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JASMONATE REGULATION OF DEFENSE RESPONSES
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GUANGHUI LIU

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Gregg Howe
Major Professor's Signature

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JASMONATE REGULATION OF DEFENSE RESPONSES IN TOMATO (*LYCOPERSICON ESCULENTUM*)

By

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ABSTRACT

JASMONATE REGULATION OF DEFENSE RESPONSES IN TOMATO
(*LYCOPERSICON ESCULENTUM*)

By

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Jasmonic acid (JA) and methyl-JA (MeJA) are fatty acid-derived cyclopentanone signals that regulate a broad range of plant defense responses against herbivores and microbial pathogens. In my thesis research, I found that the expression of a lanthionine synthetase C-like (LANCL) gene in tomato is induced in response to wounding and treatment with MeJA, indicating that *LeLANCL* expression is regulated by the JA signaling pathway. LANCL proteins are homologous to bacterial LanC, which is involved in the synthesis of lantibiotic peptides exhibiting antimicrobial properties. Thus, LeLANCL may have a role in jasmonate-mediated plant protection against biotic stress. In a second project, a tomato mutant (*jl1*) that is defective in wound-induced expression of *proteinase inhibitor* (*PI*) gene was shown to be compromised in resistance to the tobacco hornworm (*Manduca sexta*). Wounded *jl1* plants accumulate normal levels of OPDA, but are compromised in their ability to produce JA. The gene defined by *jl1* encodes an acyl-CoA oxidase (named LeACX1) that catalyzes the first and rate-limiting step of fatty acid β-oxidation in the peroxisome. This finding indicates that the final step in JA biosynthesis involves β-oxidation of 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) to JA. *LeACX1* transcripts constitutively accumulate in tomato leaves, and are further induced by wounding in a JA-dependent manner. These results show that LeACX1 plays a major role in the β-oxidation step of JA biosynthesis and induced resistance to herbivores.

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CHAPTER 1

Introduction: Jasmonate Biosynthesis, Action, and Function

Throughout their lives, plants interact with a wide array of organisms such as insects and pathogens. Plants have evolved complex traits that affect their interactions with these organisms at all levels. Although some of these relationships are mutually beneficial, many other interactions cause plants to deploy defensive strategies that protect them against invaders. To combat invasion by herbivorous insects effectively, plants make use of pre-existing physical barriers such as the cuticle, bark, and trichomes to repel or trap insect predators (León et al., 2001). Plants also accumulate high levels of pre-formed chemical compounds that are toxic to insect invaders (Wittstock and Halkier, 2002). This protection strategy can be described as constitutive, in contrast to induced defenses in which the synthesis of toxins and anti-feedants is triggered by insect attack (Harborne, 1988; Ryan, 2000; Gatehouse, 2002). Because the latter protection mechanism does not become activated until plants are attacked, the fitness cost of induced resistance is less than that involved in constitutive defense (Simms and Fritz, 1990; Gatehouse, 2002; Heil and Baldwin, 2002; Kessler and Baldwin, 2002).

An important aspect of inducible defenses is their expression occurrence both at the site of wounding and in undamaged tissues distant from the site of primary attack (Green and Ryan, 1972; Karban and Baldwin, 1997). This systemic induced response protects plants against subsequent invaders. Wound-induced defenses in tomato (*Lycopersicon esculentum*), which are typically triggered by feeding insects or mechanical wounding (Howe et al., 2000; Ryan, 2000), represent one of best examples of systemic-induced resistance to herbivores (Kessler and Baldwin, 2002). In their landmark study of wound-inducible proteinase inhibitors (PIs), which are expressed within ≈2 hrs after mechanical wounding or herbivore attack, 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. The fatty acid-derived plant hormones jasmonic acid (JA) and methyl JA (MeJA) are essential signals for the control of wound-induced defense responses (Li et al., 2002b; Turner et al., 2002; Weber, 2002). JA also plays an important role in plant developmental processes such as root growth, tendril coiling, trichome formation, seed maturation, and production of viable pollen.

I. Biosynthesis of jasmonates

JA and its volatile methyl ester, MeJA, are potent signaling molecules that are derived from fatty acids. JA and its cyclic precursors and derivatives, collectively referred to as jasmonates (JAs), occur ubiquitously in the plant kingdom (Wasternack and Hause, 2002). Following the first identification of MeJA as a major fragrance in the essential oil of jasmine plants (Demole et al., 1962), the octadecanoid pathway for jasmonate biosynthesis (Figure 1.1) was elucidated by Vick and Zimmerman (1984). The pathway starts with the release of α-linolenic acid (α-LA) from membrane lipids in the chloroplast (Narváez-Vásquez et al., 1999; Ishiguro et al., 2001). A 13-lipoxygenase (LOX) adds molecular oxygen to α-LA resulting in the production of 13S-hydroperoxylinolenic acid (HpOTrE). This hydroperoxy fatty acid is converted to an unstable allene oxide by the action of allene oxidase synthase (AOS). Allene oxidase cyclase (AOC) then transforms the allene oxide intermediate to the first cyclic compound in the pathway, 12-oxo-phytodienoic acid (OPDA). The terminal reactions of JA biosynthesis occur in the peroxisome, which is the site of fatty acid β-oxidation in plants. First, the cyclopentenone ring of OPDA is reduced by OPDA reductase (OPR3) to yield 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0). Three cycles of β-oxidation remove six carbons

Figure 1.1. The octadecanoid pathway for jasmonate biosynthesis.

The pathway originates with the release of α-LA from the chloroplast membrane by a lipase. α-LA is then converted to OPDA in the chloroplast by the sequential action of 13-lipoxygenase (LOX), allene oxidase synthase (AOS), and allene oxidase cyclase (AOC). OPDA is transferred to the peroxisome and reduced to OPC-8:0, which is converted to JA by three cycles of β-oxidation. JA can be methylated by jasmonate methyl transferase (JMT) to the volatile MeJA in the cytosol. JA can also be metabolized to other JA conjugates such JA-Ile.

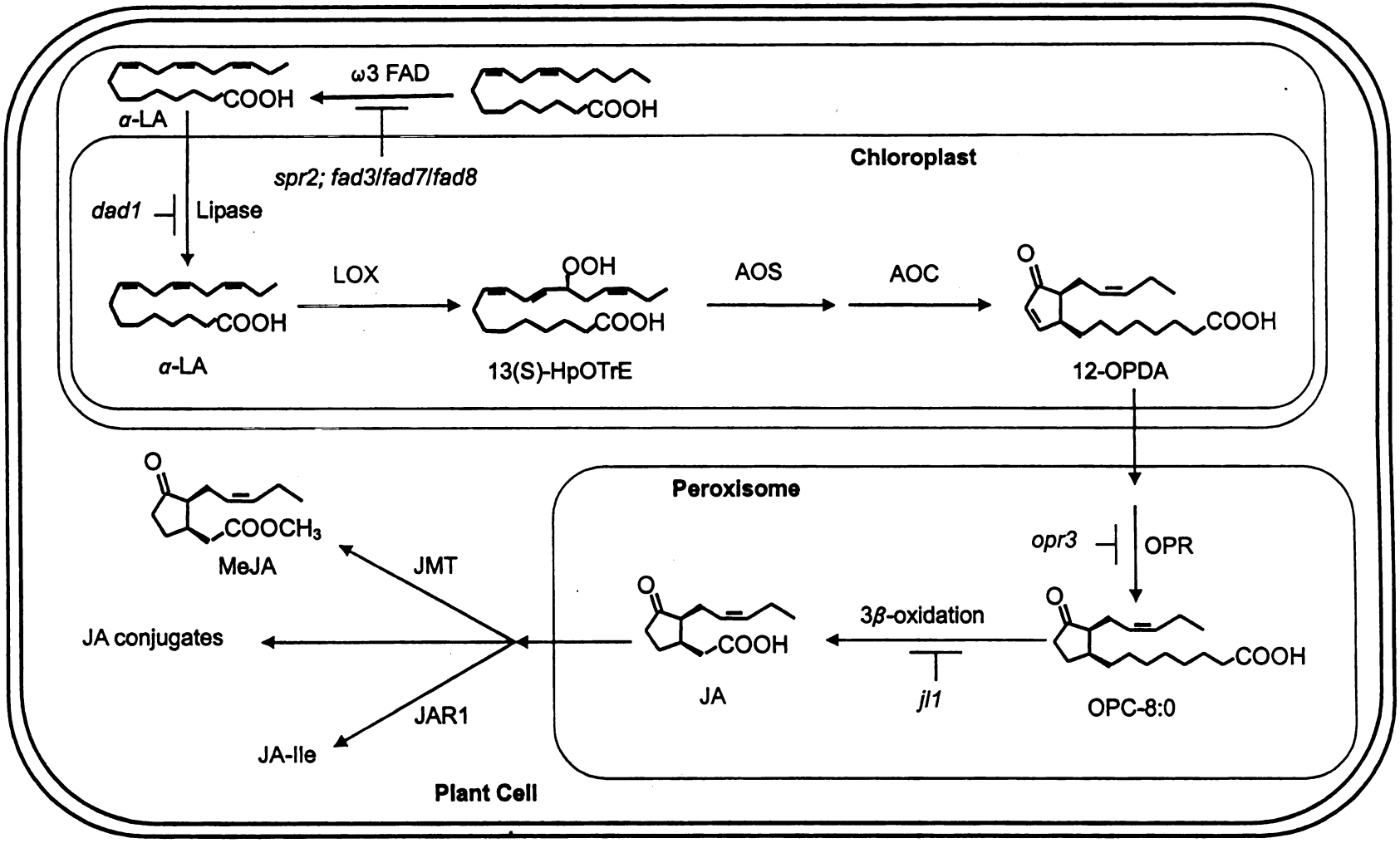

Figure 1.1. The octadecanoid pathway for jasmonate biosynthesis.

from the carboxyl side chain, completing the biosynthesis of JA. JA also occurs in a variety of modified forms, including MeJA, glycosyl esters, and amide-linked conjugates with various amino acids (Sembdner and Parthier, 1993; Staswick and Tiryaki, 2004).

I.1. Linolenic acid and lipases

The critical requirement of α-LA in JA biosynthesis was explicitly demonstrated by the analysis of an Arabidopsis "triple" fatty acid desaturase (FAD) mutant (*fad3/fad7/fad8*), which produces no trienoic fatty acid and is completely deficient in JA (Wallis and Browse, 2002). Although photosynthesis and vegetative growth of the mutant are unaffected, the triple mutant is male sterile. Exogenous α-LA and JA restore fertility to the mutant (McConn and Browse, 1996), indicating the JA plays an essential role in plant reproductive development. Additional evidence for the role of ω3-FADs in JA biosynthesis comes from the characterization of the *spr2* mutant of tomato (Li et al., 2003). This mutant is defective in anti-herbivore defense in response to wounding and the 18-amino-acid peptide wound signal, systemin (Howe and Ryan, 1999). The *spr2* gene encodes a chloroplast fatty acid desaturase (LeFAD7) that is homologous to Arabidopsis FAD7/8. *spr2* plants are severely deficient in α-LA and JA accumulation, indicating that chloroplast pools of α-LA are required for wound- and systemin-induced JA biosynthesis in tomato (Li et al., 2003).

Recent evidence indicates that phospholipases (PLs) that release fatty acid precursor from membrane lipids play a critical role in JA biosynthesis. The male sterile *delayed in anther dehiscence 1* (*dad1*) mutant of Arabidopsis is deficient in JA accumulation in floral tissues (Ishiguro et al., 2001). *DAD1* encodes a PLA1 that presumably liberates α-

LA from membrane lipids (Figure 1.1). Localization of DAD1 to the cholroplast is consistent with the notion that it generates fatty acid substrate for the plastid-localized enzymes of the octadecanoid pathway. The capacity of *dad1* mutant plants to accumulate normal levels of JA in wounded leaves (Ishiguro et al., 2001) indicates that other lipases are invloved in JA biosynthesis in response to insect and pathogen attack. PLA2 and PLD have also been implicated in wound-induced JA biosynthesis (Creelman and Rao, 2002; Howe and Schilmiller, 2002; Turner et al., 2002).

I.2. Lipoxygenase (LOX)

LOXs catalyze the conversion of fatty acids to their corresponding hydroperoxy derivatives. Plant LOXs oxygenate α-LA at the 9 or 13 positions to generate 9- or 13-HpOTrE, respectively. LOXs involved in JA biosynthesis produce 13-HpOTrE, and are called 13-LOX (Feussner and Wasternack, 2002). Evidence for the involvement of 13-LOX in JA biosynthesis came from the observation that plants treated with LOX inhibitors or transgenic plants with suppressed LOX activity exhibited reduced JA levels in response to wounding (Peña-Cortés et al., 1993; Bell et, al., 1995; Royo et al., 1999; Halitschke and Baldwin, 2003).

I.3. Allene oxide synthase (AOS)

Production of JA requires the metabolism of 13-HpOTrE to an unstable epoxide intermediate by AOS. AOS is a cytochrome P450 enzyme (CYP74A) that contains an N-terminal plastid targeting sequence (Laudert et al., 1996). AOS activity and protein has been localized to the plastid outer envelope (Bleé and Joyard, 1996; Froehlich et al., 2001). Tomato has two chloroplast-targeted 13-AOSs (Howe et al., 2000; Sivasankar et

al., 2000). AOS knockout mutants of Arabidopsis, which has a single AOS gene, are completely defective the wound-induced JA accumulation and the activation of wound response genes (Park et al., 2002; von Malek et al., 2002). Wounding increases *AOS* expression and AOS activity (Laudert et al., 1996; Laudert and Weiler, 1998) in both wounded and systemic leaves. The reaction catalyzed by AOS is the first committed step in JA biosynthesis. Therefore, regulation of *AOS* expression and activity is considered a major control point for JA biosynthesis (Laudert and Weiler, 1998). Overexpression of *AOS* in transgenic Arabidopsis plants did not alter the basal JA levels, but these transgenic plants produced more JA than wild-type plants in response to wounding (Laudert et al., 2000; Park et al., 2002). Therefore, it appears that *AOS* expression limits JA levels in wounded plants. The production of JA in unwounded plants appears to be limited by substrate availability (Laudert et al., 2000; Ziegler et al., 2001).

I.4. Allene oxide cyclase (AOC)

AOC catalyzes the stereospecific cyclization of the unstable allene oxide to OPDA. A single *AOC* gene in tomato encodes a protein that is targeted to the chloroplast by an N-terminal transit peptide (Ziegler et al., 2000). In tomato, *AOC* expression is highest in roots, flower buds, flower stalks, with a lower expression in stems, young leaves, and pistils (Hause et al., 2000). *AOC* transcript is transiently induced in tomato leaves in response to wounding or treatment with JA or systemin. *AOC* expression is primarily confined to the vascular bundle tissues, specifically in companion cells and sieve elements (Hause et al., 2000; 2003; Stenzel et al., 2003). Vascular bundle-specific localization of *AOC* transcript coincides with the spatial accumulation of JA in main veins of tomato leaves (Stenzel et al., 2003).

I.5. 12-Oxo-phytodienoic acid reductase (OPR)

OPR catalyzes the reduction of OPDA to OPC-8:0. Both Arabidopsis and tomato contain three OPR isozymes. However, only OPR3 has the capacity to reduce the 9S, 13S-stereoisomer of OPDA, which is the biologically relevant precursor of JA (Müssig et al., 2000; Strassner et al., 2002). This was confirmed in studies demonstrating that an *opr3* null mutant lacks JA and is male sterile (Wallis and Browse, 2002). *OPR3* transcript is induced by JA (Müssig et al., 2000) and wounding (Strassner et al., 2002; Li et al., 2004). Localization of OPR3 to the peroxisome (Strassner et al., 2002) provides strong support for the hypothesis that the later phase of JA formation occurs in this organelle. The spatial separation of the octadecanoid pathway into two distinct compartments (chloroplast and peroxisome) implies that transport processes should be existed to shuttle OPDA from the chloroplast to the peroxisome.

I.6. β-Oxidation

Shortening of the side chain of OPC-8:0 to form JA is achieved by three rounds of β-oxidation. These reactions are thought to occur in the peroxisome, which is the exclusive site of fatty acid β-oxidation in plants (Gerhardt et al., 1983; Strassner et al., 2002). However, until recently, there has been little direct evidence for the involvement of β-oxidation in the biosynthesis of JA. The enzymatic activities involved in fatty acid β-oxidation are acyl-CoA oxidase (ACX), multifunctional enzyme showing enoyl-CoA isomerase and hydroxyacyl-CoA dehydrogenase activity, and thiolase (Gerhardt, 1992). Castillo et al. (2004) identified specific β-oxidation enzyme encoding genes that were expressed in wounded leaves of Arabidopsis. Wound-activated synthesis of JA was

reduced in *acyl-CoA oxidase 1* (*ACX1*) and *3-ketoacyl-CoA thiolase 2* (*KAT2*) antisense plants, suggesting that ACX1 and KAT2 play a role in the biosynthesis of JA in Arabidopsis (Castillo et al., 2004).

