr2* scions (Figure 5.4, lanes 7-8), at a level comparable to that observed in wild-type scions (Figure 5.4, lanes 3-

4). As is also predicted from the grafting phenotypes of individual mutants, wound-induced systemic expression of *PI-II* was abolished in grafted plants that are deficient in both JA biosynthesis in stock (i.e., *spr2*) leaves and JA responsiveness in scion (i.e., *jai1*) leaves (Figure 5.4, lanes 9-10).

Roles for jasmonate biosynthesis and signaling in 35S::prosys-mediated PI expression

Previously it was shown that ectopic expression of prosystemin from a *35S::prosys* transgene leads to constitutive *PI* expression in the absence of wounding (McGurl et al., 1994). Grafting experiments presented in the same study further demonstrated that unwounded *35S::prosys* stock tissue produces a graft-transmissible signal that activates *PI* expression in wild-type scion leaves. To investigate the role of jasmonate synthesis and perception in the *35S::prosys*-mediated signaling pathway, PI-II protein accumulation was measured in *spr2* and *jai1* scions that were grafted to either wild-type or *35S::prosys* stock. As previously reported by McGurl *et al.* (1994), wild-type scion leaves accumulated high levels of PI-II in response to a signal emanating from the *35S::prosys* stock (Table 5.2). The responsiveness of *spr2* scion leaves to the *35S::prosys*-derived signal was comparable to that of wild-type scions. In contrast, *jai1* scions were completely unresponsive to the *35S::prosys*-derived signal.

Table 5.1. Proteinase inhibitor II accumulation in wild-type and *spr2* scion leaves in response to wounding.

Graft combination (Scion / Stock)	PI-II in scion (μg/ml)	
	Unwounded	Wounded
WT / WT	26 ± 10	117 ± 27
spr2 / *spr2*	3 ± 2	11 ± 13
WT / *spr2*	6 ± 1	113 ± 21
spr2 / WT	14 ± 7	17 ± 7

Three plants of each of the indicated graft combinations were constructed from four-week-old tomato plants. Three weeks after grafting, PI-II levels were measured in two newly-developed leaves of the scion, using one excised leaflet per leaf. At the same time, ten of the remaining leaflets on the scion were wounded with a hemostat. PI-II levels in four of the wounded leaflets were determined 48 h later. Values represent the mean PI-II concentration of three plants per combination ± SD. WT, wild-type.

Table 5.2. Proteinase inhibitor II accumulation in grafted tomato plants in response to a long-distance signal generated in a *35S::prosys* transgenic line.

Graft combination (Scion / Stock)	PI-II (μg/ml leaf juice)	
	Scion	Stock
WT / WT	12 ± 7	18 ± 14
35S::prosys / *35S::prosys*	229 ± 14	304 ± 45
WT / *35S::prosys*	140 ± 36	252 ± 61
spr2 / WT	14 ± 7	16 ± 9
spr2 / *35S::prosys*	150 ± 12	263 ± 10
jai1 / WT	ND	30 ± 11
jai1 / *35S::prosys*	ND	279 ± 11

Five-week-old tomato plants were grafted in the indicated combinations. Three weeks after grafting, PI-II accumulation was measured in three plants of each graft combination. PI-II levels were determined using three leaflets per leaf. Values represent the mean PI-II concentration of three plants per combination ± SD. WT, wild-type; *35S::prosys*, transgenic line that overexpresses prosystemin from the *35S::prosys* transgene. ND, not detectable.

Discussion

Systemic activation of defensive *PI* genes in tomato is orchestrated by signaling events that operate both within and between cells. A wealth of biochemical and genetic data support the original proposal (Farmer and Ryan, 1992) that (pro)systemin functions in this pathway to regulate the synthesis of JA, which in turn activates the expression of a subset of target genes including those encoding defensive PIs. However, very little is known about the relationship between systemin-induced JA synthesis and the cell non-autonomous processes by which mobile signals are produced at the wound site, transported through the plant, and perceived by target cells distal to the wound site. To address this question, we used classical grafting techniques to examine long-distance wound signaling in mutants that are deficient either in JA biosynthesis or JA signaling. A model consistent with our results and other available genetic data is shown in Figure 5.5. This model accounts for the following observations. First, a graft-transmissible signal for systemic *PI* expression is produced in response to either wounding or *35S::prosys*. Second, in non-grafted plants, *def1*, *spr2*, and *jai1* suppress both wound- and *35S::prosys*-induced *PI* expression. Third, *jai1* plants are insensitive to both systemin and JA/MeJA, whereas the JA synthesis mutants *spr2* and *def1* are insensitive to systemin but responsive to JA/MeJA. Fourth, *jai1* scions do not respond to a graft-transmissible signal generated either by wounding or *35S::prosys*, whereas *spr2* scions do. Conversely, *spr2* and *def1* stock leaves that are deficient in wound-induced JA accumulation are also deficient in wound-induced generation of a graft-transmissible signal, whereas *jai1* plants are functional in this respect. Taken together, these findings indicate that activation of the

octadecanoid pathway in damaged leaves is required for the production of a long-distance signal whose functional recognition in distal leaves requires jasmonate action.

It should be emphasized that other systemic wound responses may operate independently of or in parallel to the systemin/jasmonate pathway that regulates the synthesis of PIs and other defensive phytochemicals. For example, hydraulic signals may be involved in rapid systemic wound responses such as the activation of a wound-inducible protein kinase activity in tomato leaves (Malone, 1996; Stratmann and Ryan, 1997). There is also evidence that genes whose expression in tomato plants is rapidly and systemically induced by wounding are regulated by signaling pathways that operate independently of systemin and JA (O'Donnell et al., 1998; Howe et al., 2000; Walling 2000). Such a pathway may account for the residual signaling activity observed in wild-type scions in response to wounding of *srp2* (Figure 5.3b) or *def1* (data not shown) stock leaves. Alternatively, this residual signaling may reflect incomplete loss of function of *Spr2/Def1*.

Although systemic activation of *PI* genes clearly involves jasmonate-mediated signaling events in undamaged (scion) responding leaves, grafting experiments conducted with *spr2* and *def1* plants indicate that JA biosynthesis is likely not required in these leaves. This observation raises the question of whether *PI* expression in undamaged leaves is mediated by JA or a related octadecanoid signal. Previous studies aimed at addressing this question suggest that JA, rather than C_{18} precursors of JA, is the active signal for *PI* expression in tomato leaves (Wasternack et al., 1998). If JA is a signal for *PI* expression in undamaged leaves, our results, together with reports of wound-induced

systemic increases in JA levels in tomato (Herde et al., 1996), suggest that JA is transported from its site of synthesis in stock tissues to undamaged responding leaves. An alternative hypothesis is that the requirement for *Jai1*-dependent signaling in undamaged leaves is fulfilled by a jasmonate signal other than JA. Candidates for such a signal include OPDA and its C_{16} analog, dinor-OPDA (Weber et al., 1997). A more precise understanding of how *def1* and *spr2* affect the octadecanoid pathway should provide additional insight into this possibility, as will grafting experiments using transgenic plants that are engineered for a deficiency in specific octadecanoid pathway enzymes.

The roles of jasmonate biosynthesis and perception in wound-induced systemic signaling appear to be similar to their roles in *35S::prosys*-mediated *PI* expression (Figure 5.5). This conclusion is based on the finding that *spr2* and *def1* (L. Li and G. Howe, unpublished data) scions respond to a graft-transmissible signal generated in *35S::prosys* stock, whereas *jai-1* scions do not (Table 5.2). Because *spr2* and *def1* plants are insensitive to exogenous systemin (Howe et al., 1996; Howe and Ryan, 1999), it seems unlikely that the graft-transmissible signal produced in *35S::prosys* plants is systemin. Rather, our results suggest that this signal is a compound that acts downstream of *Spr2*/*Def1* and through *Jai1*. Possible candidates for this signal include JA/MeJA and OPDA. Measurement of these compounds in *35S::prosys* plants may help to address this question. It is interesting to note that while *spr2* and *def1* scions respond to the *35S::prosys*-derived signal, these mutations effectively suppresses *35S::prosys*-mediated signaling when present in homozygous state in the *35S::prosys* genetic background (Howe et al., 1996; Howe and Ryan, 1999). This observation indicates that normal *Spr2*

and *Def1* activity, and thus a functional octadecanoid pathway, is required for production of the *35S::prosys*-derived graft-transmissible signal.