I.7. Cellular compartmentation of JA biosynthesis

The conversion of α-LA to OPDA occurs in the chloroplast, which contains an abundance of α-LA. Localization studies using biochemical fractionation and immunocytochemical and *in vitro* chloroplast import assays have demonstrated a chloroplast location for DAD1, LOX, AOS and AOC, which together catalyze the core chloroplast reactions in the octadecanoid pathway. In addition to protein localization, the corresponding enzymatic activities are also found in the chloroplast (Bleé and Joyard, 1996; 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 occurs in the peroxisome as indicated by the peroxisome-specific compartmentation of OPR3 and the β-oxidation enzymes (Strassner et al., 2002). The spatial separation of JA biosynthesis suggests that the transfer of OPDA from chloroplast to peroxisome might be an important regulatory mechanism in JA biosynthesis. OPDA could be released from chloroplast membranes enzymatically, and this could account for the rapid transient increase in free OPDA and JA when leaves are wounded. The presence of a large pool of OPDA esterified to chloroplast galactolipids suggests that these pools might function as precursors for JA (Stelmach et al., 2001).

I.8. Regulation of jasmonate synthesis

Most genes encoding JA biosynthetic enzymes are coordinately activated in response to wounding or JA treatment (Li et al., 2004). Within 1 hr of treatment, transcripts encoding *DAD1* (Ishiguro et al., 2001), *LOX* (Heitz et al., 1997), *AOS* (Howe et al., 2000), *AOC* (Stenzel et al., 2003), and *OPR3* (Strassner et al., 2002) begin to accumulate. The *AOS* promoter contains motifs that are similar to other known stress response elements. GUS activity expressed from an AOS promoter-GUS fusion was induced by wounding and JA treatment (Kubigsteltig et al., 1999). Although these data indicate a positive feedback regulation in JA biosynthesis (Sivasankar et al., 2000), wound induced expression of octadecanoid pathway genes is not required for JA synthesis. Rather, substrate availability appears to play a major role in the regulation of JA biosynthesis (Ziegler et al., 2001).

I.9. Prosystemin and systemin

The systemin signaling pathway is a unique aspect of the wound response pathway in solanaceous plants. Systemin is an 18-amino-acid polypeptide that is produced at the wound sites of tomato leaves. Systemin has a role in the systemic regulation of defensive genes such as *PI*s (Ryan et al., 2000). Systemin is derived from a 200-amino-acid precursor called prosystemin (McGurl et al., 1992). Genetic 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 in tomato plants abrogated the systemic wound response (McGurl et al., 1992), whereas overexpression of the prosystemin cDNA resulted in constitutive activation of wound

response genes in unwounded plants (McGurl et al., 1994). Narváez-Vásquez and Ryan (2004) recently presented *in situ* hybridization and immunocytochemical evidence that wound- and MeJA-induced prosystemin mRNA and protein are exclusively found in vascular phloem parenchyma cells of minor veins and midribs of leaves, and in the bicollateral phloem bundles of petioles and stems of tomato. Prosystemin protein was also found in parenchyma cells of various floral organs, including sepals, petals and anthers. At the subcellular level, prosystemin was localized in the cytosol and the nucleus of vascular parenchyma cells. These data indicate that vascular phloem parenchyma cells are the sites for the synthesis and, presumably, proteolytic processing of prosystemin (Narváez-Vásquez and Ryan, 2004). Enzymes involved in the processing of prosystemin to systemin have not yet been identified.

The systemin receptor, SR160, is a member of the leucine-rich repeat (LRR) receptor kinase family (Scheer and Ryan, 2002). The interaction of systemin with SR160 activates an intracellular signaling cascade, including depolarization of the plasma membrane, the opening of ion channels, an increase in intracellular Ca^{2+}, activation of a MAP kinase activity and a PLA_2 activity. These rapid changes are thought to play important roles in the intracellular release of linolenic acid from membranes and its subsequent conversion to JA (Ryan, 2000).

II. The jasmonate signal transduction pathway

II.1. Perception of jasmonate

Similar to other plant hormones (Kende and Zeevaart, 1997), JA-mediated responses are thought to be transduced upon binding of JA to a receptor (Creelman and Mullet,

1997). The lipophilic and the volatile nature of JA has made the direct analysis of the JA receptor difficult (Creelman and Mullet, 1997). Isolation of JA response mutants has been used to identify a number of loci important for JA signaling (Table 1.1). Several of the genes defined by these mutants have been identified, including COI1 (a LRR-containing F-box protein; Xie et al., 1998), JAR1 (JA-amino synthetase; Staswick et al., 2004), and JIN1 (AtMYC2 transcription factor; Lorenzo et al., 2004). Neither of these appears to function as a JA receptor. It is possible that functional redundancy within the JA perception apparatus precludes the identification of the components by mutational analysis.

II.2. Role of ubiquitin-mediated proteolysis in jasmonate signaling

Selective proteolysis mediated by the ubiquitin-proteasome pathway plays a key regulatory role in numerous cellular processes in both animals and plants. Ubiquitin is a small polypeptide that is covalently attached to target proteins by three protein complexes called the ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). Ubiquitinated proteins are degraded by the 26S proteasome. The specificity and timing of substrate ubiquitination are primarily controlled by the E3 complexes (Voges et al, 1999).

The analysis of the *coronatine insensitive 1* (*coi1*) mutant of Arabidopsis, which is fully insensitive to JA, provides a link between JA signaling and the ubiquitin-proteasome pathway. The *COI1* gene encodes an F-box protein that is a component of an E3-type ubiquitin ligase complex referred to as SCF^{COI1} (named for the three major proteins of the complex: Skp1, Cullin, and F-box protein). This was confirmed by the

Table 1.1. Jasmonate response mutants of Arabidopsis.

Mutant	Phenotype	References
jar1	Reduced root growth inhibition by MeJA, enhanced susceptibility to *Pythium irregulare*; JAR1 belongs to the acyl adenylate-forming firefly luciferase superfamily, and it adenylates JA; JAR1 is a JA-amino synthetase that is required to activate JA for optimal signaling; allelic to *jin4*	Staswick et al., 1992; 1998; 2002; 2004
jin1	Reduced root growth inhibition by MeJA, increased resistance to necrotrophic pathogens; *JIN1* encodes AtMYC2, a nuclear-localized basic helix-loop-helix-leucine zipper transcription factor, and differentially regulates the expression of two groups of JA-induced genes	Berger et al., 1996; Lorenzo et al., 2004
jue1/2/3	Reduced *LOX2* mRNA level; not yet cloned	Jensen et al., 2002
coi1	Reduced root growth inhibition by coronatine, enhanced susceptibility to *Pythium irregulare* and *Alternaria brassicicola*, male sterility; COI1 is and LRR-containing F-box protein	Feys et al., 1994; Xie et al., 1998
cet1	Constitutive expression of the JA inducible gene *Thi2.1*; not yet cloned	Hilpert et al., 2001
cex1	Constitutive JA-responsive phenotypes including JA-inhibitory growth and constitutive expression of JA-regulated *AtVSP*, *Thi2.1* and *PDF1.2*; not yet cloned	Xu et al., 2001
cev1	Stunted roots with long root hairs, accumulated anthocyanin, constitutive expression of the defense-related genes *VSP1*, *VSP2*, *Thi2.1*, *PDF1.2*, and *CHI-B*, enhanced resistance to powdery mildew diseases; CEV1 encodes a cellulose synthase	Ellis et al., 2001; 2002
joe1/2	Enhanced sensitivity to MeJA; not yet cloned	Jensen et al., 2002

Abbreviations: *jar*, jasmonate resistant; *jin*, jasmonate insensitive; *jai*, jasmonate insensitive; *jue*, jasmonate underexpressing; *coi*, coronatine insensitive; *cet*, constitutive expression of the thionin gene; *cex*, constant expression of JA-inducible genes; *cev*, constitutive expression of *VSP1*; *joe*, jasmonate overexpressing; LRR, leucine rich repeat.

demonstration that AtCOI1 associates physically with AtCUL1, AtRbx1, and either of the Arabidopsis Skp1-like proteins ASK1 or ASK2 to assemble a functional SCF-type E3 ubiquitin ligase complex (Devoto et al., 2002; Xu et al., 2002). Moreover, plants deficient in other components of SCF complexes also show impaired responsiveness to JA (Devoto et al., 2002; Xu et al., 2002; Feng et al., 2003). The existence of a conserved COI1 homologue in other species has been demonstrated recently by the identification of the tomato *COI1* gene (*LeCOI1*) (Li et al., 2004). The presumed function of the SCF^{COI1} multiprotein complex is to attach ubiquitin to regulatory proteins that interact with the leucine-rich repeat domain of COI1. A model for JA signaling has been proposed (Creelman and Rao, 2002). In the absence of JA, JA-responsive genes are repressed by a negative regulator. Increased JA levels initiate a signaling cascade that results in modification (e.g. phosphorylation) of the negative regulator, such that SCF^{COI1} recognizes and targets this protein for degradation.

Π.3. OPDA pathway

A few studies indicated that OPDA was also active as signal without prior metabolism to JA (Howe, 2001). The Arabidopsis *opr3* mutant, which is defective in the conversion of OPDA to JA, exhibited full resistance to the dipteran *Bradysia impatiens* and the fungus *Alternaria brassicicola* (Stintzi et al., 2001). Several wound-inducible genes previously known to be JA-dependent were activated in *opr3* plants. In addition, exogenous OPDA powerfully upregulated several genes. These results indicate that the resistance of *opr3* plants is mediated by a signal other than JA, the most likely candidate being OPDA. This research concluded that OPDA works in concert with JA to fine-tune the expression of defense genes. Because resistance to insect and fungal attack can be

observed in the absence of JA, it was suggested that OPDA could fulfill some JA roles *in vivo* (Stintzi et al., 2001; Farmer et al., 2003).

III. Physiological function of jasmonates

Jasmonates play a dual role in regulating plant development and responses to numerous stresses (Creelman and Mullet, 1997; Turner et al., 2002; Rojo et al., 2003). Levels of endogenous JA are highest in young growing tissue (Creelman and Mullet, 1995) and increase after treatment with elicitors, wounding, UV light, water deficit, pathogen infection and ozone treatment (Rao et al., 2000; Rojo et al., 2003). Application of JA induces the expression of a larger number of genes that are responsive to these stress signals (Reymond et al., 2000; Cheong et al., 2002). The identification and analysis of mutants that are impaired in JA biosynthesis, signaling, and the analysis of transgenic plants with altered expression of JA biosynthetic genes or JA signaling factors have offered new insights into the function of JA in plants.

III.1. Role of JA in resistance to herbivores and pathogens

JAs play a central role in regulating plant defense (Kessler and Baldwin, 2002; Turner et al., 2002; Wasternack and Hause, 2002). So-called "direct" defenses are mediated by JA-regulated phytochemicals that interact directly with plant invaders to negatively affect their feeding, growth or reproduction. Classic examples are PIs and polyphenol oxidase (PPO), which reduce the digestibility of damaged leaf tissue (Ryan, 2000). Several lines of evidence demonstrate that JA is the main regulator for the activation of direct defenses. First, treatment of plants with exogenous JAs results in major re-programming of gene expression, including defense-related genes that are

activated by mechanical wounding and herbivore attack (Farmer and Ryan, 1992; Li et al., 2004). Second, endogenous levels of JA increase rapidly in response to wounding and other biotic stress (Penninckx et al., 1996; Lee and Howe, 2003). Third, mutants that are defective in either the JA biosynthesis or signaling are compromised in resistance to herbivores (Howe et al., 1996; McConn et al., 1997; Li et al., 2003; 2004). By contrast, constitutive activation of JA signaling results in enhanced resistance to herbivores (Li et al., 2002a). In addition to anti-herbivore defense, genetics studies in Arabidopsis showed that JA signaling promotes direct defense against fungal pathogens (Wallis and Browse, 2002).

JA also plays an important role in "indirect" defenses (Kessler and Baldwin, 2002). One of the best examples of this fascinating type of self-protection is the production of plant volatiles (e.g. terpenoids) in response to fatty acid amide elicitors found in the oral secretions of foraging lepidopteran herbivores. These emitted volatile chemicals attract natural enemies of the herbivore. Increasing evidence indicates that production and emission of volatiles in response to herbivore attack involves the host plant's JA signaling pathway (Thaler, 1999; Kessler and Baldwin, 2002). The ability of JA to induce the production of extrafloral nectar which will attract ants to fend off insect herbivores is another remarkable example of a JA-mediated indirect defense (Heil et al., 2001).

There is also some evidence to suggest that JA mediates plant-to-plant signaling (Farmer, 2001). MeJA released from sagebrush induced the expression of *PI*s in neighboring tomato plants (Farmer and Ryan, 1990), supporting the hypothesis that MeJA is a natural wound signal for interplant communication of defense responses (Karban et al., 2000). This view is supported by the demonstration that MeJA released from *A.*

tridentate inhibited the germination of nearby *N. attenuata* plants (Preston et al., 2002).

III.2. Role of JA in systemic signaling

Many induced plant responses occur both locally at the damaged site and systemically in undamaged tissues (Green and Ryan, 1972; Ryan, 2000). This implies that signals generated at the wound site travel through the plant and activate defense responses in unwounded tissue. Several chemical signals such as systemin, OPDA, JA, and MeJA, have been implicated in the systemic wound response (Ryan, 2000; León et al., 2001; Farmer et al., 2003). Although all of them induce PI accumulation in distal untreated leaves after application to one leaf (Farmer and Ryan, 1992; Ryan, 2000), grafting experiments indicate that JA is likely a long-distance wound signal for activation of defense gene expression (Li et al., 2002b; Lee and Howe, 2003). Tomato JA biosynthesis mutants (e.g. *spr2*), JA perception mutants (e.g. *jai1*), and systemin perception (e.g. *spr1*) mutants are all impaired in wound-induced systemic *PI* expression (Lightner et al., 1993; Lee and Howe, 2003; Li et al., 2003; 2004). Classical grafting techniques were used to determine whether a particular mutant is defective in the production of the systemic wound signal in wounded leaves or the recognition of that signal in unwounded leaves. Analysis of systemic wound signaling in reciprocal grafts between wild-type, *spr2*, and *jai1* plants revealed that jasmonate synthesis is needed to produce the systemic signal in wounded leaves, but is not required in systemic undamaged tissues. Conversely, JA perception is required for recognition, but not production, of the transmissible wound signal (Li et al., 2002b). These results suggest that a signaling compound derived from the octadecanoid pathway acts as a transmissible wound signal.

What then is the role of systemin in systemic wound signaling? Grafts between wild-type and *spr1* plants that are insensitive to systemin showed that systemin functions at or near the wound site to amplify jasmonate synthesis, but is likely not a long-distance signal by itself (Lee and Howe, 2003). This was confirmed by grafting experiment showing that *spr2* scions which are insensitive to systemin, can perceive the signal generated in wild-type rootstock leaves (Li et al., 2002b). These results suggest that jasmonate acts as the long-distance signal (Stratmann, 2003).

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

Exogenous JAs exert both inductive and inhibitory effects on a variety of plant developmental processes (Creelman and Mullet, 1997; Wasternack and Hause, 2002). Mutants that are defective in JA biosynthesis or perception provide an opportunity to assay the function of JA in specific development processes. One of the most pronounced effects of exogenous JA is general inhibition of growth. JA-mediated inhibition of root growth has been used as a phenotype to screen mutants having reduced sensitivity to JA (Staswick et al., 1992; Lorenzo et al., 2004) or constitutive JA signaling (Ellis et al, 2001). Another effect of JA is decreased expression of genes involved in photosynthesis and reduction in chlorophyll content. JA-mediated chlorosis and increased abscission suggest that JA may play a role in promoting senescence (Wasternack and Hause, 2002). This is consistent with the demonstration that senescence of Arabidopsis leaves is correlated with increased expression of JA biosynthetic genes and increased JA levels (He et al., 2002). However, JA biosynthesis or signaling mutants of Arabidopsis and tomato do not show obvious delayed-senescence phenotypes, suggesting that JA is not strictly required for the normal process of senescence, or that senescence-like effects induced by exogenous JA

do not accurately reflect the normal senescence program. The abnormal development of glandular trichomes in tomato *jai1* plants suggest a role of JA signaling in the promotion of glandular trichome-based defense (Li et al., 2004). Trichome production in leaves of Arabidopsis is stimulated by mechanical wounding and exogenous JA (Traw and Bergelson, 2003), indicating that trichome development is affected by JA.