The grafting experiments reported herein demonstrate that jasmonate biosynthesis and action, while both required for long-distance activation of *PI* genes, operate at distinct spatial positions along the systemic signaling pathway. More specifically, we propose that jasmonate biosynthesis is required for the generation of a long-distance wound signal, whereas jasmonate action is involved in the recognition of this signal in responding leaves. The most straightforward interpretation is that jasmonate is an essential component of the transmissible wound signal. This is consistent with other reports implicating JA/MeJA as intercellular signals for stress-induced gene expression (Farmer et al., 1992; Parchmann et al., 1997; Zhang and Baldwin, 1997; Löbler and Lee, 1998; Ryan, 2000; Seo et al., 2001). Such a scenario raises the question of the role of systemin in the systemic response. As discussed previously (Farmer et al., 1992; Hause et al., 2000; Ryan, 2000), localized production of systemin at the site wounding may induce the synthesis of JA/MeJA, which in turn could promote gene expression in neighboring cells. Although this model suggests that JA/MeJA act in a paracrine fashion analogous to eicosanoid signals in animal cells, it is conceivable that jasmonates exert their effects over much longer distances. Alternatively, systemin-induced activation of the octadecanoid pathway could further amplify the signaling cascade through positive feedback on (pro)systemin production or action (Jacinto et al., 1997; Scheer and Ryan, 1998). Identification of mutants that are defective in (pro)systemin perception may provide additional insight into the role of this polypeptide in the systemic wound response, and the mechanism by which it regulates the octadecanoid pathway.

Figure 5.5. Genetic model for the role of jasmonate synthesis and signaling in the systemic activation of wound-responsive *PI* genes in tomato plants. Wounding or expression of *35S::prosys* leads to the production of a long-distance signal that activates *PI* gene expression in distal leaves (i.e., scion). A functional octadecanoid pathway for jasmonate biosynthesis (Hatched line), which is disrupted by *def1* and *spr2*, is required for the production of the long-distance signal (X). Jasmonate signaling, which is blocked by *jai1*, is required for the recognition of the long-distance signal.

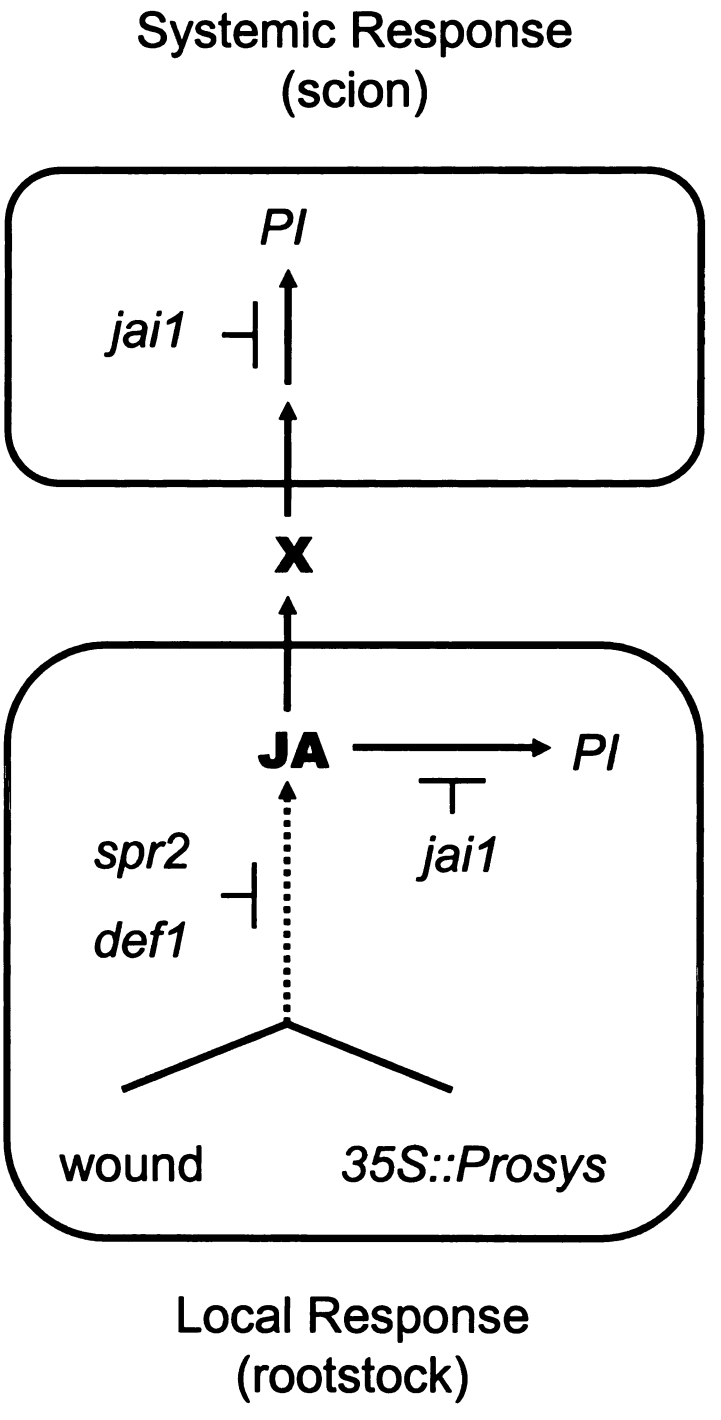
Systemic Response
(scion)
PI
jai1
X
JA
PI
spr2
def1
jai1
wound
35S::Prosys
Local Response
(rootstock)

Figure 5.5

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CHAPTER 6

Molecular Cloning of Jail

Abstract

The *jai1-1* mutant, isolated from a fast-neutron irradiated Micro-Tom population, is impaired in jasmonate signaling. The mutation results in compromised wound responses, increased susceptibility to herbivores, defects in glandular trichome development, and female sterility. In a separate screen for ethyl methane sulfonate (EMS) induced mutations that suppress prosystemin-mediated responses, a second *jai1* allele (*jai1-2*) was identified. Using a candidate gene approach, the *jai1* mutations were found to be lesions in the tomato homolog of the Arabidopsis *CORONATINE INSENSITIVE1* (*AtCOI1*) gene that encodes an F-box protein involved in ubiquitin/proteasome-dependent proteolysis. Stable transformation of *jai1-1* plants with the tomato *COI1* (*LeCOI1*) cDNA restored MeJA-induced expression of defense genes, fertility, and glandular trichome development. These findings demonstrate that *LeCOI1* is an essential component of the jasmonate signaling pathway governing defense and developmental processes in tomato.

Introduction

Tomato (*L. esculentum*) provides an excellent model system in which to study the role of jasmonates in defense- and development-related processes. In our efforts to characterize the systemic wound response pathway in tomato, we conducted various genetic screens that led to the identification of jasmonate-insensitive (*jai1*) mutants that are deficient in jasmonate signaling. The *jai1-1* mutant was isolated from a fast-neutron mutagenized Micro-Tom population (Li et al., 2001; Howe et al., 2002). In a separate genetic screen for mutations that suppress prosystemin-mediated responses, an EMS-induced allele of *jai1* called *jai1-2* was recovered (Howe and Ryan, 1999; Li et al., 2001). Characterization of the *jai1-1* mutant showed that *Jai1* is required for MeJA-mediated root growth inhibition, and anthocyanin accumulation in germinating seedlings (Chapter 3). In leaves, *Jai1* was found to be essential for wound-, MeJA-, and herbivore-induced defense gene activation and defense against hervibores (Chapter 3; Howe et al., 2002). Furthermore, the critical importance of *Jai1* for pollen viability, trichome development on green fruits, female fertility, and constitutive expression of defense genes in flowers was evident by the defects of *jai1-1* plants in these processes (Chapter 3, Li et al., 2001). Taken together, these results indicated that *Jai1* is a positive regulator of jasmonate signaling in most if not all tomato organs.

In Arabidopsis, extensive genetic screens have revealed a number of loci required for the function of the jasmonate signaling pathway (see Table 1.2). One of the genes identified was *AtCOI1* encoding a leucine rich repeat (LRR) containing 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). Based on the biochemical function of F-box proteins from yeast and Drosophila (Deshaies, 1999), it was postulated that COI1 serves as a receptor in the ubiquitin ligase complex to recruit a phosphorylated substrate protein for ubiquitination (Creelman, 1998; Xie et al., 1998). Subsequent degradation of the substrate, which was proposed to be a repressor of transcription, by the 26S proteasome, would then allow transcriptional activation of jasmonate-responsive genes (Creelman, 1998; Xie et al., 1998).

Mutation of *AtCOI1* has global effects on jasmonate signaling that are reminiscent of the phenotypes of tomato *jai1* plants. Arabidopsis *coi1* mutants are unresponsive to root growth inhibition by the bacterial toxin coronatine that is structurally related to JA (Figure 1.1), fail to express jasmonate-regulated genes, are male sterile, and highly susceptible to insect herbivores and fungal pathogens (Feys et al., 1994; Benedetti et al., 1995; McConn et al., 1997; Thomma et al., 1998). Further, exhaustive genetic screens in Arabidopsis for mutants that affect jasmonate responses in both defense and development have only yielded alleles of *coi1* (Ellis and Turner, 2002). This finding suggests that COI1 plays a central role in jasmonate signaling and that there is genetic redundancy in other components of the pathway. Given that the ubiquitin/proteasome pathway is well-conserved among all eukaryotes, we hypothesized that *Jai1* corresponds to the tomato ortholog of *AtCOI1*.

In this chapter, a candidate gene approach was used to test whether *jai1* is a mutation in the *LeCOI1* gene. Analysis of the full-length *LeCOI1* cDNA indicated that it encodes a 603 amino acid protein that is 67% identical to AtCOI1. DNA sequencing of *LeCOI1* from *jai1-2* plants revealed a single base-pair mutation that results in the

Gly_{261}→Cys amino acid substitution. The *jai1-1* mutation was found to be a 6.2-kb deletion in *LeCOI1* that completely abolished expression of the gene. *Agrobacterium*-mediated transformation of *jai1-1* plants with the *LeCOI1* cDNA driven by the CaMV 35S promoter restored MeJA-induced *PI* expression, fertility and glandular trichome development. These findings demonstrate that jasmonate signaling and jasmonate-regulated processes in tomato require LeCOI1.

Materials and Methods

Plant material and treatments

Tomato seedlings (*Lycopersicon esculentum* Mill cv Micro-Tom and cv Castlemart) were grown under 17 h days at 27 °C with light at 200 μmol m^{-2} sec^{-1} and 7 h at 16 °C in darkness. Because the *jai1* mutants are sterile, they are maintained as heterozygotes (crossed with wild-type). The *jai1-1* plants used in this study were derived from the original mutant 406A (Li et al., 2001; Howe et al., 2002) backcrossed three times using wild-type plants (cv Micro-Tom) as the recurrent pistillate parent. The *jai1-2* plants were derived from the original 124A mutant (Howe and Ryan, 1999; Li et al., 2001) backcrossed twice using wild-type plants (cv Castlemart) as the recurrent pistillate parent. All experiments involving *jai1* mutants were performed with homozygous lines. For selection of *jai1-1* mutant plants in F_2 populations, resistance to inhibition of root growth and anthocyanin accumulation by MeJA was assayed as described in the *Materials and Methods* of Chapter 3. Homozygous *jai1-2* plants were selected from the F_2 populations by assaying wound-induced PI-II accumulation in cotyledons of twelve-day-old plants. MeJA treatment of adult plants (three-week-old wild-type plants or transgenic plants after transferring to soil for three weeks) was performed as described in the *Materials and Methods* of Chapter 3.

Molecular biological techniques

A 1.1-kb tomato EST clone (AI482978) that shows significant sequence identity with the 3' end of *AtCOI1* was used to screen a Bacterial Artificial Chromosome (BAC) library (Budiman et al., 2000). A single BAC clone (BAC249O9) was identified. A 9.5-kb sequence was obtained from this BAC clone by sequencing several polymerase chain reaction (PCR) products designed to cover the *LeCOI1* locus. Two open reading frames (ORFs) in this 9.5-kb region were revealed by the GENSCAN program (http://genes.mit.edu/GENSCAN.html): one is *LeCOI1* and the other is annotated as a putative Myb transcription factor (AI488165) that is transcribed in the opposite direction relative to *LeCOI1*.

Two primers, C1 (5'- CGG GAT CCC TCT CCT CCA TCT TCT TCA A) and C2 (5'- CGA GCT CAT ACA TAT GGA CAA GAC ACC T), were designed according to the *LeCOI1* sequence to amplify the cDNA by reverse transcription-PCR (RT-PCR). Five μg of total RNA isolated from tomato leaf tissue was reverse transcribed using the Enhanced Avian HS RT-PCR-20 Kit (Sigma) and C2 primer as recommended by the manufacturer. cDNA products of the reaction were used as template for a PCR reaction that employed the primers C1 and C2. The resulting 2,044-kb PCR products were ligated into pGEM-T vector (Promega) to generate plasmid pGEM-COI1. The *COI1* cDNA insert was sequenced in its entirety by primer walking. To obtain the *COI1* cDNA from *jai1-2*, the same set of primers, C1 and C2, was used for RT-PCR. The PCR products were cloned into vector pGEM-T for sequencing. Three primers, C5 (5'- GAG GCA ATA TGT GGA TTT GAT GGA), C6 (5'- CCA CAC CGT GTT CTT TTG AAG TGG

A) and C7 (5'- GGA GAC GAT ATG TTG AGA CTA AGT) and the arbitrary primer AD2 were used in a thermal asymmetric interlaced-PCR (TAIL-PCR) reaction to clone a fragment of DNA from *jai1-1* plants. This fragment was cloned into pGEM-T and sequenced.

Transformation

The pGEM-COI1 plasmid harboring the full-length *LeCOI1* cDNA was digested with *Bam*HI and *Sst*I. The resulting 2,040-bp fragment containing the cDNA was cloned into the *Bam*HI and *Sst*I sites of the binary vector pBI121 (Clontech), replacing the GUS reporter gene. The resulting construct was introduceded into *Agrobacterium tumefaciens* strain AGLO (Lazo et al., 1991). Transformation of cotyledon explants (cv Micro-Tom) was performed as previously described (Li and Howe, 2001). Twenty-eight independent primary transformants (T_1) were regenerated on kanamycin-containing medium and transferred to soil. Introduction of the transgene was confirmed by a PCR assay using a primer set of C3 (5'- CTG CAA GTT AGG GCT GAA GAT CTT) and C4 (5'- GGC CAA GCA CTT CCA ATC CTC TAT). These primers were designed to amplify a 1116-bp and a 433-bp product from the endogenous *COI1* gene and the *35S::LeCOI1* transgene, respectively. T_1 plants were then tested for MeJA-induced PI-II accumulation and were subsequently transferred to the greenhouse for collection of T_2 seeds.

RNA and DNA blot analyses

Total RNA was isolated from tomato tissue and analyzed by RNA blot hybridization as described in the *Materials and Methods* of Chapter 2. The full-length *LeCOI1* cDNA was used as a probe for detection of the corresponding transcripts. cDNAs for the *Prosystemin* and *eIF4* genes were used as loading controls. Genomic DNA purification and blot analysis were also performed as described in the *Materials and Methods* of Chapter 2. Five μg aliquots of DNA were digested with either *Bgl*II or *Eco*RI and hybridized with either the full-length *LeCOI1* cDNA or different portions of the *LeCOI1* gene as indicated in the text.