JA biosynthesis and signaling mutants of Arabidopsis are all male sterile, indicating that JA is essential for male gametophyte development in this plant (Berger, 2002; Wallis and Browse, 2002). JA appears control multiple aspects of male fertility including development of viable pollen, timing of anther dehiscence, and elongation of the anther filaments. Jasmonate application experiments further demonstrated that JA is necessary and sufficient to promote these reproductive processes. However, comparable JA biosynthesis mutants in tomato display normal fertility (Li et al., 2003), and a JA signaling mutant is female sterile (Li et al., 2001; 2004). These phenotypes are consistent with the fact that wild-type tomato flowers accumulate high levels of JA (Wasternack and Hause, 2002), and that tomato JA biosynthetic mutants have significant levels of JA in floral tissue. These observations indicate that male fertility in tomato is not strictly dependent on JA. The apparent differences in the roles of JA in reproductive development in tomato and Arabidopsis indicate that JA signaling pathway regulates distinct development processes in different plants (Li et al., 2004).

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CHAPTER 2

A Lanthionine Synthetase C-Like Gene (*LeLANCL*) Is Regulated by the Jasmonate Signaling Pathway in Tomato

The cDNA Microarray analysis was done by Dr. Youfu Zhao.

Abstract

Jasmonic acid (JA) and methyl-JA (MeJA) are fatty acid-derived cyclopentanone signals that regulate a broad range of plant defense responses against herbivores and microbial pathogens. We used cDNA microarray analysis to identify and characterize genes in *Lycopersicon esculentum* (tomato) that are regulated by the JA signaling pathway. Here, we report the identification and preliminary characterization of a novel gene (*LeLANCL*) that is predicted to encode a new member of the lanthionine synthetase C-like (LANCL) family of proteins found in both plants and animals. These proteins appear to be homologous to bacterial LanC, which is part of a membrane-bound complex involved in the synthesis and transport of lantibiotic peptides that exhibit potent antimicrobial properties. We found that *LeLANCL* transcripts accumulated to their highest level in reproductive tissues of healthy tomato plants. In vegetative tissues, *LeLANCL* expression was highly induced in response to mechanical wounding and treatment with MeJA. Wound-induced expression of the gene was blocked in tomato mutants that are defective in JA biosynthesis or JA perception. These results indicate that *LeLANCL* expression is regulated by the JA signaling pathway, and further suggest that the gene could serve a role in defense against herbivores or pathogens. The physiological function of LANCL in plant growth and development is being investigated by analysis of tomato plants that are altered in *LANCL* expression.

Introduction

Unlike animals, plants are anchored to the ground and thus are unable to easily avoid injuries caused by chewing insects or larger herbivores. Pre-existing physical barriers such as the cuticle, bark, trichomes, and thorns provide one line of defense against herbivores (León et al., 2001). However, plants rely mainly on chemical barriers for protection against insect attack. Plants constitutively synthesize a broad range of secondary metabolites, including alkaloids and terpenoids, which are toxic to herbivores and pathogens (Wittstock and Gershenzon, 2002). Also, plant cells become competent for the activation of chemical defenses in responses to initial damage by an herbivore. These wound-activated responses play a role in healing of the damaged tissues and the production of chemicals that negatively affect herbivore performance. Wound-inducible genes encode proteins that have one of the following functions: (i) anti-feedant proteins such as proteinase inhibitors (PIs) and polyphenol oxidase (PPO); (ii) activation of wound-induced signaling pathways for defense; and (iii) metabolic changes such as alkaloid and terpenoid production (Kessler and Baldwin, 2002). Wounding of a single leaf activates defense mechanisms both in the tissues directly damaged (local response) and in the undamaged tissues (systemic response) (Green and Ryan, 1972). Wound-activated gene expression requires the synthesis, accumulation, and perception of jasmonic acid (JA) (Ryan et al., 1993; Li et al., 2002a), which is synthesized by the octadecanoid pathway (Vick and Zimmerman, 1984).

Lantibiotics are *lan*thionine-containing an*tibiotic* peptides (Sahl and Bierbaum, 1998). These antimicrobial compounds are characterized by the presence of the unusual amino acids lanthionine and β-methyllanthionine. These amino acids form intramolecular

thioether rings that originate from posttranslational modification of serine, threonine, and cysteine residues (Schnell et al., 1988; Sahl et al., 1995). Lantibiotics are synthesized from ribosomally made prepeptides which are encoded by the structure gene *LanA* (Meyer et al., 1995; Siegers et al., 1996). The LanA prepeptides have N-terminal leader peptide and C-terminal prepeptide regions in which the Ser, Thr, and Cys residues are posttranslationally modified to the rare amino acids by a two-step reaction (Figure 2.1) (Ingram, 1970; Sahl and Bierbaum, 1998). First, the hydroxyl amino acids Ser and Thr are dehydrated to yield didehydroalanine (Dha) and didehydrobutyrine (Dhb), respectively (Weil et al., 1990). Second, the thioethers are formed by an intramolecular Michael addition that involves the thiol groups of neighboring Cys residues and the double bonds of the didehydroamino acids. The lantibiotic modification enzyme LanB is involved in the dehydration reaction, whereas LanC catalyzes thioether formation (Figure 2.1) (Meyer et al., 1995; Koponen et al., 2002). The leader peptide is removed proteolytically from the prepeptide after the modification reactions have been completed, thus releasing the mature peptide.

Members of the lanthionine synthetase C-like (LANCL) protein family have been identified in animals and plants (Bauer et al., 2000; Mayer et al., 2001a). These peripheral membrane proteins are homologous to the bacterial LanC (Bauer et al., 2000). Two LANCLs have been characterized in detail. LANCL1 was originally isolated from human erythrocyte membranes and is mainly expressed in the brain and testis (Mayer et al., 1998). This peripheral membrane protein has also been identified in mouse and rat (Mayer et al., 2001a). Sequence analysis revealed that LANCL is similar to the bacterial LanC (Bauer et al., 2000), which is a part of membrane-associated complex involved in

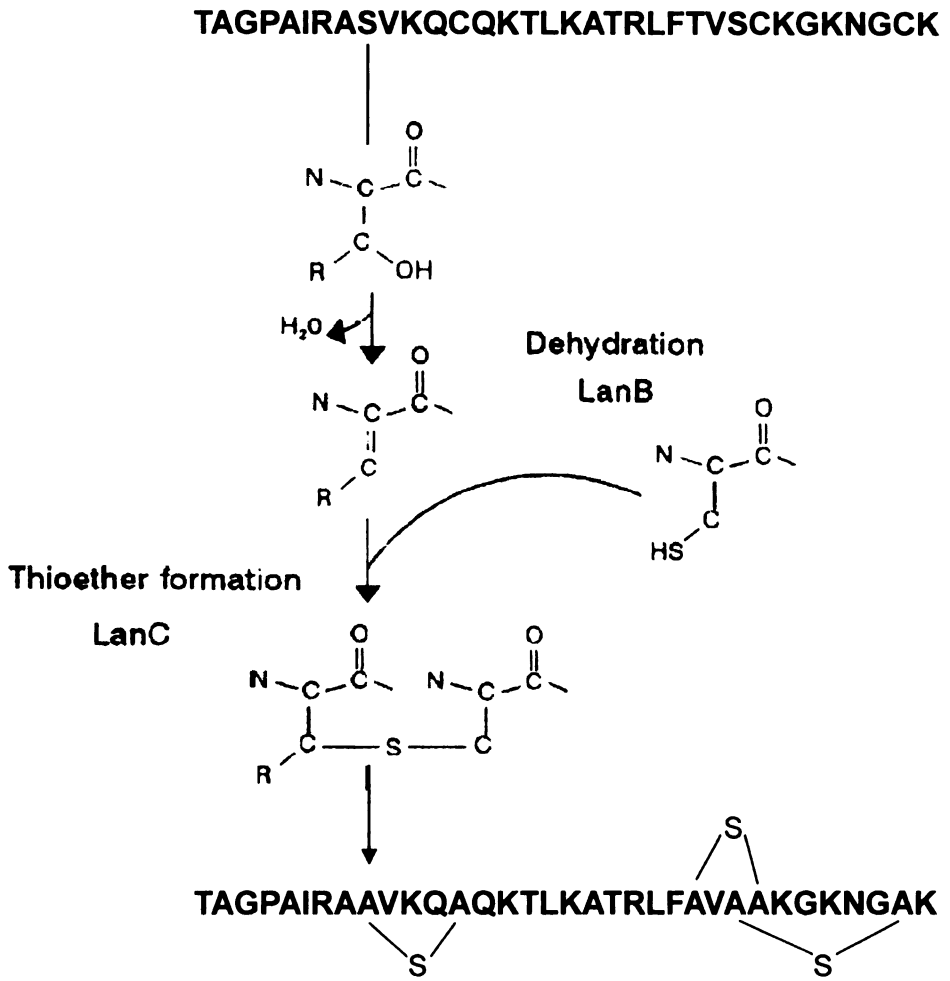


Figure 2.1. Schematic representation of the posttranslational modification of Pep5. Top, the unmodified Pep5 prepeptide. Middle, the dehydration of a hydroxyl amino acid (R=H for Ser or R=CH_3 for Thr) and the formation of the thioether (R=H for Lan or R=CH_3 for MeLan). Bottom, mature Pep5 (Modified from Sahl and Bierbaum, 1998).

the modification of peptides (Siegers et al., 1996; Kiesau et al., 1997). LANCL2 is also highly expressed in testis and brain (Mayer et al., 2001b). Its expression increases cellular sensitive to adriamycin, which is used as an anticancer drug.

The function of LANCL proteins family is not known. Based on the limited homology to bacterial LanC, most notably the seven GXXG repeats, these proteins are thought to be peptide-modifying enzyme components in eukaryotic cells. In eukaryotes, the GXXG is a signature motif within the KH module, which is a sequence motif found in a number of proteins that are found in close association with RNA (Musco et al., 1996). Structural studies suggest that the GXXG loop functions as a DNA/RNA-binding surface (Musco et al., 1996). The presence of this conserved sequence in LANCL suggests that it may function as a single-strand nucleic acid binding protein (Park and James, 2003). The high expression of LANCLs in testis and brain, organs separated by blood-tissue barriers, may hint at a role in the immune surveillance of these organs. *LANCL2* is coamplified and overexpressed with epidermal growth factor receptor (EGFR) in glioblastoma, which is the most aggressive form of primary brain tumors (Wang et al., 1998; Eley et al., 2002). Recently, Park and James (2003) reported that LANCL2 increased cellular sensitivity to adriamycin by decreasing the expression of P-glycoprotein (P-gp).

Here, we describe the identification and preliminary characterization of tomato LeLANCL, a new member of LANCL protein family. We found that *LeLANCL* expression in tomato leaves was highly induced in response to mechanical wounding and treatment with MeJA. Wound-induced expression of the gene was blocked in mutants that are defective in JA biosynthesis or JA perception. *LeLANCL* transcripts accumulated to their highest level in reproductive tissues of healthy tomato plants. These results indicate

that *LeLANCL* expression is regulated by the JA signaling pathway, and further suggest that the gene could serve a role in defense against herbivores or pathogens. The physiological function of LANCL in plant growth and development is being investigated by analysis of tomato plants that are altered in *LeLANCL* expression.

Material and Methods

Plant material and growth conditions

Lycopersicon esculentum Mill cv Castlemart and cv Micro-Tom were used as wild-type. Tomato seeds were placed on a piece of water-saturated filter paper in a shallow Tupperware box and allowed to germinate in the dark at room temperature for 4-5 days. At this time (emerging radicals were 1-1.5 cm in length), the seedlings were transferred to Jiffy peat pots (Hummert International, Earth City, MO). Seedlings were grown in a growth chamber maintained under 17 hr days at 28 ˚C with light (200 μmol $m^{-2}sec^{-1}$) and 7 hr at 16 ˚C in the darkness.

All experiments involving the *jai1* mutant were performed with homozygous (*jai1/jai1*) lines. Homozygous *jai1* seedlings were selected from F2 populations as previously described (Li et al., 2004).

Wound- and MeJA- response assay

Two-leaf-stage plants (18-day-old for cv Castlemart and 20-day-old for cv Micro-Tom) containing two fully-expanded leaves and a third emerging leaf were wounded with a hemostat across the midrib of all leaflets (typically three) on the lower leaf. Three hrs later, the same leaflets were wounded again, proximal to the petiole. Wounded plants

were incubated under standard growth conditions. At different time point after wounding, the wounded leaf (local response) and the upper, unwounded leaf (systemic response) were harvested separately for extraction of RNA. Quantification of local and systemic PI-II protein levels were measured by radial immunodiffusion assay (Ryan, 1967; Trautman et al., 1971) 24 hrs after wounding. Briefly, a 5-µl aliquot of expressed leaf juice was placed into a well (0.5 mm diameter) of an agar plate (2% (w/v) Noble agar, 0.9% (w/v) NaCl, 20 mm Tris, pH 8.5) containing 1% (v/v) polyclonal antiserum obtained from a goat that was immunized with tomato PI-II. One day later, the diameter of the immunoprecipitate ring that results from the antibody-antigen interaction was measured and used to calculate the amount of PI-II per milliliter of leaf juice. Based on a standard curve obtained using purified PI-II, the detection limit of the assay was estimated to be about 5 µg PI-II per ml of leaf juice.

MeJA treatment of tomato plants was performed by incubating two-leaf-stage plants in a sealed Lucite box in which 2 µl pure MeJA was dissolved in ethanol and distributed to several evenly spaced cotton wicks. For each time point of sampling, six plants were removed from the box for isolation of total RNA from leaf tissues. PI-II level in leaves was measured by radial immunodiffusion assay 24 hrs after MeJA treatment.

RNA isolation and gel blot analysis

RNA was isolated from tomato leaves or other tissues and analyzed by gel blot hybridization as described previously (Li et al., 2002a). RNA concentration was determined by absorbance at 260 nm. Gels were run in duplicate, with one set stained with ethidium bromide to verify RNA quality and to check for equal loading of samples.

A cDNA for tomato translation initiation factor *eIF4a* (cLED1D24) was used as the loading control. DNA probes were isolated and radiolabeled with [^{32}P-α]dCTP as described previously (Howe et al., 2000). The cDNA insert in EST clone cTOA14M17 was amplified by polymerase chain reaction (PCR) in a 100-µl reaction volume with pBluescript SK(-) primers T3 and T7. PCR products were precipitated with ethanol and re-suspended in 50 µl of elution buffer. 1 µl of this PCR product was used for probe labeling. Hybridization results were visualized by autoradiographic exposure of hybrized blots to Kodak XAR-5 film.

Identification of MeJA-induced LANCL gene

cDNA microarray experiments performed with the12K element TOM1 slide (Cornell University) identified 288 genes that are differently regulated (3-fold or greater) by the JA signaling pathway (Zhao and Howe, unpublished data). One of the genes, represented by two EST clones (cTOA14M17 and cLED6G16), was among those chosen for further analysis. The Institute for Genomic Research (TIGR) and Solanaceae Genomics Network (SGN) designation for this gene is TC101615 and SGN-U147059, respectively. EST clone cTOA14M17 was obtained from the Clemson University Genomics Institute (Clemson, SC, USA). The identity of cTOA14M17 was verified by single pass DNA sequencing at the MSU Genomics Technology Support Facility (MSU GTSF). The DNA sequence of cTOA14M17 showed that it contained a full-length cDNA fragment. We designated it as *LeLANCL*. Two genomic DNA fragments (1461-bp and 3370-bp) containing part of the *LeLANCL* gene were amplified from wild-type genomic DNA. Primers GCRF (5'- TTT CAG TTC CAT TTT CAG GAA-3') and 14M17-496R (5'-CCA TAA AGG AGG TCA TAT GAC AT) generate 3370-bp product, and primers 14M17-524

(5'-GCC CTT CCT GTT GGA CCT GAA-3') and GCRR (5'-CCT TCT GAT TGA CCT TAT ATA-3') generate 1461-bp product. After ligation into pGEM-T vector (promega), these clones were sequenced. These two overlapping fragments encompassed the full-length *LeLANCL* gene. The intron and exon organization of *LeLANCL* gene was deduced by comparison of cDNA and genome sequences.