Results

The tomato COI1 gene

Identification of AtCOI1 as an essential component of jasmonate signaling implies that the well-conserved ubiquitin-mediated proteolysis pathway plays an important role in transducing the jasmonate signal. Many phenotypes of the *jai1-1* plants, such as altered expression of JA-responsive genes, alleviated root growth inhibition by MeJA, compromised resistance to herbivores, and reduced pollen viability in *jai1-1* plants, are similar to those of the Arabidopsis *coi1* mutants. These observations prompted us to hypothesize that *Jai1* corresponds to the tomato ortholog (*LeCOI1*) of *COI1*. As a first step to test this, we isolated a full-length *LeCOI1* cDNA. The 2,050 bp cDNA contained a 1,812 nucleotide open reading frame (ORF) that is predicted to encode a 603 amino acid protein. The deduced primary sequence of LeCOI1 shares 67.2% sequence identity with AtCOI1 (Figure 6.1). The region of conservation is spread throughout the protein. The secondary structure of LeCOI1 is virtually identical to that of AtCOI1, as both contain a degenerate F-box motif at the N-termini and 16 imperfect LRRs that comprise almost the entire remainder of the proteins (Figure 6.1; Xie et al., 1998).

Extensive DNA blot analyses (Figure 6.2b and data not shown) and EST database searches (www.tigr.org) suggest that *LeCOI1* is a single copy gene in tomato. Screening of a bacterial artificial chromosome (BAC) library of tomato genomic DNA (Budiman et al., 2000) yielded a single positive clone (BAC249O9). DNA sequence obtained from BAC249O9 indicated that *LeCOI1* is composed of 3 exons and 2 introns (Figure 6.2a), similar to the structure of *AtCOI1*. The size of LeCOI1 exons (482, 508, and 822 bp for

Figure 6.1. Sequence alignment of AtCOI1 and LeCOI1. Identical amino acids are shown in black boxes while similar residues are shaded. The F-box motif is underlined. * indicates the position (residue 261) of the mis-sense *jai1-2* mutation.


LeCOI1 1
AtCOI1 1
LeCOI1 58
AtCOI1 61
LeCOI1 118
AtCOI1 121
LeCOI1 178
AtCOI1 181
LeCOI1 238
AtCOI1 241
LeCOI1 298
AtCOI1 292
LeCOI1 358
AtCOI1 352
LeCOI1 418
AtCOI1 412
LeCOI1 478
AtCOI1 472
LeCOI1 538
AtCOI1 532
LeCOI1 596
AtCOI1 589

Figure 6.1

exon 1, 2, and 3, respectively) is similar to that of *AtCOI1* exons, but the *LeCOI1* introns (3,078 and 683 bp for intron 1 and 2, respectively) are much larger than their AtCOI1 counterparts. Located approximately 3 kb downstream of the *LeCOI1* stop codon is a small ORF annotated as a Myb transcriptional factor, whick is transcribed in the opposite direction relative to *LeCOI1* as indicated by the GENESCAN program (Figure 6.2a).

jai1 plants harbor mutations in LeCOI1

As shown in Figure 6.2b, DNA blot analysis using full-length *LeCOI1* cDNA as a probe detected three *Bgl*II-derived restriction fragments with an additive size of ~ 12 kb from wild-type DNA. In *jai1-1* plants, a single 5.5 kb band was detected. This result indicated a partial deletion of *LeCOI1* in *jai1-1* plants. Further DNA blot analyses using different portions of the *LeCOI1* gene as a probe revealed the presence of the first exon and the downstream intergenic region but not the remainder of the gene in *jai1-1* plants. This observation prompted us to perform TAIL-PCR reactions from *jai1-1* DNA using forward primers annealing to the middle region of intron 1. We expect the PCR product to contain sequences flanking the deletion. Indeed, the 1.3-kb PCR product thus obtained contained sequence identical to part of wild-type intron 1 fused with sequence located near the putative *Myb* gene (Figure 6.2a). The sequence of the wild-type DNA in this region indicated that *jai1-1* corresponds to 6,243 bp deletion, which removes 3′ half of intron 1, exon 2, intron 2 and exon 3, is deleted in *jai1-1* plants (Figure 6.2a). Consistent with with this scenario, RNA blot analysis showed that accumulation of *LeCOI1* trnascript was completely abolished in *jai1-1* plants, which was readily detected in all

Figure 6.2. *jai1-1* plants harbor a 6,243 bp deletion in the *LeCOI1* locus. (a) Schematic diagram of *LeCOI1* on chromosome 5. E1, E2, and E3, *LeCOI1* exon1, exon2, and exon3. Myb, an open reading framing encoding a putative *Myb* gene located adjacent to *LeCOI1*. (b) DNA blot analysis of wild-type (1), *jai1-1* (2), and F_1 hybrid (3) plants. *Bgl*II-digested DNA was probed with the full-length *COI1* cDNA (A), exon1 (B), exon3 (C), the intergenic space between exon3 and Myb (D), and the Myb ORF (E). The position of migration of molecular weight standards (kb) is shown on the left.

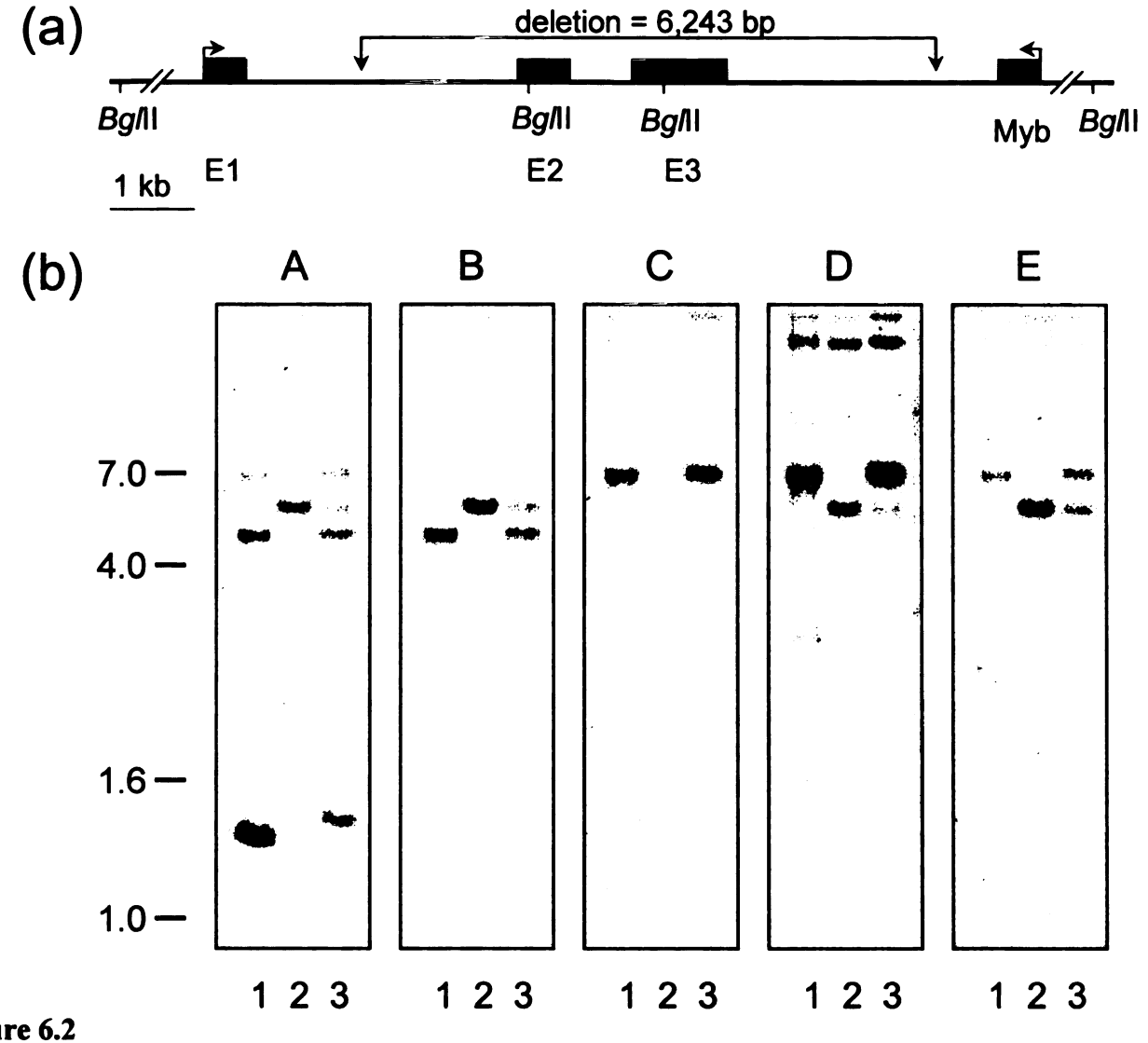
(a)
deletion = 6,243 bp
BglII
BglII
BglII
Myb
BglII
1 kb
E1
E2
E3
(b)
A
B
C
D
E
7.0
4.0
1.6
1.0
1 2 3
1 2 3
1 2 3
1 2 3
1 2 3