Agrobacterium tumefaciens–mediated transformation

EST clone cTOA14M17 was digested with *XbaI* and *XhoI* to release the full-length (1.7-kb) *LeLANCL* cDNA. This fragment was subsequently cloned in sense orientation into the *XbaI* and *XhoI* sites of the binary vector pBITONY under the control of the *Cauliflower mosaic virus* (*CMV*) 35S promoter. Similarly, a cDNA fragment was released from EST cTOA14M17 by digestion of *XhoI* and *SacI* and cloned in antisense orientation into the *XhoI* and *SacI* sites of pBITONY under control of the CMV 35S promoter. These two constructs were designed as S-LANCL and AS-LANCL, respectively. The inserts and cloning junctions were sequenced to verify the construction. The S-LANCL and AS-LANCL constructs were transformed into *Agrobacterium tumefaciens* strain AGLO (Lazo et al., 1991). *Agrobacterium*-mediated transformation of tomato cotyledon explants was performed as described previously (Li and Howe, 2001; Li et al., 2003). The presence of the transgene in independently regenerated kanamycin-resistant transformants (T0) was confirmed by PCR with primer set GRCF and GCRR or primer set 14M17-524 and GCRR. The former primer set amplified 1.4-kb product corresponding to the transgene. The later primer set amplified 1.48- and 0.9-kb products corresponding to the endogenous *LeLANCL* gene and transgene, respectively. T0 lines were potted into standard soil mix and grown in a growth chamber under standard conditions. Approximately 3 weeks after

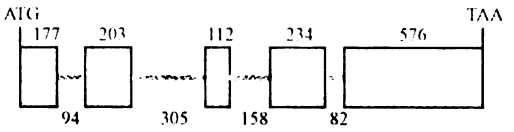
transfer to soil, plants were assayed for wound-induced PI-II accumulation. T1 seed was collected after T0 plants were transferred to greenhouse.

Results

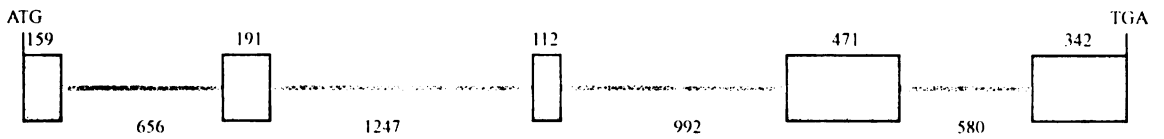
Identification of a LANCL gene in tomato

The full-length *LeLANCL* cDNA contained a 1275-base pair (bp) open reading frame (ORF) predicted to encode a 424-amino-acid protein. The genomic sequence of *LeLANCL* was determined by analysis of genomic PCR products. Comparison of cDNA and genomic sequences showed that the gene contained 5 exons and 4 introns (Figure 2.2). A BLAST search (Altschul et al., 1990) of the protein database at National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) showed that LeLANCL is similar to several lanthionine synthetase C-like (LANCL) proteins. Proteins in this family are homologous to bacterial lanthionine synthetase (LanC) (Sahl and Bierbaum, 1998), which is part of a membrane-bound complex involved in the synthesis and transport of lantibiotic peptides exhibiting potent antimicrobial properties (Siegers et al., 1996; Kiesau et al., 1997; Bauer et al., 2000; Mayer et al., 2001a).

The amino acid sequence of tomato LANCL shows significant similarity to LANCL1 and LANCL2 in human and mouse, which have been characterized in detail. A key feature of all LANCL proteins is seven repetitive hydrophobic domains containing a GXXG motif (Figure 2.3) (Mayer et al., 1998; Bauer et al., 2000), which is proposed to be a single-strand nucleic acid binding surface (Musco et al., 1996). This general repetitive structure of the seven hydrophobic domains is identical in all LanC-like protein family members and most of LanC proteins in prokaryotes (Figure 2.3). Several



At5g65280 (genome 1941bp/cDNA 1302 bp)



LeLANCL (genome 4750 bp/cDNA 1275 bp)

Figure 2.2. Gene structure of *LeLANCL* and its homolog in Arabidopsis.

Intron and exon sequences are indicated by horizontal lines and closed boxes, respectively, and are drawn to same scale. Numbers above and below each gene denote the number of nucleotides in each intron and exon, respectively. Only the translated region of the first and last exon of each gene is shown, together with the start (ATG) and stop (TAA or TGA) codons within the respective exon.

Figure 2.3. Amino acid sequence of LeLANCL and multiple alignment with other members of the LanC-like protein family and four prokaryotic LanC proteins.

Amino acid sequences were aligned by CLUSTAL W method and shaded by DNASTAR (MegAlign 5.06, DNASTAR Inc.). Identical residues are shown as white text on black background. Similar residues are shown as black text on grey background. Conserved residues are marked by black background. Two conserved glycine residues that are essential for the function of the LanC protein EpiC from *Staphylococcus epidermidis* are labeled 'G'. Two cysteine residues suggested to form part of the active site of LanC enzymes are labeled 'C'. The name, species, and GenBank access number of each proteins aligned are: LeLANCL (tomato), 7-TM-GPCR (potato, putative 7-transmembrane G-protein-coupled receptor, AAF75794), At5g65280 (*A. thaliana*, NP_201331), At1g52920 (LANCL2, *A. thaliana*, NP_175700), At2g20770 (LANCL1, *A. thaliana*, NP_850003), OSJNBa0074L08 (OSJNBa0074L08.13, rice, CAD41202), LANCL (rice, NP_922162), LANCL1 (human LANCL1, CAA72205), LANCL1 (mouse LANCL1, AAH58560), LANCL1 (rat LANCL1, XP_343585), LANCL1 (zebrafish, CAC39613), LANCL (frog, AAH51600), LANCL2 (human LANCL2, AAH70049), LANCL2 (mouse, AAH16072), EpiC (LanC, *Staphylococcus epidermidis*, S23417), NisC (LanC, *Lactococcus lactis*, Q03202), PepC (LanC, *Staphylococcus epidermidis*, S58361), SpaC (LanC, *Bacillus subtilis*, P33115).

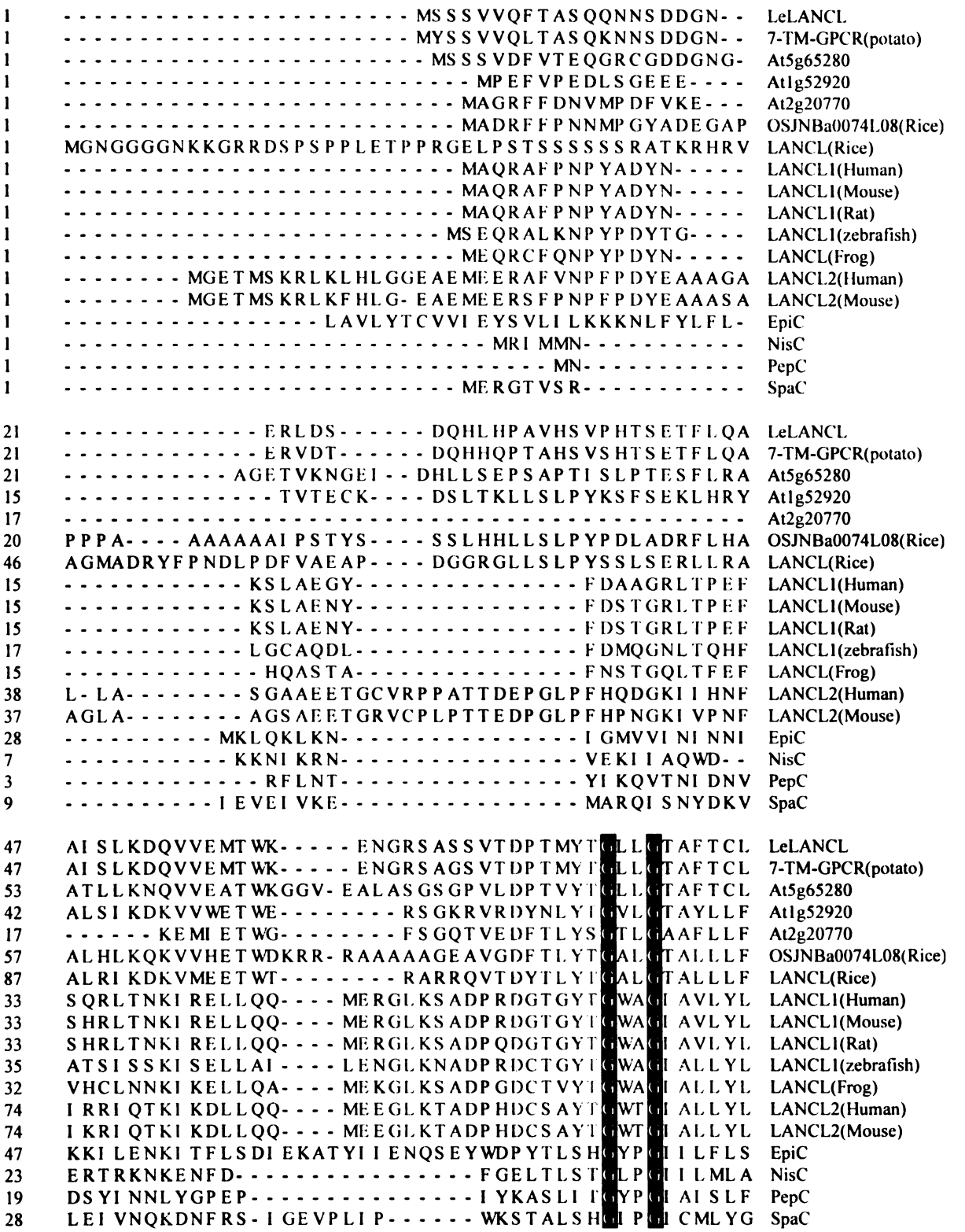


Figure 2.3. Amino acid sequence of LeLANCL and multiple alignment with other members of the LanC-like protein family and four prokaryotic LanC proteins.

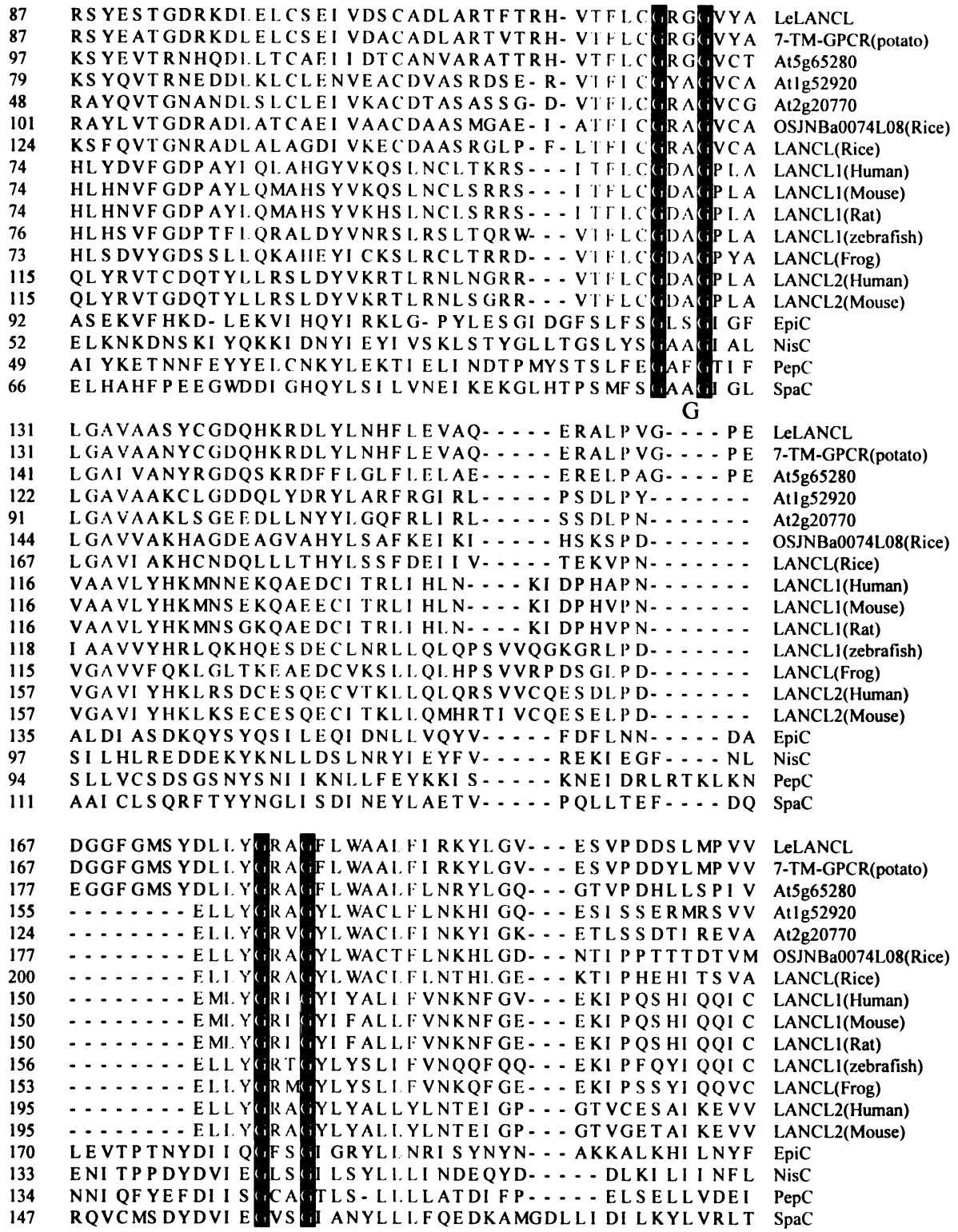
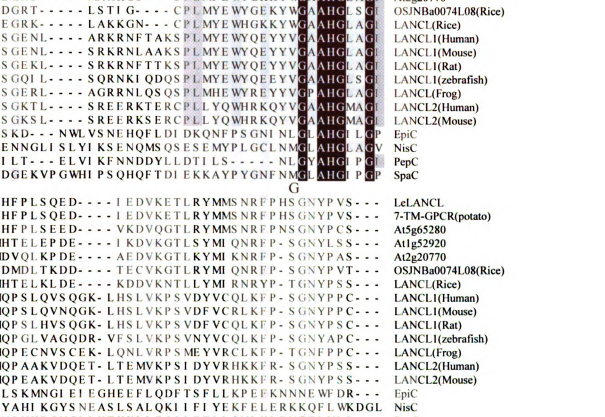


Figure 2.3. (cont'd)



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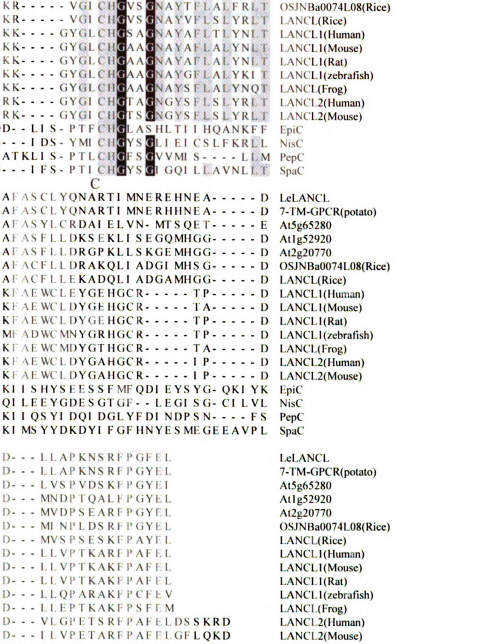


Figure 2.3. (cont'd)

residues are conserved within the single repeats (Figure 2.3). It is likely that these residues are important for the function of LanC-related proteins. The conserved Gly residues in repeats 2 and 4 (marked by "G" in Figure 2.3) were shown by mutation analysis to be essential for the function of the LanC protein EpiC (Kupke and Gotz, 1996). The Cys residues in repeats 5 and 6 (marked by 'C' in Figure 2.2) were suggested to play a role in the active site of LanC enzymes (Kupke and Gotz, 1996; Okeley et al., 2003). However, these two Cys residues are not conserved in LeLANCL (Figure 2.3). The high degree of evolutionary conservation within the LanC-like protein family suggests that these proteins play a fundamental role in animals and plants (Mayer et al., 2001a).

LeLANCL is most closely related to a gene from *Solanum chacoense* (potato; 94% identity) that is annotated as a putative 7-transmembrane G-protein-coupled receptor protein (7-TM-GPCR) (Figure 2.3 and 2.4). In Arabidopsis, three genes are similar to *LeLANCL* (Figure 2.4). Two of these, At2g20770 and At1g52920, are known to be related to LANCL1 and LANCL2, respectively (Mayer et al., 2001a). The third one, At5g65280, is more related to LeLANCL (63% identity) (Figure 2.4). Two cysteine residues in repeats 5 and 6 are not conserved in LeLANCL, At5g65280, and the *S. chacoense* protein (Figure 2.3).

LeLANCL expression is highly induced in response to MeJA and mechanical wounding

RNA gel blot analysis was used to confirm the cDNA microarray analysis showing that *LeLANCL* is positively regulated by JA. *LeLANCL* transcripts began to accumulate

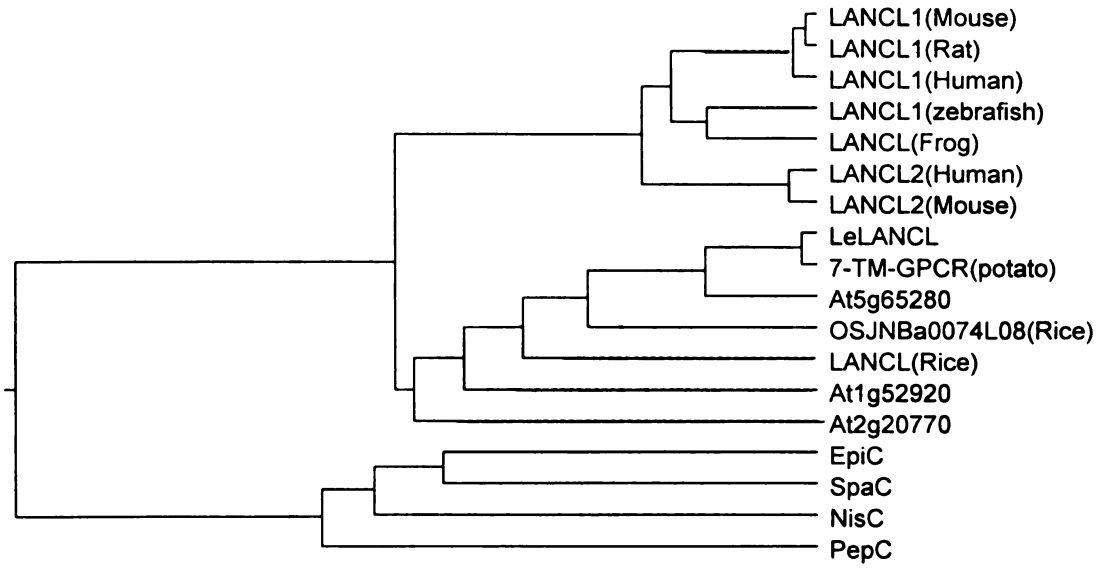


Figure 2.4. Phylogenetic relationship of LeLANCL to other LanC and LanC-like proteins.

A rooted phylogenetic tree was constructed with the MegAlign 5.06 (DNASTAR Inc.) based on the alignment by CLUSTAL W method.

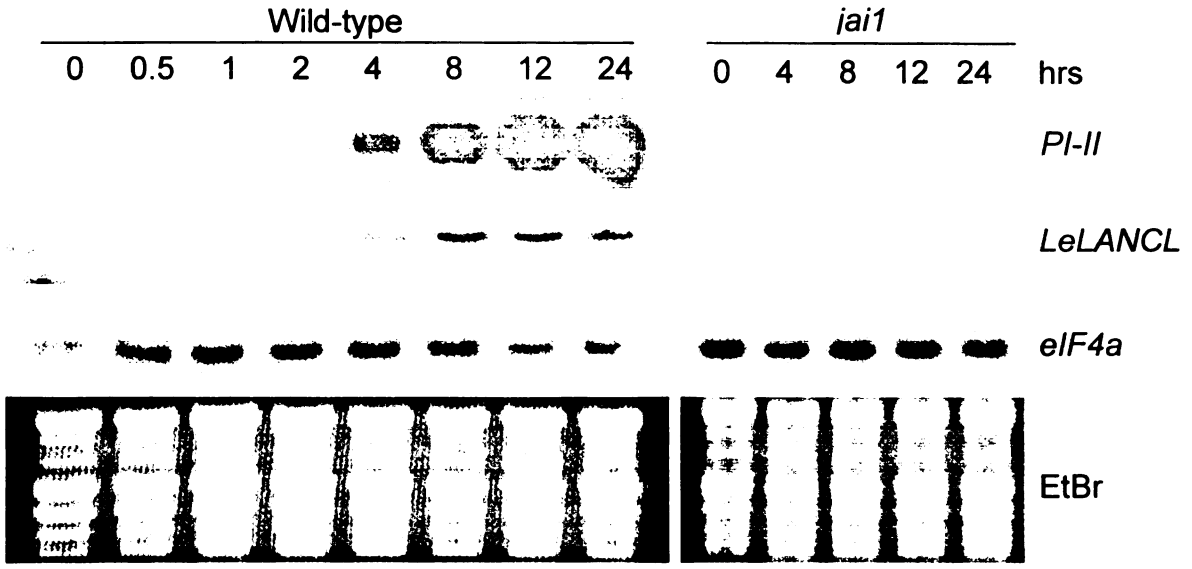


Figure 2.5. *LeLANCL* expression in response to exogenous MeJA in wild-type and *jai1* plants.

Three-week-old wild-type (cv Micro-Tom) and *jai1* (JA-insensitive mutant) plants were exposed to MeJA vapor for various lengths of time (hrs) in an enclosed box. Leaves from plants of the same genotype were pooled and harvested for RNA isolation at different time points. RNA isolated from untreated plants (0-hr time point) also was analyzed as a control. RNA gel blots were hybridized to the *LeLANCL* cDNA and the well-characterized JA response gene, *PI-II*. Blots also were hybridized to an *eIF4a* probe as a loading control. Ethidium bromide (EtBr) staining was used to verify the quality of RNA.

within 1 hr of MeJA treatment and remained elevated for at least 24 hrs (Figure 2.5). *LeLANCL* expression was not detected in untreated leaf tissue. These results indicate that the expression of *LeLANCL* in tomato leaf tissue is JA-inducible. To determine whether induced *LeLANCL* expression in leaves is dependent on a functional JA signaling pathway, we measured the expression of LeLANCL in a tomato mutant (*jai1*) that lacks COI1 (Li et al., 2004). The results showed that *jai1* plants are deficient in MeJA-induced expression of *LeLANCL* (Figure 2.5). We conclude that the expression of *LeLANCL* is induced by JA in tomato leaves in a COI1-dependent manner.

LeLANCL expression also was highly induced by mechanical wounding (Figure 2.6). *LeLANCL* was expressed 0.5 hr after wounding and transcripts reached a maximum level 1 hr after wounding. After 8 hr, expression declined to a low level. To determine whether wound-induced expression of *LeLANCL* depends on JA, expression of the gene was analyzed in the *spr2* mutant that is defective JA biosynthesis (Li et al., 2003). The results showed that *LeLANCL* expression was undetectable in *spr2* plant after wounding. Consistent with a previous study showing that *spr2* plants are fully responsive to exogenous JA and its metabolic precursors (Li et al., 2003), expression of *LeLANCL* was induced by exogenous MeJA in *spr2* plants (data not shown).

Tissue-specific expression of LeLANCL

RNA gel blot analysis was used to determine the expression pattern of *LeLANCL* in various tissues of tomato. In wild-type plants, *LeLANCL* transcripts were relatively abundant in flower, especially in mature unopened flowers and young flower buds (Figure 2.7). Lower expression was observed in stems and fruits. This highest expression

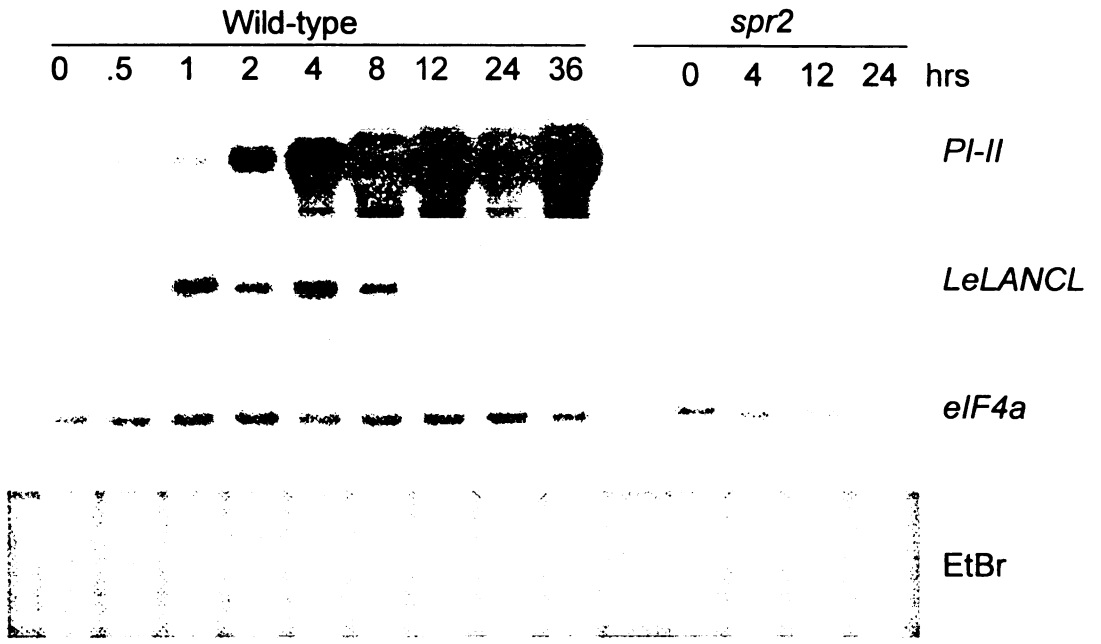


Figure 2.6. *LeLANCL* expression in response to wounding in wild-type and *spr2* plants.

Two-leaf-stage wild-type (cv Micro-Tom) and *spr2* (JA biosynthesis mutant) plants were mechanically wounded on both lower and upper leaves. Total RNA was extracted from the wounded leaf tissue at different times after wounding. RNA isolated from unwounded plants (0-hr time point) also was analyzed as a control. RNA gel blot was analyzed as described in Figure 2.5.

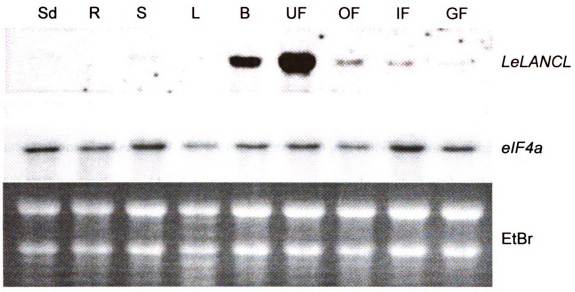


Figure 2.7. Expression pattern of *LeLANCL* in different tissues.

Blots containing total RNA from seedlings (Sd), roots (R), stems (S), leaves (L), developing flower buds (B), mature unopened flowers (UF), mature opened flowers (OF), small (<0.5cm) immature green fruits (IF), and mature green fruits (GF) from Micro-Tom plants were hybridized to a *LeLANCL* cDNA probe. Blots also were hybridized to a probe for *eIF4a* as a loading control. EtBr staining was used to verify RNA quality.

level in reproductive tissues is consistent with the fact that almost all tomato ESTs corresponding to *LeLANCL* were identified in cDNA libraries constructed from either flower or ovary mRNA (TIGR, http://www.tigr.org).

Construction and preliminary characterization of transgenic plants altered in LeLANCL expression

To begin to assess the function of LANCL in plants, we constructed transgenic tomato plants that overexpress the *LeLANCL* cDNA in either the sense or antisense orientation. The *LeLANCL* cDNA was ligated into pBITONY vector in either sense (S-LANCL) or antisense (AS-LANCL) orientation under the control of the CMV 35S promoter. *Agrobacterium*-mediated transformation was used to transform the constructs into Micro-Tom plants. Thirty-one regenerated primary (T0) lines of S-LANCL and 23 lines of AS-LANCL were obtained. PCR analysis indicated that most T0 plants contained the transgene (Figure 2.8). In 31 overexpression lines, only 2 of them (# 4 and 22) had no *LeLANCL* transgene. In 23 antisense lines, 5 of them (# 3, 10, 18, 19, and 23) had no transgene. T1 lines homozygous for the transgene were identified and characterized. PCR analysis was performed to determine the presence of transgenes in T1 plants (Figure 2.9), which showed that transgenes were separated in some T1 plants from the same T0 line. However, in other T1 plants (S-LANCL-6 and AS-LANCL-1) from same T0 line transgenes were not separated. This maybe due to the multicopy of transgenes in these T0 lines.

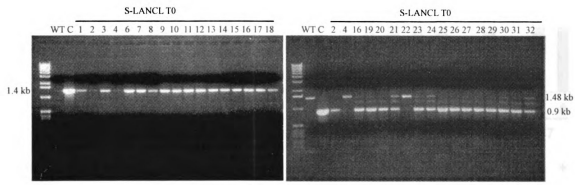
To determine whether the transgenic plants are affected in the normal wound response, wound-induced systemic PI-II expression was measured in transgenic plants.

Figure 2.8. PCR-based detection of transgenes in S-LANCL and AS-LANCL transformants.

The presence of the transgene in regenerated primary transformants (T0) was verified by PCR with primer sets GCRF and GCRR (Figure A), or 14M17-524 and GCRR (Figure B and C) in S-LANCL lines (Figure A and B) and AS-LANCL line (Figure C). Micro-Tom served as the wild-type (WT) control. S-LANCL (Figure A and B) and AS-LANCL (Figure C) constructions in pBITONY served as positive control (C) for PCR reaction. Primer set GCRF and GCRR amplified 1.4-kb products corresponding to S-LANCL transgene. The endogenous genomic PCR product was not detectable, presumably due to its large size (4.7 kb) (Figure A). Primer set 14M17-524 and GCRR amplified 1.48- and 0.9-kb products corresponding to the endogenous *LeLANCL* and transgenes (both S-LANCL and AS-LANCL), respectively (Figure B and C).

(A)

(B)



(C)

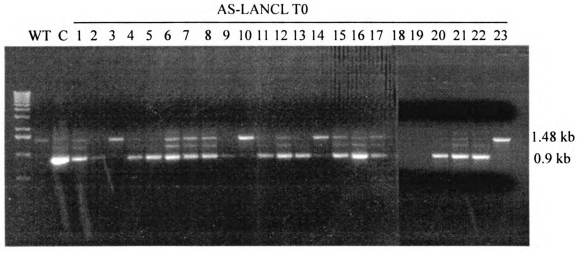


Figure 2.8. PCR-based detection of transgenes in S-LANCL and AS-LANCL transformants.

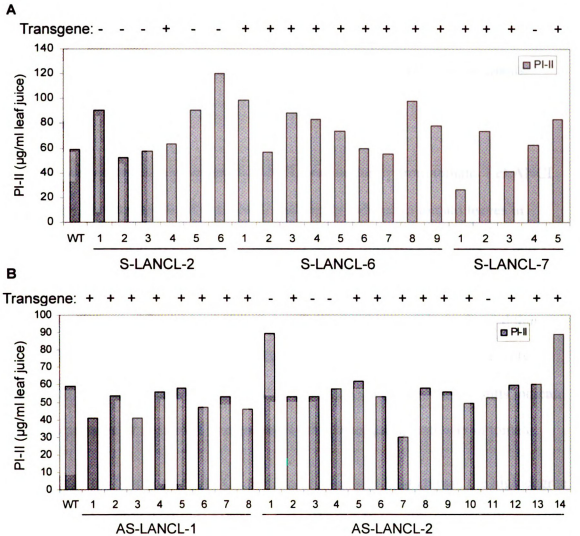


Figure 2.9. PI-II levels in *LeLANCL* transgenic plants.

Three-week-old T1 plants harboring S-LANCL (A) or AS-LANCL (B) transgenes were wounded with a hemostat across the midrib of all leaflets (typically three) on the lower leaf. Twenty-four hours later, PI-II levels were measured in upper unwounded leaves by radial immunodiffusion assay. The T1 transgenic plants were scored for the presence (+) or the absence (-) of the *LeLANCL* transgene by the PCR assay described in Figure 2.8.

Upon wounding, all S-LANCL lines and AS-LANCL lines showed wound response similar to that observed in wild-type plants (Figure 2.9). These results suggest that *LeLANCL* is not involved in regulating PI-II expression in wounded tomato plants.

Discussion

Here, we report a new member of LANCL protein family from tomato, LeLANCL. Interestingly, the expression of LeLANCL was highly induced in tomato leaves in response to mechanical wounding and treatment with applied MeJA (Figure 2.5 and 2.6). Wound-induced expression of the gene was blocked in tomato mutants that are defective in JA biosynthesis (*spr2*) or JA perception (*jai1*). RNA gel blot analysis showed that *LeLANCL* mRNA accumulated to high levels in flower tissue, which constitutively accumulates high levels of JA (Hause et al., 2000). Taken together, these results indicate that the expression of *LeLANCL* is regulated by the JA signaling pathway in tomato.