Figure 6.2

tissues examined in wild-type plants (Figure 6.3). The *jai1-1* deletion is not likely to affect the putative *Myb* gene, as the deletion occurs more than 500 bp away from the stop codon of the *Myb* gene (Figure 6.2a).

The *jai1-2* mutant was identified from a genetic screen for suppressors of *35S::prosys*-mediated responses (Howe and Ryan, 1999; Li et al., 2001). *jai1-2* plants were insensitive to jasmonates, deficient in wound responses, and female sterile (Howe and Ryan, 1999; Li et al., 2001). To determine whether *LeCOI1* is affected in *jai1-2* plants, we obtained the *LeCOI1* cDNA from these plants by RT-PCR. Sequence comparison between the *jai1-2 COI1* cDNA and that of wild-type identified a mis-sense change (G→T) that convertes Gly^{261} in the 12^{th} LRR to a Cys (Figure 6.1). This polymorphism was confirmed by sequencing PCR products amplified from wild-type and *jai1-2* genomic DNA (data not shown). The *jai1-2* mutation did not abolish the expression of *LeCOI1* as indicated by the RT-PCR reaction (data not shown). Nevertheless, *jai1-2* exhibited complete insensitivity to MeJA treatment regarding defense gene activation (Figure 5.1), indicating that residue Gly^{261} is critical for the function of LeCOI1. Taken together, these results demonstrate that the *jai1-2* allele is a mis-sense mutation in the *LeCOI1* gene.

Figure 6.3. RNA blot analysis of *LeCOI1* transcript in tissues of wild-type and *jai1-1* plants. R, roots; P, petioles; L, leaves; F, flower buds, S, sepals; and G, green fruits. Probes representing the prosystemin gene (*Prosys*) and *eIF4* were used as loading controls.

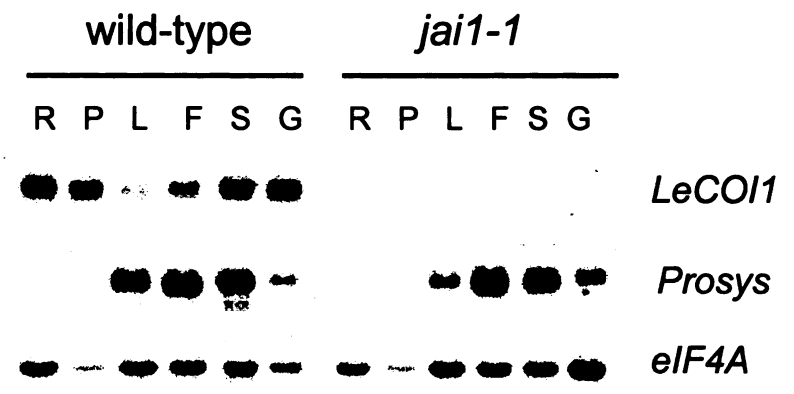

Figure 6.3

Table 6.1. Phenotypes of the primary (T_1) *jai1-1* transgenic plants transformed with the *35S::COI1* transgene.

Lines	01	02	03	04	05	06	07	08	09	10	11	12	13	14
Transgene [a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MeJA-induced PI-II [b]	-	+	-	+	+	+	+	+	+	+	+	+	+	-
Trichome on fruits [b]	-	-	-	+	+	+	-	+	-	+	-	+	+	-
Seed set [b]	-	-	-	+	+	+	-	+	+	+	+	+	+	-

Table 6.1. cont'd.

Lines	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Transgene[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
MeJA-induced PI-II[b]	+	-	+	+	+	-	-	+	+	+	+	+	+	-
Trichome on fruits[b]	-	-	+	-	-	-	-	+	+	-	-	-	-	-
Seed set[b]	-	-	+	-	+	-	-	+	-	-	-	-	-	-

a A PCR assay was used to distinguish the 35S::*COI1* transgene and the endogenous *COI1* (see text for details of the PCR assay). + and -, presence or absence of the transgene-specific PCR product in samples prepared from the transgenic plants.
b These phenotypes of the transgenic plants were scored. + indicates transgenic plants with a particular phenotype similar to wild type plants; - indicates a phenotype similar to the *jai1-1* mutant plants. See text for detail.

Molecular complementation of the jai1-1 mutation by LeCOI1

To confirm that *LeCOI1* corresponds to *Jai1*, the wild-type *LeCOI1* cDNA was cloned into the plant transformation vector pBI121 under control of the CaMV 35S promoter. The resulting construct (*35S::LeCOI1*) was introduced into *jai1-1* plants by *Agrobacterium*-mediated transformation. A total of 28 independent primary transgenic plants (T_1) were obtained. The presence of the *35S::LeCOI1* transgene in these plants was confirmed by a PCR assay that detects a transgene-specific PCR product (Table 6.1). Upon treatment of T_1 plants (four-week-old) with MeJA, seven of the 28 T_1 plants showed no accumulation of PI-II in their leaves. PI-II accumulation in the other 21 T_1 plants ranged from 30 to 220 μg/ml leaf juice (Table 6.1). As a control for this experiment, *jai1-1* plants grown under the same condition produced no PI-II in response to MeJA treatment, whereas wild-type plants accumulated around 160 μg PI-II/ml leaf juice (data not shown). These resultes indicated that MeJA-induced PI-II accumulation was restored in these 21 lines. All the 28 primary transgenic plants flowered and set fruit. However, glandular trichomes were observed on developing fruits from only 10 of these plants (Table 6.1; also see Figure A1). Fertility was restored in 12 of the primary transgenic plants as determined by the recovery of mature seed from these plants (Table 6.1). Taken together, these experiments demonstrate that the *jai1* mutant phenotypes are caused by the loss-of-function of *LeCOI*. All seven plants that lacked MeJA-induced PI-II were sterile and defective in trichome development. Thus, it is likely that the transgene is not expressed in these lines.

To determine the copy number of the transgene in representative primary transgenic lines, four T_1 plants in which all *jai1-1* phenotypes were complemented were subjected to DNA blot analysis. When genomic DNA from wild-type plants was digested with *Eco*RI and probed with the full-length *LeCOI1* cDNA, two hybridizing bands were detected (Figure 6.4). Since the 3′ half of *COI1* is deleted in *jai1-1* plants, only the higher molecular weight *Eco*RI band corresponding to the 5′ half of *LeCOI1* was detected (Figure 6.4). The four transgenic plants (in *jai1-1* background) all contained hybridizing bands in addition to the *jai1-1*-specific band, indicating that they all contained the transgene (Figure 6.4). The different hybridizing pattern of the four lines further indicated that these transgenic plants were derived from independent transformation events. As there is no internal *Eco*RI site within the *LeCOI1* cDNA (i.e. the transgene), the number of these additional bands should correspond to the copy number of the transgene. As shown in Figure 6.4, T_1-08 and T_1-13 appear to each have two copies of the transgene, whereas T_1-10 and T_1-19 appeare to contain multiple copies of the transgene. Therefore, T_1-08 and T_1-13 were chosen for further analysis.