Although the function of LANCL proteins in eukaryote is unknown, the high degree of conservation within the LanC-like protein family, notably within the seven hydrophobic repeats, suggests that these proteins play a fundamental role in animals and plants (Mayer et al., 2001a). The presence of the GXXG motif in LANCL proteins suggests that those proteins may function as single-stranded nucleic acid-binding proteins (Musco et al., 1996; Park and James, 2003). Based on its homology to the LanC protein, and its wound- and JA-inducible expression in tomato leaves, LeLANCL may function as a peptide-modifying enzyme in plant defense against herbivores or pathogens.

Two Cys residues in LanC (marked by 'C' in Figure 2.3) were suggested to play a role in the active site of LanC enzymes (Okeley et al., 2003). In a working model, Okeley

et al. (2003) suggested that the two conserved cysteines may provide two of the ligands to zinc, which may function to activate the Cys thiol of the peptide substrate toward intramolecular Michael addition of the dehydroalanine and dehydrobutyrine residues. However, these two Cys residues are not found in LeLANCL (Figure 2.3). These results indicate that LeLANCL maybe have a different function than LANCL proteins in eukaryotes and LanC proteins in bacteria.

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CHAPTER 3

Characterization of the Tomato *jl1* Wound Response Mutant

Map-based cloning of *ACX1* was done by Dr. Chuanyou Li.

Dr. Sastry Jayanty measured the level of JA.

Dr. Bonnie McCaig provided the RNA used for the Northern blot in Figure 3.5.

We thank Dr. Yuichi Kobayashi for kindly providing the OPC-8:0 used in this study.

Abstract

The activation of proteinase inhibitor (PI) expression in tomato plants in response to mechanical wounding and herbivore attack is mediated by jasmonic acid (JA). JA is biosynthesized from linolenic acid (LA) by the octadecanoid pathway. Here, we report the characterization of a tomato mutant (*jl1*) deficient in wound-induced expression of PIs. Insect feeding assays showed that *jl1* plants are compromised in defense against the tobacco hornworm (*Manduca sexta*). Using a map-based cloning approach, we demonstrated that the gene defined by *jl1* encodes an acyl-CoA oxidase (ACX; named LeACX1), which catalyzes the first and rate-limiting step of fatty acid β-oxidation in the peroxisome. A function for ACX in JA biosynthesis is consistent with the widely held assumption that the final step in JA biosynthesis involves β-oxidation of 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) to JA. Consistent with this, wounded *jl1* plants accumulated normal levels of OPDA, but are deficient in the production of JA. *LeACX1* transcripts constitutively accumulated in tomato leaves, and were further induced by wounding in a JA-dependent manner. Taken together, these results show that LeACX1 plays a major role in the β-oxidation step of JA biosynthesis and resistance to herbivores.

Introduction

Many plants respond to insect attack and wounding by activating the expression of genes involved in herbivore deterrence, wound healing, and other defense-related processes. The synthesis of wound-induced phytochemicals is regulated by signal transduction pathways that act locally at the site of wounding and systemically in unwounded leaves (Green and Ryan, 1972). Several lines of evidence demonstrate that jasmonic acid (JA) plays a central role in the regulation of wound-induced defense responses (Farmer and Ryan, 1992; Conconi et al., 1996; Howe et al., 1996; Li et al., 2002b). The peptide signal systemin, which is a unique component of the wound response pathway in solanaceous plants, regulates the biosynthesis of wound-inducible defensive proteinase inhibitors (PIs) through the JA pathway (Ryan, 2000; León et al., 2001). Systemin initiates the wound signaling by binding to a 160-kD plasma membrane-bound receptor called SR160 (Scheer and Ryan, 2002). Binding of systemin to SR160 is thought to trigger the release of linolenic acid (LA) from membrane lipids (Narváez-Vásquez et al., 1999). Wound-induced production of JA in tomato can also occur by a systemin-independent pathway (Lee and Howe, 2003). The LA is metabolized to JA via the octadecanoid pathway (Farmer and Ryan, 1992; Ryan, 2000; Li et al., 2001), which is initiated in the chloroplast by addition of molecular oxygen to LA. The resulting 13S-hydroperoxylinolenic acid (13(S)-HpOTrE) is converted to 12-oxo-phytodienoic acid (12-OPDA) by the action of allene oxide synthase (AOS) and allene oxide cyclase (AOC). Subsequent reduction of OPDA in peroxisomes by OPDA reductase (OPR3) yields 3-oxo-2(2'[Z]-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0), which is shortened by three cycles of β-oxidation to yield JA (Figure 3.1).

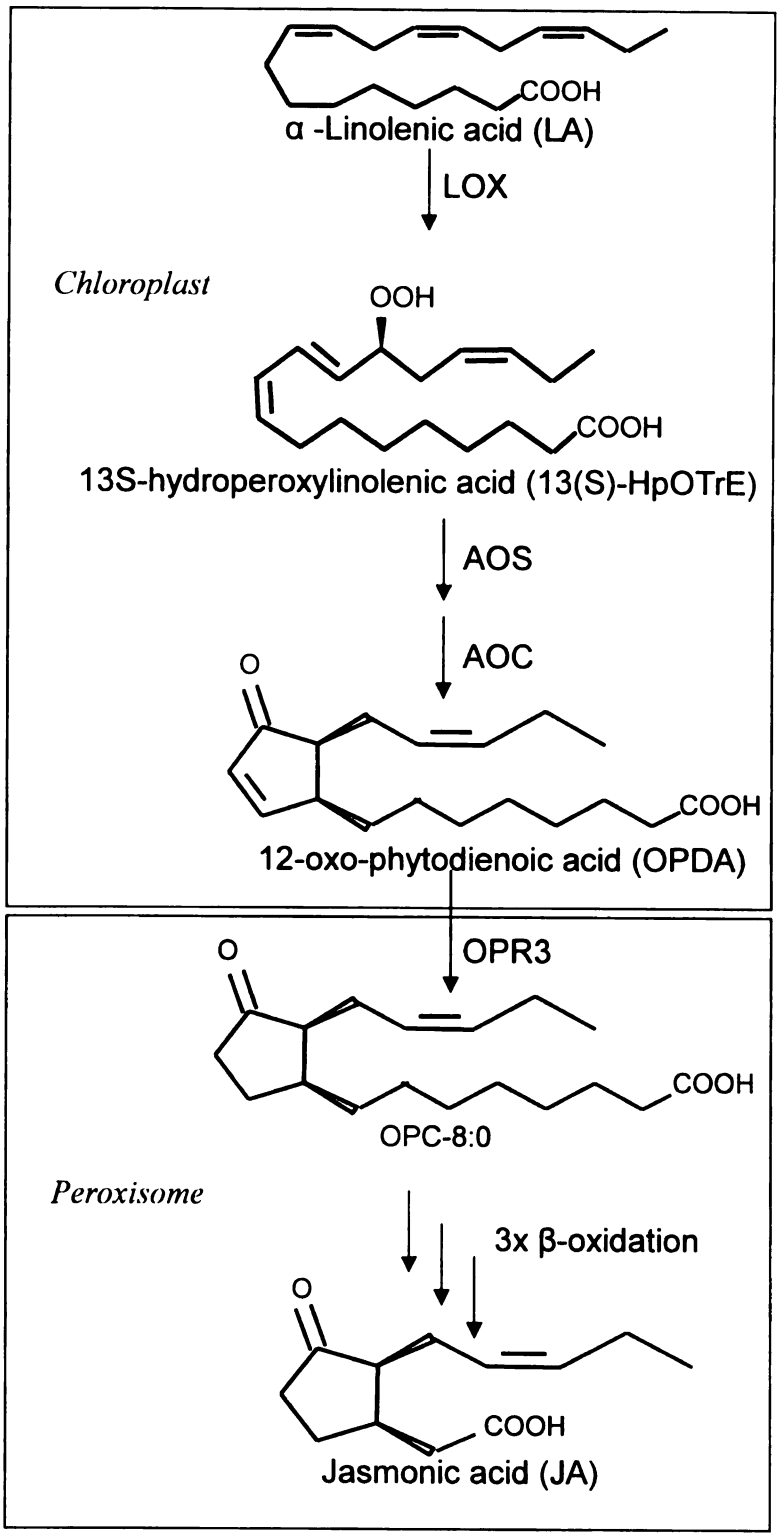


Figure 3.1. Octadecanoid pathway for JA biosysthesis.

Most enzymes in the JA biosynthesis pathway have been well studied, and the corresponding genes have been cloned and characterized (Creeman and Mullet, 1997; Ishiguro et al., 2001; Schaller, 2001; Feussner and Wasternack, 2002; Turner et al., 2002; Li et al., 2003). The exception to this is the β-oxidation enzymes in the peroxisome. It has long been proposed that OPC-8:0 undergoes three cycles of β-oxidation in the peroxisome to produce JA (Vick and Zimmerman, 1984). However, direct evidence for a role of peroxisomal fatty acid β-oxidation in JA biosynthesis is lacking.

The *jl1* mutant line of tomato was identified in a screen of EMS-mutagenized tomato plants for individuals that are defective in PI accumulation in response to mechanical wounding (Lighter et al., 1993). Exogenous MeJA was shown to restore the production of PIs in *jl1* plants, suggesting that *jl1* is defective in the biosynthesis of JA. Experiments conducted in the Howe lab showed that *jl1* plants failed to elevate JA levels in response to wounding; however, OPDA levels in wounded and unwounded *jl1* plants were comparable to those in wild-type (Lee and Howe, unpublished data). These results indicate that *jl1* plants synthesize OPDA but appear to be defective in its conversion to JA. Map-based cloning showed that *jl1* plants have a defective *ACX1* gene, which encodes an acyl-CoA oxidase involved in fatty acid β-oxidation in the peroxisome (Li and Howe, unpublished data). Here, we report the phenotypic characterization of the tomato *jl1* mutant.

Material and Methods

Plant material and growth conditions

Tomato (*Lycopersicon esculentum*) Mill cv Castlemart was used as wild-type except

where otherwise indicated. Plants were grown and maintained as described in Chapter 2. A *jl1* homozygous line was back-crossed three times to cv Castlemart as the recurrent parent.

Identification of LeACX1

The *LeACX1* gene was identified by a map-based cloning approach described previously (Li et al., 2003). Briefly, a BC1 mapping population was constructed from a cross between a homozygous *jl1* mutant (*L. esculentum*) and the wild tomato species *L. pennellii*, followed by backcross of a resulting F1 plant to *jl1*. Bulked segregant analysis (Michelmore et al., 1991; Li et al., 2003) was performed to identify amplified fragment length polymorphism (Vos et al., 1995) markers linked to *jl1*. In a mapping population of 1200 BC1 plants, the gene defined by *jl1* was mapped to a region flanked by makers GP40 and cLED14K7 on the long arm of chromosome 8. TG510 marker, which co-segregated with the target gene in all 1200 BC1 plants, was then used to screen a tomato bacterium artificial chromosome (BAC) library constructed from *L. cheesmanii* genomic DNA. Two overlapping BAC clones (166B24 and 232L13) were identified. Hybridization of BAC end sequences was used to determine the orientation and relative position of the BACs. Fine mapping studies localized the target gene to BAC232L13, which was shot-gun sequenced to five-fold coverage. Basic local alignment search tool (BLAST) searches were performed to identify candidate genes in the BAC. The strongest candidate identified was *LeACX1*.

A full-length *LeACX1* cDNA was amplified by reverse transcription-PCR (RT-PCR) of RNA isolated from wild-type and *jl1* plants. The primers were TCP1 (5'-CTG AGA

GTA AGA GAG ATG GAG-3') and TCP8 (5'-CTG GGA GGA AAA GAA GCC AAA-3'), which were designed based on *LcACX1* sequence information. The resulting RT-PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced.

Wound response assay

Two-leaf-stage plants (18-day-old seedlings containing two full expanded leaves and a third emerging leaf) were wounded with a hemostat as described in Chapter 2. At different time points after wounding, wounded leaves were harvested for isolation of RNA. PI-II levels were measured in wounded leaves (local response) and unwounded leaves (systemic response) by radial immunodiffusion assay (Ryan, 1967) 24 hrs after wounding, as described in Chapter 2.

RNA isolation and gel blot analysis

RNA was isolated from tomato leaves and analyzed by gel blot hybridization as described in Chapter 2. Gels were also stained with ethidium bromide (EtBr) to verify RNA quality. A cDNA for tomato translation initiation factor *eIF4a* (cLED1D24) was used as the loading control. A DNA fragment was amplified by PCR from EST clone cLES14H13, which contains the full-length *LeACX1* cDNA. The PCR primers were P1 (5'-GCT CTA GAG CGT AAG AGA GAT GGA GGG T-3') and P2 (5'-CGA GCT CGA ACA GTT TGC TGC AGC TCT CG-3'). The resulting ~2 kb PCR product was gel-purified and labeled with [^{32}P-α]dCTP. To directly compare transcript levels in wild-type and *jl1* plants, blots containing RNA from both genotypes were hybridized in the same container, washed under same condition, and exposed to film for the same length of time.

Elicitor feeding experiments

Systemin was provided by Dr. Ryan (Washington State University). 13(S)-HpOTrE and 12-OPDA were purchased from Cayman Chemical (Ann Arbor, MI). OPC-8:0 was provided by Dr. Kobayashi (Tokyo Institute of Technology, Japan; Ainai et al., 2003). (±) JA was purchased from Sigma (St. Louis, MO). These compounds were supplied to tomato plants through their cut stems. Briefly, two-leaf-stage plants were excised at the base of the stem with a double-edge razor blade (Ted Pella, Inc.), and immediately placed into 0.5 ml centrifuge tubes containing various amounts of the elicitor diluted in 300 µl of 15 mM sodium phosphate buffer (pH 6.5). Two excised plants were placed in one tube. Because 13(S)-HpOTrE, OPDA, OPC-8:0 and JA were originally dissolved in ethanol, the mock control solution contained same amount of ethanol. When 90% of the elicitor solution was imbibed (approximately 50 mins), the plants were transferred to glass vials containing ~20 ml of distilled water. Plants were kept in a sealed Lucite box in a growth chamber under normal conditions. Leaves were harvested at different time points after elicitor treatment for isolation of total RNA. PI-II levels in leaves were measured 24 hrs after treatment.

Tobacco hornworm feeding trials

Tobacco hornworm (*Manduca sexta*) eggs and Ready-To-Use Hornworm Diet were obtained from Carolina Biological Supply Company (Burlington, NC). Eggs were hatched at 27°C under continuous light as recommended by the supplier. Hatched larvae were reared on the artificial diet for 3 days before being transferred to tomato plants.

In experiment 1, 11-newly hatched larvae were placed on leaves of each of 5

separately potted 6-week-old wild-type and *jl1* plants. The average weight of larvae at the beginning of the feeding trial was 18 mg. After 10 days of feeding, larvae were recovered from wild-type and *jl1* plants, and the weight of each larva was measured. PI-II levels in damaged and undamaged leaf tissue were measured at that time.

In experiment 2, 60-newly hatched larvae were placed randomly on leaves of 20 4-week-old plants of each genotype. Each plant genotype was grown in a separate flat. The average weight of larvae at the beginning of the feeding trial was 15 mg. Larvae were allowed to move freely between plants of the same genotype. After 3 days of feeding, larvae were recovered from each genotype. PI-II levels and larvae weight were measured as described in experiment 1. Damaged and undamaged leaf tissue was collected for RNA isolation.

Measurement of jasmonic acid

Tomato leaf tissues were collected and immediately frozen in liquid nitrogen. JA was extracted and as according to the method described by Schmelz et al. (2003) and Engleberth et al. (2003). Dihydrojasmonic acid was added to samples as an internal standard. Methylated carboxylic acids from plant samples were volatilized and collected on volatile collection traps® (VCT) (Analytical Research Systems Gainesville, Florida). Samples were eluted from the VCT resin by methylene chloride and subsequently analyzed by gas chromatography-mass spectroscopy (GC-MS). GC-MS analysis was performed by selected ion monitoring, with isobutane chemical ionization as described (Schmelz et al., 2003). The GC-MS system consisted of a 6890 Network GC connected to a 5973 inert Mass Selective Detector (Agilent, Palo Alto, CA, USA).Compounds were

separated on a HP5MS column (30m x 0.25mm x 0.25um). The temperature regime for GC was 40°C for a minute after injection, followed by sequential temperature ramps of 25 °C/min to 150 °C, 5 °C/min to 200 °C, 10 °C ramp to 240 °C. The 240 °C temperature was maintained for 10 minutes.

Results

The jl1 mutant of tomato has a defective ACX1 gene

In a map-based cloning experiment performed by Dr. Chuanyou Li, two genes (designated as *ACX1* and *ACX2*, respectively) were considered to be candidates for the gene that is defective in *jl1* plants (Figure 3.2.A; B). cDNA sequences corresponding to *ACX1* were obtained from wild-type *L. esculentum* and *jl1* by reverse transcription-PCR (RT-PCR). Sequence comparison of cDNA clones revealed that *jl1* plants harbor a single base mutation in *ACX1* that changes the nucleotide at position 414 of the ORF from a C to a T. This mutation is predicted to change Thr139 to an isoleucine (I). This mutation was confirmed in genomic sequences from wild-type and *jl1* plants. Alignment of ACX proteins from various plants and animals showed that Thr139 is conserved in all ACXs (Figure 3.2.C), suggesting a functional importance of this residue (Nakajima et al., 2002). Indeed, the crystal structure of rat ACX showed that this Thr is involved in binding the FAD co-factor (Nakajima et al., 2002). Comparison between genomic and cDNA sequences showed that the *LeACX1* gene contains 14 exons and 13 introns (Figure 3.2.B). The C-to-T substitution in the mutant occurs within the fourth exon (Figure 3.2.B).

Expression of wound response genes is reduced in jl1 plants

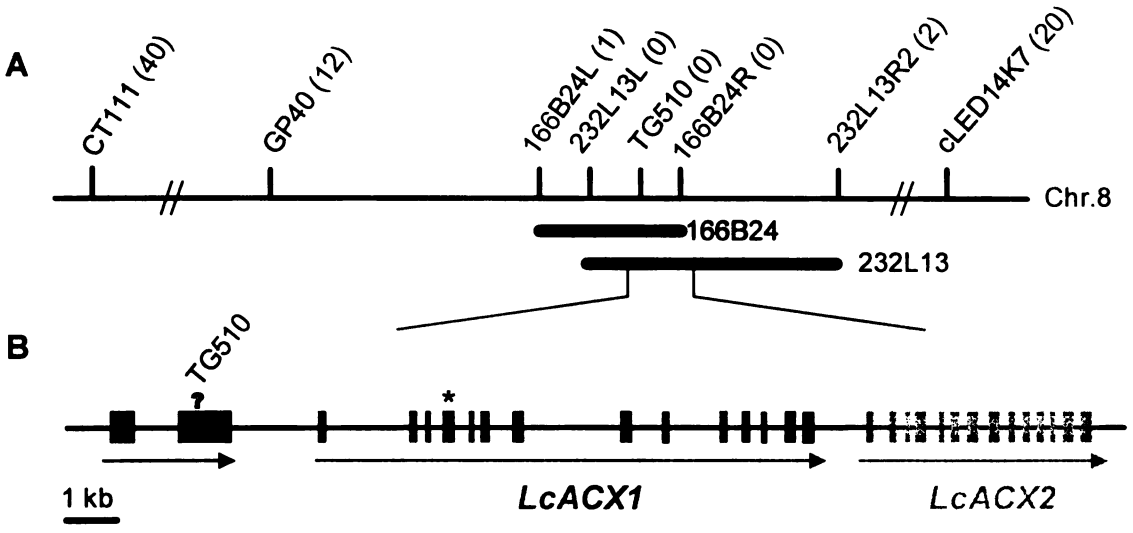
Wound responsive genes in tomato can be divided into two classes based on their

Figure 3.2. Map-based cloning of the *ACX* gene.

(A) Genetic and physical map of *LeACX1*. The mutation was mapped to a region between RFLP markers GP40 and cLED14K7 on chromosome 8. The gene's location was narrowed down to a region encompassed by overlapping BAC clones (166B24 and 232L13). Numbers in parentheses indicate the number of recombinants identified between markers and the target gene.

(B) Structure of three genes (designated TG510, *LcACX1* and *LcACX2*) identified on BAC323L13. Filled boxes represent exons and lines between boxes represent introns or intergenic regions. TG510 is a RFLP marker that co-segregates with LeACX1 in 1200 BC1 plants.

(C) Alignment of the FAD binding region of various ACXs from plants and animals. The *jl1* mutation changes the threonine (T) to an isoleucine (I).



C

Tomato ACX1	131	IIGCYAQ	T	ELGHGSNVQGLETTATFD 156
Arab ACX1	131	IIGCYAQ	T	ELGHGSNVQGLETTATFD 156
Pumpkin ACOX	177	YPGCFAM	T	ELHHGSNVQGLQTTATFD 202
Rat ACO	132	ITGTYAQ	T	EMGHGTHLRGLETTATYD 157
Human BACO	147	IIATYAQ	T	ELGHGTYLQGLETEATYD 172
Yeast ACO	183	IYGCFAM	T	ELGHGSNVAQLQTRAVYD 208
Human MCDH	154	LMCAYCV	T	EPGAGSDVAGIKTKAEKK 179
Human LCDH	166	CIGAIAM	T	EPGAGSDLQGIKTNAKKD 191
Human VLCDH	210	TVAAFCL	T	EPSSGSDAASIRTSAVPS 235

Figure 3.2. Map-based cloning of the *ACX* gene.

temporal and spatial pattern of induction (Ryan, 2000; Lee and Howe, 2003). Transcripts of so-called "early" response genes accumulate rapidly (within 1 hr) and transiently in response to wounding. These include genes encoding JA biosynthetic enzymes such as lipoxygenase D (LoxD; Heitz et al., 1997), allene oxide synthase 2 (AOS2; Howe et al., 2000), 12-OPDA reductase 3 (OPR3; Strassner et al., 2002), as well as other signaling components such as prosystemin (PSYS; Jacinto et al., 1997). By contrast, mRNA transcribed from "late" response genes begins to accumulate locally and systemically about 2 hrs after wounding. These genes mostly encode defense related proteins, including proteinase inhibitor I and II (PI-I, PI-II; Graham et al., 1986), and cathepsin D inhibitor (CDI; Hildemann et al., 1992). To investigate the expression pattern of wound-induced genes in *jl1* plants, we determined the temporal expression pattern of representative "early" and "late" genes, as well as *LeACX1*. Local expression of the late response genes (*PI-II* and *CDI*) was detected in wild-type plants within 2 to 4 hrs of wounding, with transcript levels reaching a maximum 12 hrs after wounding (Figure 3.3). Transcripts representing two early response genes (*LoxD* and *OPR3*) accumulated in wild-type plants 0.5 to 1 hr of wounding, and the expression level declined 2 hrs after wounding. Similar to *LoxD* and *OPR3*, *LeACX1* mRNA accumulated in wild-type leaves 1 hr after wounding, and reached maximal levels 2 hrs after wounding. *LeACX1* transcripts declined to basal level 12 hrs after wounding. These results indicated that *LeACX1* behaves as an "early" wound response gene, consistent with its role in JA biosynthesis. Wound-induced expression of late response genes was undetectable (*CDI*) or was reduced to a very low level (*PI-II*) in *jl1* plants. Early response gene transcripts (*LoxD*, *OPR3*, *LeACX1*) were induced by wounding in *jl1* plant, although at a level that

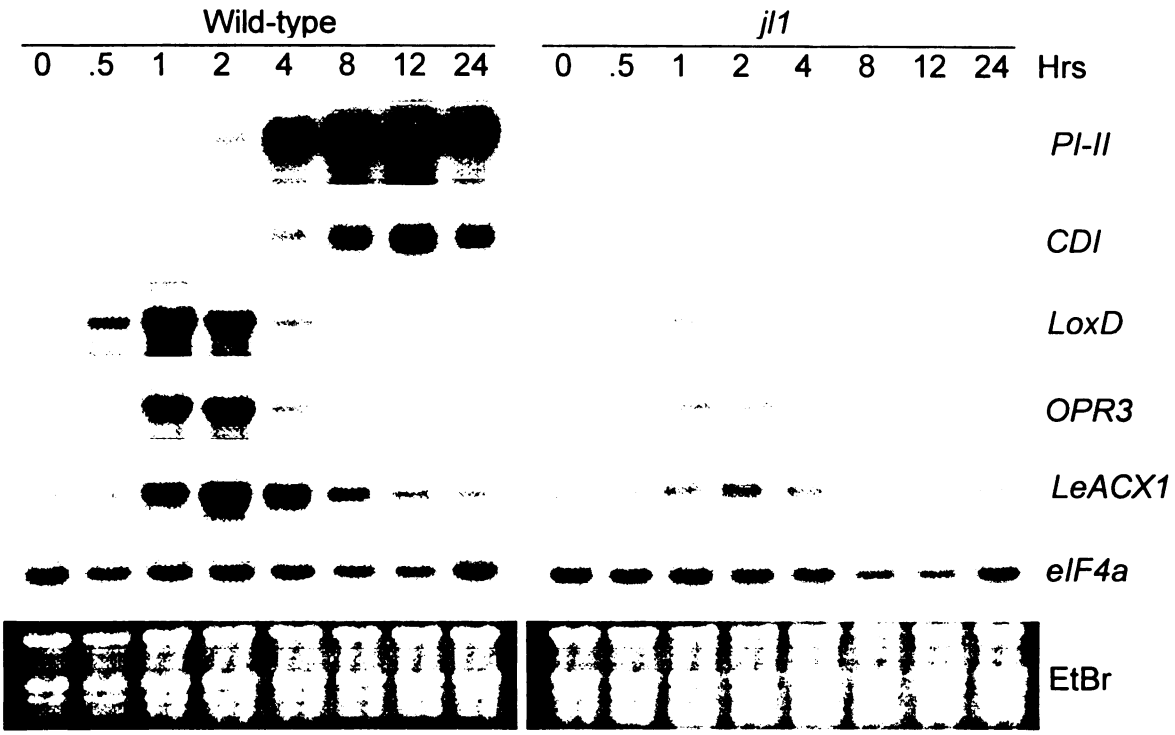


Figure 3.3. Gene expression in wild-type and *jl1* plants in response to mechanical wounding.

Tomato seedling (cv Castlemart and *jl1*) at the two-leaf-stage were wounded with a hemostat on both lower and upper leaves. Total RNA was isolated from the wounded leaves at various times after wounding. RNA was prepared from unwounded plants (0 time) as a control. cDNA probes representing different classes of wound responsive genes and *LeACX1* were used for hybridization, as shown on the right of the figure. *eIF4a* was the loading control. EtBr staining was used to verify the quality of RNA.

was approximately 20% of wild-type. These results are consistent with the expression pattern of wound response genes in the tomato *jai1* mutant that lacks JA perception (Li et al., 2004), and indicate that the expression of early genes is controlled by both JA-depended and JA-independed pathway.

The jl1 mutant is defective in resistance to tobacco hornworm

Previous studies have established that the octadecanoid pathway for JA biosynthesis plays an important role in defense of tomato against a broad spectrum of herbivores (Howe et al., 1996; Li et al., 2002a; 2003). The inability of *jl1* plants to express significant levels of defensive PIs in response to mechanical wounding suggested that this mutant might be compromised in resistance to herbivorous insects. To test this possibility, 6-week-old (experiment 1) or 4-week-old (experiment 2) wild-type and *jl1* plants were challenged with tobacco hornworm larvae. After termination of the feeding trial, we assessed the weight of larvae, the amount of leaf damage, and the level of PI-II in leaves of both genotypes. Hornworm feeding on wild-type plants resulted in accumulation of high levels of PI-II in both damaged and undamaged leaves (Table 3.1). In contrast, little or no PI-II accumulation was detected in damaged and undamaged *jl1* leaves. RNA gel blot analysis showed that the expression of defense-related genes (*CDI* and *PI-II*) was induced by hornworm attack both locally (damaged) and systemically (undamaged) in wild-type but not in *jl1* plants (Figure 3.4). Similar to mechanical wounding, *jl1* plants did express early response genes (*LoxD* and *OPR3*) locally and systemically when attacked by hornworm larvae, although the level of expression was much lower than that in wild-type.

Table 3.1. Tobacco hornworm feeding assay with wild-type and *jl1* plants.

Experiment[a]	Genotype	Damaged leaf PI-II (μg/ml)	Undamaged leaf PI-II (μg/ml)	Larval weight (g)[b]
1	Wild-type	225 ± 47	199 ± 66	0.99 ± 0.41 (n=38)
	jl1	0	0	3.45 ± 1.68 (n=42)
2	Wild-type	141 ± 73	151 ± 26	0.10 ± 0.04 (n=38)
	jl1	15 ± 24	0	0.19 ± 0.05 (n=41)

[a] In experiment 1, 11 newly-hatched larvae were placed on leaves of each of 5 separately potted 6-week-old wild-type and *jl1* plants. In experiment 2, 60 newly-hatched larvae were placed randomly on leaves of 20 4-week-old plants of each genotype, in separate flats. Larvae were allowed to move freely between plants of the same genotype. Experiment 1 and 2 were terminated 10 and 3 days after the start of feeding trial, respectively. At that time, larvae were recovered from plants and their weight was measured. PI-II levels in damaged and undamaged leaf tissue also were measured. Data represent mean ± standard deviation.

[b] In both experiments, the weight of larvae grown on wild-type and mutant plants was significantly different at $P<0.001$ (Student's *t* test).

Interestingly, hornworm feeding activated *LeACX1* expression locally and systemically in both wild-type and *jl1* plants (Figure 3.4). To determine whether the induction of *LeACX1* in response to hornworm attack was dependent on JA signaling, *LeACX1* expression was assessed in the JA insensitive *jai1* mutant that was challenged with hornworms for 4 days. RNA gel blot analysis showed that *jai1* plants accumulated a basal level of *LeACX1* transcript, and this accumulation was not affected by hornworm attack (Figure 3.5). These results indicate that the basal expression of *LeACX1* is controlled independently of JA, whereas induced expression by mechanical wounding or hornworm attack requires a functional JA signaling pathway.

In addition to these effects on gene expression, we also found that *jl1* plants were defoliated by tobacco hornworms much faster than wild-type plants (Figure 3.6). The average weight of larvae grown on *jl1* plants was about 3.8-fold (experiment 1) and 2-fold (experiment 2) greater than that of larvae reared on wild-type plants for the same period of time (Table 3.1). These results indicate that *jl1* compromises the tomato's defense against herbivorous insects. As a result, foliage from *jl1* plants is a better food source for hornworm larvae.

Response of jl1 plants to exogenous signaling compounds

Previous studies have shown that exogenous systemin and various intermediates in the octadecanoid pathway activate the biosynthesis of JA leading to accumulation of defensive PIs (Farmer and Ryan, 1992; Lee and Howe, 2003). The defect in ACX1 in *jl1* plants suggested that elicitors acting upstream of JA would be unable to induce the accumulation of PIs in *jl1* plants. To test this prediction, we supplied plants with systemin

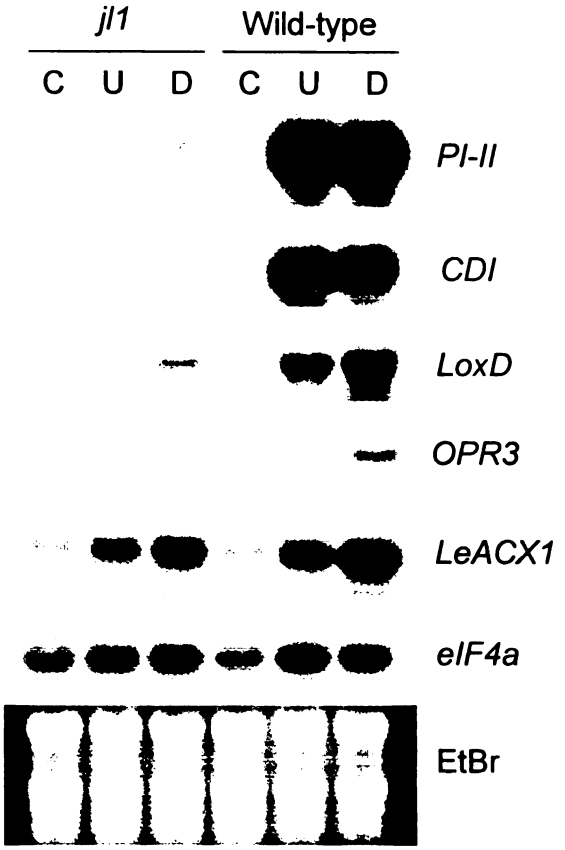


Figure 3.4. Accumulation of wound-induced transcripts in response to tobacco hornworm attack.

Newly hatched tobacco hornworm larvae were allowed to feed on wild-type and *jl1* plants for 3 days (experiment 2 in Table 3.1). Total RNA was isolated separately from hornworm damaged (D) and undamaged (U) leaves of the same genotype. RNA from leaf tissue of unattacked plants was prepared as control (C). RNA blots were hybridized to cDNA probes indicated on the right. *eIF4a* was used as loading control, and EtBr staining was used to verify the quality of RNA.