The progeny (T_2) from T_1-08 and T_1-13 each segregated for plants with and without the transgene as revealed by the PCR assay (data not shown). RNA blot analysis showed that T_2 plants containing the *35S::LeCOI1* transgene also accumulated *LeCOI1* transcript, whereas T_2 plants without the transgene did not. Further inspection by RNA blot analysis revealed that T_2 plants containing the transgene accumulated high levels of

PI-II transcript in response to MeJA treatment whereas T_2 plants that lacked the transgene were deficient in MeJA-induced *PI-II* expression (Figure 6.5). These results established a correlation between the presence of the transgene and the MeJA-induced *PI-II* expression in the T_2 generation, and thus confirmed that the *jai1* phenotypes are caused by the loss of function of *LeCOI1*.

Figure 6.4. DNA blot analysis of the primary *jai1-1* transgenic plants expressing *35S::LeCOI1*. Genomic DNA from wild-type, *jai1-1*, and four representative T_1 plants was digested with *Eco*RI, and probed with the full-length *LeCOI1* cDNA. The position of migration of molecular weight standards (kb) is shown on the left.

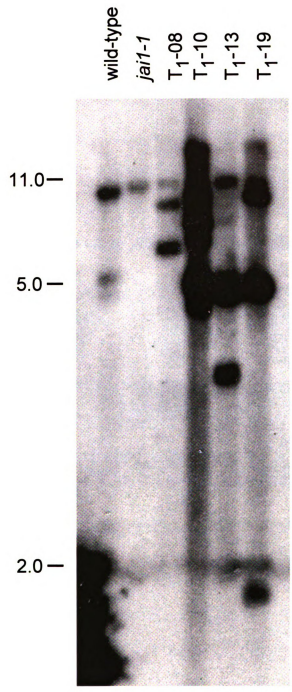

Figure 6.4

Figure 6.5. RNA blot analysis of representative T_2 transgenic plants. Total RNA was prepared from wild-type (WT), *jai1-1* (*jai1*), and T_2-08 and T_2-13 plants before (-) and after (+) exposure to MeJA vapor for 12 h. The T_2 transgenic plants were scored for the presence (+) or the absence (-) of the *35S::LeCOI1* transgene by the PCR assay prior to MeJA treatment. cDNAs for *PI-II* and *LeCOI1* were used as probes. *eIF4A* was the loading control.

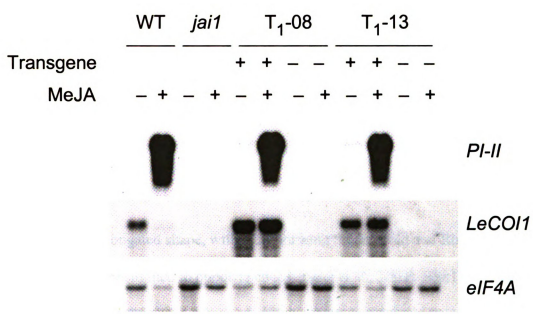

Figure 6.5

Discussion

Selective proteolysis performed by the ubiquitin-proteasome pathway plays a key regulatory role in numerous cellular processes in both animals and plants. In this system, ubiquitin serves as a reusable tag to target proteins for degradation by the proteasome (Voges et al., 1999). Covalent attachment of ubiquitin to substrate proteins involves a cascade of three protein complexes called ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3; Voges et al., 1999). SCF type of E3 enzymes represent one of the best characterized classes, which are composed of four core subunits: a Skp1-like adaptor protein; the cullin subunit Cul1; an F-box containing protein; and a RING-finger protein called Rbx1 (Deshaies, 1999). The structure of SCF^{Skp2} (superscript denotes the F-box protein) has been recently determined. The complex adopts an elongated shape, with Cul1 forming the scaffold and Rbx1 and the Skp1/Skp2 complex positioned to opposite ends (Zheng et al., 2002). Rbx1 and Cul1 constitute the heterodimeric core, which can interact with distinct substrate-recognizing units (such as the Skp1/F-box protein complex) to form a large number of E3 complexes (Deshaies, 1999; Zheng et al., 2002).

Isolation and characterization of the *coi1* mutants in Arabidopsis established an important role of *COI1* in jasmonate signaling. Identification of COI1 as a LRR-containing F-box protein implied its participation in the formation of SCF^{COI1} (Xie et al., 1998). This hypothesis was later supported by biochemical experiments demonstrating the direct physical association of AtCOI1 with AtCUL1, AtRbx1, and either of the

Arabidopsis Skp1-like proteins ASK1 or ASK2 (Devoto, et al., 2002; Xu et al., 2002). Recent demonstration of the physical association of SCF^{COI1} with the COP9 signalosome and the genetic requirement of the signalosome in jasmonate signaling further sustained the notion that SCF^{COI1} is the central mediator of jasmonate signaling (Feng et al., 2003).

In the current chapter, we reported that *Jai1* corresponds to the tomato ortholog of *AtCOI1*. This conclusion was based on the fact that LeCOI1 shares high sequence identity with AtCOI1 and that mutations in either gene affect jasmonate signaling. Interestingly, mutations in the *COI1* gene in both tomato and Arabidopsis affect jasmonate signaling in both vegetative and floral tissues (Chapter 3; Feys et al., 1994; Xie et al., 1998). These observations indicated that the core components of jasmonate signaling pathway are conserved in leaves and floral organs. Based on the biochemical function of SCF^{COI1}, a model for jasmonate signaling has been proposed in which transcriptional repressors of jasmonate-responsive genes would be targeted for degradation by SCF^{COI1} in the presence of jasmonates, allowing transcription of these genes (Creelman, 1998; Xie et al., 1998). Were this model to hold true, it can be proposed that in vegetative and floral tissues, COI1 selectively removes different transcriptional repressors to activate different transcriptomes that control diverse physiological events.

A major gap in understanding the jasmonate signal transduction pathway regards the identification of COI1-interacting proteins that are presumably targeted for

ubiquitination and subsequent degradation in the 26S proteasome. Efforts have been made in Arabidopsis using the yeast two-hybrid technique to isolate COI1-interacting proteins (Devoto et al., 2002). This study identified the SKP1 homologues and a histone deacetylase, though the functional relevance of this histone deacetylase in the context of jasmonate signaling has yet to be determined (Devoto et al., 2002). With the adoption of protein-complex purification methods such as the Tandem Affinity Purification-tag system (Rigaut et al., 1999) in plants, it can be foreseen that future biochemical approaches will help bridge the gap and provide new insights into the jamonate signal transduction pathway.

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CHAPTER 7

Conclusions and Future Directions

The past decade saw the transformation of JA from a little regarded secondary metabolite to a well-recognized phytohormone with a dual role in plant defense and development. While the large volume of literature on JA has solidified our understanding of the chemistry and biosynthetic pathway of JA (reviewed by Creelman and Mullet, 1997; Walling, 2000; Schaller, 2001; Berger, 2002; Turner et al., 2002; Wasternack and Hause, 2002; Webber, 2002), additional research is needed to elucidate the mechanisms that regulate the production and action of jasmonates in response to developmental and environmental cues. The current dissertation, with the use of tomato (*L. esculentem*) as a model system, is focused on the identification of components of the jasmonate signaling pathway using a forward genetic approach, and on exploring JA perception mutants to dissect roles of jasmonates in plant defensive and developmental processes.

Two allelic JA insensitive mutants, called *jai1*, are described (Chapter 3, 4, and 6). Several lines of evidence indicated that *Jai1* corresponds to the tomato ortholog (called *LeCOI1*) of *AtCOI1*. First, the deduced *LeCOI1* sequence shares high identity with that of *AtCOI1* (Figure 6.1). Second, both *jai1-1* and *jai1-2* were found to be mutations in *LeCOI1* (Figure 6.1). Third, stable transformation of *jai1-1* plants with the *LeCOI1* cDNA restored all of the defective phenotypes of the mutant (Figure 6.5, A1, and Table 6.1). Identification of COI1 as an F-box protein implies its participation of formation of SCF^{COI1} (Xie et al., 1998). This hypothesis has been supported by biochemical experiments that demonstrate direct physical association of AtCOI1 with other SCF subunits: AtCUL1, AtRbx1, and either of the Arabidopsis Skp1-like proteins ASK1 or ASK2 (Devoto, et al., 2002; Xu et al., 2002). Molecular cloning of *LeCOI1* and

phenotypical analysis of the *jai1* mutants suggest that SCF^{COI1} is also a key mediator of jasmonate signaling in tomato.

Future experiments aimed at identifying COI1-interacting proteins (CIPs) will likely shed new light on the jasmonate signal transduction pathway. Previous experiments using the yeast two-hybrid technique have implicated a histone deacetylase as a CIP, though the functional relevance of this histone deacetylase in the context of jasmonate signaling remains to be determined (Devoto et al., 2002). An attractive alternative approach to identify CIPs is to use the Tandem Affinity Purification-tag system (TAP-tag; Rigaut et al., 1999) to purify COI1-containing protein complexes from transgenic tomato plants that express a TAP-tagged derivative of COI1. The major advantage of the TAP-tag system is the ability to highly purify multiprotein complexes under mild conditions (Rigaut et al., 1999). CIPs purified in this manner can then be identified by mass spectrometry-based methods. Expression and transgenic analysis of the *CIP* genes can be sought to confirm a role for CIPs in jasmonate signaling.

Using the *jai1-1* allele, a significant amount of new information regarding the wound response pathway in tomato has been obtained. Based on studies of the *jai1-1* mutant, the wound responsive genes in tomato can be classified into three groups (Figure 3.6). The first group represented by *WIPK*, is rapidly induced by wounding locally and systemically in a *Jai1*-independent manner. In contrast, wound induction of the so-called "late response genes" (Ryan 2000) is completely abolished in *jai1* plants (Figure 3.5). The third group, or the so-called "early response genes" (Ryan, 2000), requires *Jai1* for maximum wound induction. This classification is meaningful in that it fits well with the

assumed function of these genes. *WIPK* is likely involved in the initial phase of the wound response during which JA production is triggered (Seo et al., 1995; 1999). The early response genes that include JA biosynthetic genes and *prosystemin* may function to modulate JA levels. The late response genes, wichi encode proteins with defensive properties (Bergey et al., 1996; Ryan 2000), are then activated in response to elevated levels of JA. Additional experiments comparing the expression of these different classes of wound-inducible genes in response to various elicitors of the wound response may help to elucidate the signal transduction events associated with the wound response.

We demonstrated that *jai1-1* plants are compromised in resistance to both chewing insects (e.g. tobacco hornworm, Chapter 3) and cell-content-feeding arachnid herbivores (e.g. two-spotted spider mite; Howe GA, unpublished data). These results indicate that jasmonate signaling is necessary for cellular processes that are essential for protection of tomato to a broad spectrum of arthropod herbivores. This adds to our knowledge that the octadecanoid pathway for JA biosynthesis is important in defense of cultivated tomato against herbivores (Howe et al., 1996; Li et al., 2002). Together, these findings establish a central role of jasmoantes in mediating tomato resistance to herbivores. Future experiments using the *jai1* mutants to identify all the target genes that require COI1 for JA- or herbivore-induced expression might reveal novel aspects of the defense process. For instance, microarray analysis can be conducted to compare the expression profiles of wild-type and *jai1-1* plants subjected to wounding or MeJA treatment. Identification of novel genes that are differentially regulated in wild-type and *jai1-1* plants will likely provide clues to the cellular and metabolic processes that are regulated in a COI1-dependent manner.

The *jai1* mutant was used to gain insight into the role of JA in systemic wound signaling. In one set of experiments, wild-type, *jai1* and *spr2* (defective in JA synthesis; Howe and Ryan, 1999) plants were reciprocally grafted. Analysis of wound-induced *PI* expression in these chimeric plants showed that *jai1* plants lack the capacity to recognize the graft-transmissible wound signal, but are not affected in the generation of the signal. In contrast, *spr2* displayed the reciprocal phenotype; they can perceive the long-distance signal but are unable to generate it (Figure 5.3, 5.4). Thus, it appears that JA biosynthesis in response to wounding is required for the production of a mobile signal whose functional recognition is distal leaves depends on JA signaling (Figure 5.5). This finding is consistent with the hypothesis that a member of the jasmonate family of oxylipins acts as an essential component of the transmissible wound signal. Future grafting experiments using other JA biosynthetic mutants as they become available (e.g. the tomato *opr3* mutant) may help pinpoint which jasmonate species is involved in the transmission of the the long-distance wound signal.

The finding that jasmonates could act as a mobile wound signal for defense gene activation further demonstrated the genetic complexity of wound signaling in tomato. Incorporating this new information, the most recent model of the systemic wound response pathway proposes that both systemin and JA are released at wound sites, with systemin being processed from prosystemin and JA initially produced from the degradation of membranes (Ryan and Moura, 2002). Given that both systemin (McGurl et al., 1992) and JA (Chapter 5) are important for systemic wound signaling, and that systemic and JA could trigger the production of each other, it is proposed that systemin and JA would both move away from the wound site and modulate the production of each

other thought positive feedback that results in the mutual amplification of systemin and JA as a cascade along the stems and petioles (Ryan and Moura, 2002). The process would eventually be limited by the presence of extracellular systemin-inactivating enzymes (Janzik et al., 2000). Elevated levels of JA in the vascular bundle cells in the distal leaves could then diffuse to nearby palisade and mesophyll cells where the defense proteins are synthesized and compartmented (Ryan 2000; Ryan and Moura, 2002).

The role for jasmonates in regulating several developmental processes in tomato was revealed by analyzing the *jai1* mutants. The surface of wild-type tomato leaf, stem, sepal and developing fruit contains abundant glandular trichomes that provide an important constitutive anti-herbivore defense (Figure A1; Kennedy, 2003). However, *jai1-1* immature fruit is completely devoid of trichomes whereas trichome density on the *jai1-1* green tissues is significantly reduced (Figure 3.1). This phenotype likely contributed to the compromised resistance of *jai1* plants to herbivores. *Agrobacterium*-mediated transformation of *jai1-1* with the *LeCOI1* cDNA complemented the trichome defects in the mutant plants (Figure A1 and Table 6.1). These results demonstrated, for the first time, a role of the JA/*COI1* pathway in trichome development. In this context, analysis of the effects of the *jai1-1* mutation in the wild species *L. hirsutum*, which has different types of glandular trichomes, may provide further information regarding the role of jasmonate in trichome development and in trichome-based defense mechanisms.

Another novel phenotype of *jai1* plants is female sterility. Fruit production and development on *jai1-1* plants depended on pollination with either wild-type or mutant pollen, though the fruit lacked viable seed. Plants homozygous for *jai1-2* also exhibited female sterility, albeit with reduced severity (Chapter 4). Although *jai1* pollen showed

reduced germination and viability, reciprocal crosses between wild-type and *jai1* plants unambiguously demonstrated that sterility of the mutant resulted from a defect in female reproductive development (Chapter 3, 4). The fact that ripened *jai1* fruit contained numerous undeveloped seeds suggests that pollination and early embryonic development of *jai1* plants are normal. Rather, defects in late embryonic development or seed filling might contribute to the sterility phenotype. The sporophytic nature of *jai1* sterility suggests that jasmonate-regulated processes in maternal tissue are required for embryo development in tomato.

A proposed role for JA in female reproductive development in tomato contrasts the well-documented studies in Arabidopsis where JA biosynthesis and perception are essential for male, but not female, gametophyte development (McConn and Browse, 1996; Feys et al., 1994; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002; von Malek et al., 2002). Significantly, we found that *jai1* flower buds and ovaries were completely deficient in the expression of *PI* and other defense gene, indicating that constitutive activation of defense-related genes in reproductive tissues requires *COI1* (Figure 3.4). The expression pattern of *PI* is in keeping with the level of endogenous JA, which is high in young apical sink tissues and reproductive structures but inducible in older parts of the plant (Creelman and Mullet, 1997; Hause et al., 2000). Thus, *PI*s provide a convenient biochemical marker for the activity of the JA signaling pathway, and suggest a scenario in which defense gene activation is common to the physiological processes controlled by *COI1* in both vegetative and reproductive organs. Interestingly, this group of genes, including *PI*s and several other defense genes (Bergey et al., 1996), is not found in Arabidopsis (van der Hoeven et al., 2002). One possibility is

that this cocktail of defense proteins could serve as seed storage proteins during embryo development. Alternatively, some of the JA/COI1 regulated hydrolytic enzymes might be required for nutrient release during seed development.

The range of physiological processes controlled by jasmonates may ultimately reflect the function of specific genes whose expression is regulated by the hormone in a tissue- or cell type-specific manner. The well-characterized wound response pathways of tomato, together with the availability of jasmonate signaling mutants, should provide useful tools to investigate the molecular mechanisms by which jasmonates regulate diverse physiological processes. Future experiments aimed at identifying all *COI1*-dependent jasmonate-responsive genes and COI1-interacting proteins in tomato will no doubt add to our understanding of the function of jasmonate signaling. Comparison of this information with that obtained from other plant systems would provide greater insight into the evolution of plant defense signaling. This knowledge would have an enormous potential to shape our understanding of the biological characteristics of plant species found in various ecosystems. This knowledge may also be applied in ways that will enhance agronomic traits that exploit natural defense strategies.

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APPENDIX

Glandular trichomes are specialized multi-cellular appendages occurring on many vascular plants that produce and secrete secondary metabolites (Dell and McComb, 1978; Kelsey et al., 1984; Wagner, 1991; Phillips and Croteau, 1999). These phytochemicals have been implicated in attracting pollinators, reducing leaf temperature or water loss in desert plants, repelling or intoxicating pests, and tritrophic interactions (Kelsey et al., 1984; Kennedy, 2003). In wild tomato species (e.g. *L. hirsutum* and *L. pennellii*), glandular trichomes have been extensively implicated in the resistance to various anthopod insects (Williams et al., 1980; Carter and Snyder, 1985; Snyder and Carter, 1985; Weston et al., 1989; Kennedy 2003). In particular, type VI trichomes have been found to physically entrap small insects such as aphids (McKinney, 1938). The tips of type VI trichomes contain various compounds with anti-herbivore properties. For instance, sesquiterpenes from type VI trichomes of *L. hirsumtum f. typicum* were found to contribute to resistance against beet armyworm and Colorado potato beetle (Carter et al., 1989; Eigenbrode et al., 1996). The phenolic compounds in trichomes, when released by insect damage, can be toxic to the insect or can reduce the nutritive value of leaves (Duffey and Isman, 1981; Felton and Duffey, 1991). The most potent trichome toxin identified is the methyl ketones 2-tridecanone (Williams et al., 1980). Comprising up to 90% of the tip contents of type VI trichome of the accession PI134417 of *L. hirsutum*, 2-tridecanone has been found to be acutely toxic to a wide range of herbivores (Williams et al., 1980; Dimock and Kennedy, 1983).

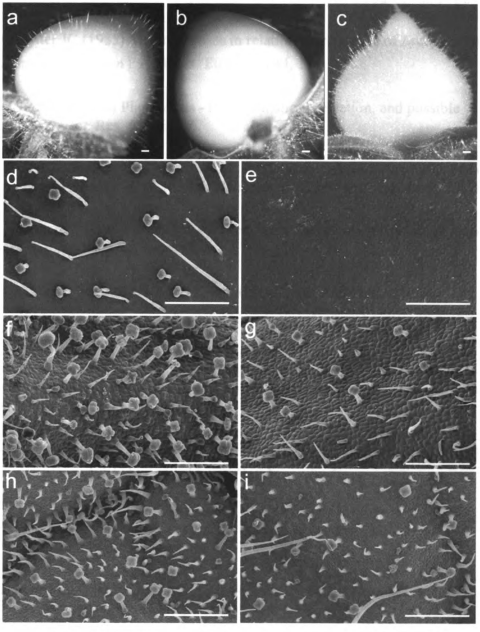
Contrasting our knowledge of the physiological roles of glandular trichomes, the molecular mechanism by which glandular trichome development and content are regulated is obscure. Some studies have found that traits controlling 2-tridecanone levels

are linked to those controlling trichome density (Nienhuis et al., 1987; Kauffman and Kennedy, 1989). Here we report that a tomato mutant (*jai1-1*) defective in the perception of jasmonates (Li et al., 2001; Li et al., 2002) is defective in the production of glandular trichomes. Molecular cloning of the gene defined by *jai1-1* identified it as the tomato ortholog (*LeCOI1*) of the Arabidopsis *CORONATINE INSENSITIVE1* gene that encodes an F-box protein involved in ubiquitin-dependent proteolysis (Xie et al., 1998; Xu et al., 2002). These results establish a role for the jasmonate signaling pathway in glandular trichome development and suggest a strategy to genetically modify the content of gland-derived compounds, which are widely used in products such as pesticides, medicines, flavor and aroma substances, and cosmetic ingredients (Kelsey et al., 1984; Duke et al., 1999; Mahmoud and Croteau, 2001).

Two types of glandular trichomes (type I and VI) are present on the aerial surfaces of cultivated tomato (*L. esculentum*). The type I trichome has an elongated stalk and a unicellular gland, whereas the type VI trichome has a relatively short stalk and a four-celled glandular head (Figure A1). Type VI trichomes are the most conspicuous glandular type on stems, leaves (Figure A1; Snyder and Carter, 1985) and sepals (Figure A1). On the surface of immature green fruit, both type I and VI are abundant (Figure A1). Strikingly, neither type of trichome is present on *jai1-1* fruits, resulting in the glabrous appearance of the fruits (Figure A1). The density of type VI on *jai1-1* sepals and leaves is reduced to approximately 30% that of wild-type tissues (Figure A1). In addition, the size of type VI trichome on *jai1-1* sepals and leaves of was smaller and their stalks shorter as compared with those of the wild-type (Figure A1). To determine whether the *jai1-1* deletion is responsible for the trichome-related phenotypes of the mutant, *Agrobacterium-*

mediated transformation was used to introduce a *35S::LeCOI1* transgene into *jai1-1* plants. As shown in Table 6.1 and Figure A1, the *LeCOI1* cDNA restored trichome development. These results provide conclusive evidence that COI1 plays an essential role in glandular trichome development in cultivated tomato.

Figure A1. Scanning electron micrograph of wild-type and *jai1-1* plants. Scanning electron micrography (SEM) was performed using 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 representative tissues (5x5 mm) were fixed in 4% glutaraldehyde in 20 mM sodium phosphate buffer (pH 7.4), dehydrated through an ethanol series, critical point dried in CO_2, and coated with gold using an EMSCOPE SC500 sputter coater (Ashford, UK). (a) Developing wild-type fruit containing both type I and type VI glandular trichomes. (b) Picture of green fruit from jai1-1 plants, which appears glabrous due to the lack of trichomes. (c) Complementation of the glabrous phenotype in transgenic *jai1-1* plants expressing the *35S::COI1* transgene. (d, e) SEM picture of the surface of wild-type (d) and *jai1-1* (e) green fruit. (f, g) SEM showing wild-type (f) and *jai1-1* (g) sepals. (h, i) SEM picture of the surface of wild-type (h) and *jai1-1* (i) young leaves.



Bar = 400 μm

Figure A1

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