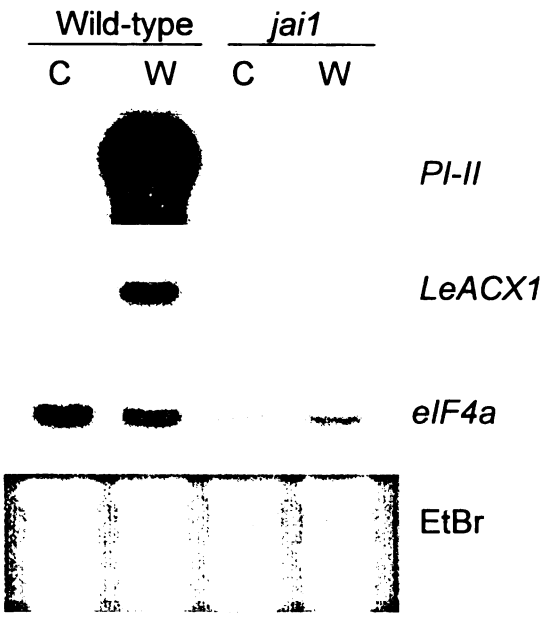


Figure 3.5. Expression of *LeACX1* in wild-type and *jai1* plants in response to tobacco hornworm attack.

Newly-hatched tobacco hornworm larvae were grown on 6-week-old wild-type (cv Micro Tom) and *jai1* plants for 4 days. Total RNA was isolated from hornworm damaged plants (W, both damaged and undamaged leaves) of the same genotype. RNA from leaf tissue of unwounded plants was prepared as control (C). *eIF4a* was used as a loading control, and EtBr staining was used to verify the quality of RNA.

Figure 3.6. Challenge of wild-type and *jl1* plants with tobacco hornworm larvae.

Eleven newly-hatched larvae (about 18 mg each) were placed on leaves of each of 5 separately potted wild-type and *jl1* plants (6-week-old). Larvae were allowed to feed for 10 days.

(A) Representative wild-type and *jl1* plants at the end of the feeding trail.

(B) Hornworm larvae recovered from wild-type and *jl1* plants at the end of feeding trail.

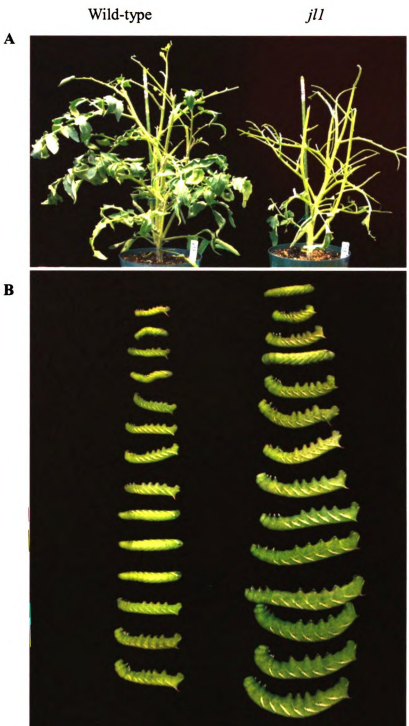


Figure 3.6. Challenge of wild-type and *jl1* plants with tobacco hornworm larvae.

(Image in this thesis is presented in color)

or with other octadecanoid pathway compounds including 13-HpOTrE, OPDA, OPC-8:0 and JA. Surprisingly, all elicitors tested induced the accumulation of PI-II in *jl1* plants (Table 3.2). This was confirmed by RNA gel blot analysis, which showed that the *PI-II* transcript accumulated in *jl1* plants supplied with OPDA (Figure 3.7). We also found that the early wound response gene *LoxD* was induced by OPDA treatment in both wild-type and *jl1* plants, but the level in *jl1* was much lower than those in wild-type. mRNA accumulation observed in buffer-treated plants reflects the effect of cutting (Lee and Howe, 2003). Notably, *jl1* plants showed no expression of PI-II in response to cutting.

To test the effect of different concentration of JA precursors on induction of PI-II expression in *jl1* plants, we supplied wild-type and *jl1* plants with different amounts of OPDA and OPC-8:0. Five nmol of each elicitor per plant was sufficient to trigger the accumulation of PI-II in both wild-type and *jl1* plants (Figure 3.8). PI-II levels in wild-type and *jl1* plants were generally correlated with the concentration of elicitors (Figure 3.8). These results indicate that exogenous OPDA and OPC-8:0 induce PI expression in *jl1* plants, despite the fact that this mutant is defective in LeACX1.

To determine if OPDA induction of PI-II in *jl1* plants is JA-dependent or JA-independent, we measured the level of JA after OPDA feeding through the cut stem. Wild-type plants accumulated low levels of JA in response to buffer, but produced 198.1 ± 9.6 pmol JA/g FW within 1 hr after application of OPDA. This level declined to the basal level 3 hrs after feeding. However, JA levels in OPDA treated *jl1* plants were <5% of that in wild-type (Figure 3.9). These finding indicated that ACX1 is strictly required for JA accumulation in response to exogenous OPDA.

Table 3.2. PI-II levels in leaves of wild-type and *jl1* plants in response to different JA precursors.

Genotype	Buffer	Systemin	HpOTrE	OPDA	OPC-8:0	JA
Wild-type	18 ± 10	123 ± 60	88 ± 32	96 ± 26	118 ± 22	187 ± 25
jl1	3 ± 4	66 ± 29	67 ± 34	122 ± 25	83 ± 43	177 ± 19

18-day-old wild-type and *jl1* seedlings were supplied through their cut stems with a buffer control (15 mM sodium phosphate, pH 6.5), or with elicitors dissolved in the buffer. The elicitors concentration used were as follows: systemin (5 nmol/plant), HpOTrE (25 nmol/plant), OPDA (10 nmol/plant), OPC-8:0 (25 nmol/plant), and JA (10 nmol/plant). Excised plants were incubated in the elicitor solution for about 50 min to allow uptake of the elicitor, and then transferred to glass vials containing distilled water. PI-II levels (μg/ml leaf juice) in leaves were measured 24 hrs after treatment. Values represent the mean and standard deviation of 6 plants.

The difference in the response between wild-type and *jl1* plants was not statistically significant (P value for buffer control is 0.03; P values range from 0.1 to 0.4 for elicitor feeding.)

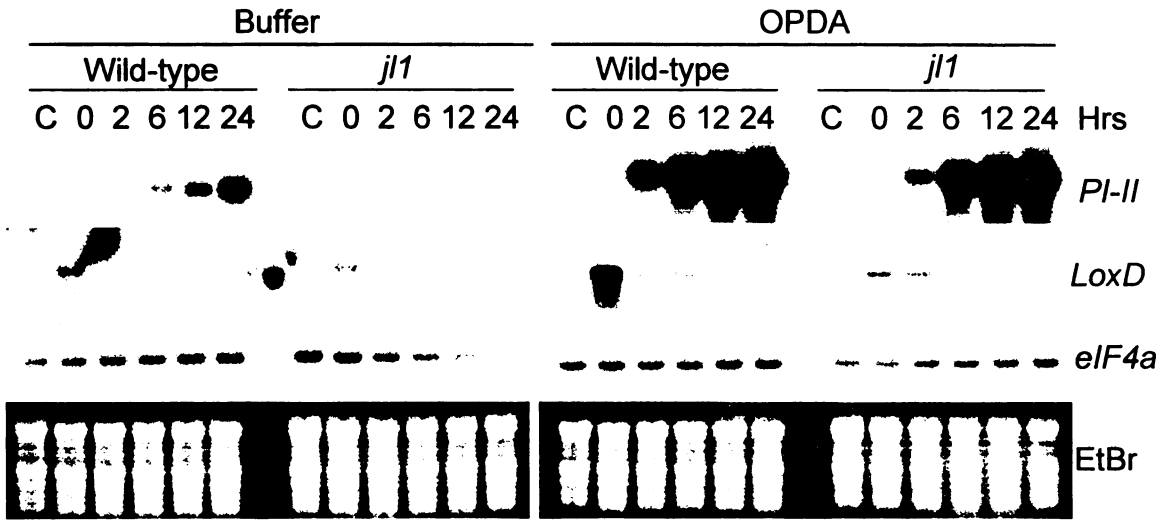


Figure 3.7. Effect of OPDA feeding on the expression of various wound-responsive genes.

Eighteen-day-old wild-type and *jl1* seedlings were supplied through their cut stems with a buffer control (15 mM sodium phosphate, pH 6.5) or OPDA (25 nmol/plant, dissolved in buffer) as described in Table 3.2. Leaf tissue of 8 plants was harvested and pooled at each sampling time point after OPDA treatment. RNA was prepared from untreated plants (C) as a control. *LoxD* and *PI-II* cDNA probes were used for hybridization. *eIF4a* was the loading control, and EtBr staining was used to verify the quality of RNA.

Figure 3.8. Dose effect of OPDA and OPC-8:0 on induction of PI-II in wild-type and *jl1* plants.

Eighteen-day-old wild-type and *jl1* seedlings were supplied with phosphate buffer (15 mM sodium phosphate, pH 6.5) containing various amounts of OPDA (A) and OPC-8:0 (B) as described in Table 3.2. PI-II levels in leaves were measured 24 hrs after treatment. Date represent the mean and standard deviation of 6 plants.

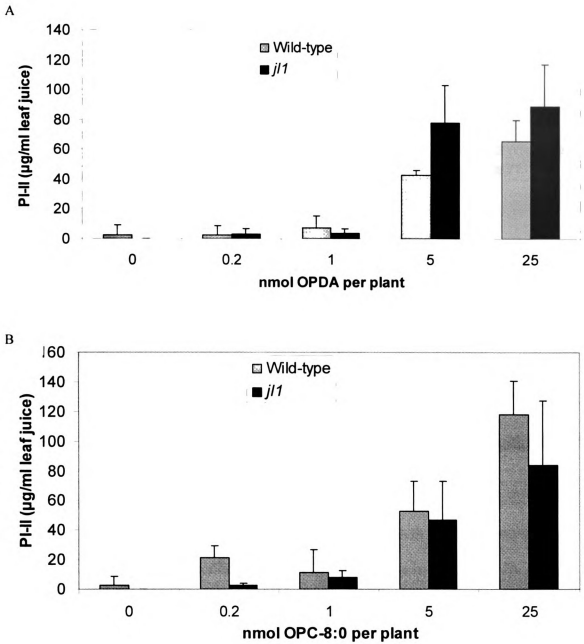


Figure 3.8. Dose effect of OPDA and OPC-8:0 on induction of PI-II in wild-type and *jl1* plants.

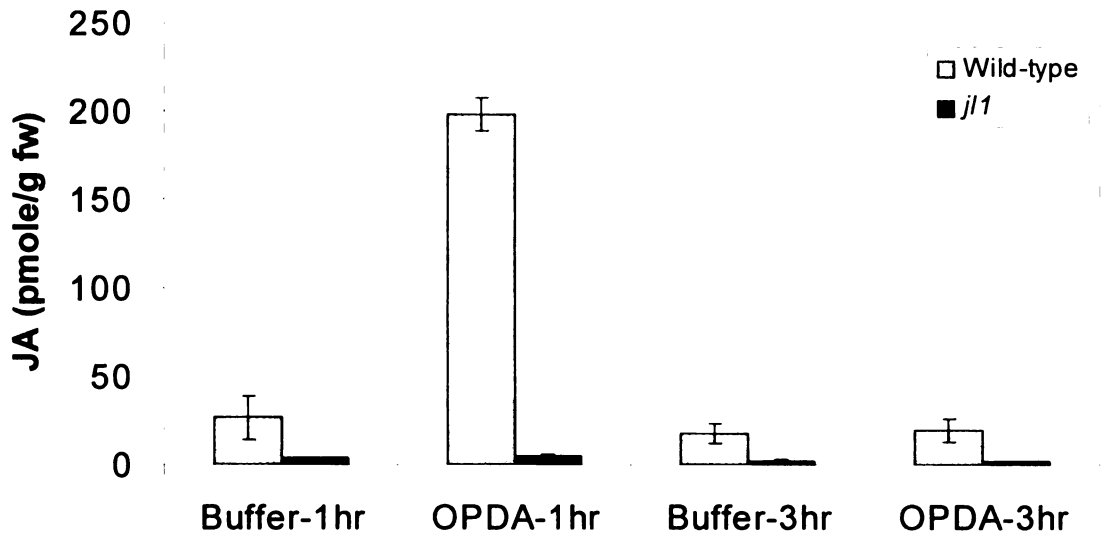


Figure 3.9. JA accumulation in wild-type and *jl1* plants in response to application of exogenous OPDA.

Eighteen-day-old wild-type and *jl1* seedlings were supplied with OPDA (20 nmol per plant) through cut stem as described in Table 3.2. Leaves were harvested for JA extraction at 1 hr and 3 hr after OPDA application. The amounts of JA in plant extracts were quantified by GC-MS. Data represent the mean and standard deviation of three independent replicates.

Discussion

LeACX1 is required for wound-induced JA biosynthesis

We demonstrated that the tomato *jl1* mutant has a defective ACX1 gene and, as a consequence, is deficient in wound-induced JA production. The expression of both early and late wound response genes was also highly reduced in the *jl1* mutant in response to mechanical wounding and hornworm attack (Figure 3.3; 3.4). The wound response of the *jl1* mutant could be rescued by exogenous JA (Lightner et al., 1993), which is consistent with the conclusion that *LeACX1* is required for the biosynthesis of JA. These data indicate that β-oxidation is needed for wound-induced JA biosynthesis. As expected, a deficiency in β-oxidation results in increased plant susceptibility to insect attack. Castillo et al. (2004) recently showed that reduced expression of the *ACX1* gene in Arabidopsis caused a defect in wound-activated synthesis of JA and reduced expression of JA-responsive genes. Also, induced expression of JA-responsive genes by exogenous application of JA was unaffected in those transgenic plants.

LeACX1 is expressed constitutively in tomato leaves and induced in response to wounding

It is generally agreed that JA biosynthetic enzymes accumulate constitutively in unwounded tomato leaves (Stenzel et al., 2003a). Here, we show that *LeACX1* is also expressed constitutively at a basal level in leaves of wild-type, *jl1*, and *jai1* plants (Figure 3.3; 3.4; 3.5). However, transcripts of *LeACX1* accumulate to a high level in response to mechanical wounding and hornworm attack, in a manner that depends on JA signaling. A similar result was found in Arabidopsis (Castillo et al., 2004). These results lead us to

conclude that the expression of *LeACX1* is controlled by both JA -dependent and -independent mechanisms. This conclusion is consistent with previous a cDNA microarray study showing that *ACX1* is more highly induced by wounding in wild-type plants than in *coi1* plants (Reymond et al., 2000), which are insensitive to JA. The JA -independent basal expression of JA biosynthetic genes suggests that the respective gene products serve an important function in the absence of stress conditions that trigger JA signaling. For example, the role of ACX1 in fatty acid β-oxidation suggests that this enzyme is involved in germination and early postgerminative development in higher plants. Uncoupling of the basal expression level of JA biosynthetic enzymes from JA signaling might provide a mechanism to ensure that the amplitude and timing of JA biosynthesis in response to stress is sufficient to activate downstream target genes (Li et al., 2004). This hypothesis is consistent with the observation that wound-induced activation of JA biosynthetic genes such as *LoxD*, *OPR3*, and *ACX1* occurs later than the wound-induced accumulation of JA (Figure 3.3) (Stenzel et al., 2003b; Castillo et al., 2004). Thus, wound-induced JA synthesis does not depend on the induced expression of JA biosynthetic genes (Miersch and Wasternack, 2000; Ziegler et al., 2001).

Role of JA and OPDA in plant defense

Unexpectedly, *jl1* plants expressed PI-II in response to octadecanoid intermediates that proceed the action of ACX1 in the of JA biosynthesis pathway (Table 3.2; Figure 3.8). This response of *jl1* contrasts with the near complete lack of PI expression in wounded *jl1* plants. The fact that *jl1* plants still respond to JA precursors such as OPDA and OPC-8:0 likely means that JA is synthesized when these precursors are supplied exogenously to *jl1* through the transportation stream. We hypothesize that high concentrations of ACX

substrate (e.g. OPC:8-CoA) generated in response to exogenous intermediates are converted to JA by the action of other ACXs. In the *Arabidopsis* genome, there are six *ACX* genes that differ in their expression pattern, subunit composition, and substrate specificity (Graham and Eastmond, 2002; Rylott et al., 2003). AtACX1 prefers medium- to long-chain saturated acyl-CoAs, AtACX2 displays activity against long-chain unsaturated acyl-CoAs (Hooks et al., 1999), AtACX3 is a medium-chain (C_{10}-C_{14}) acyl-CoA oxidase (Eastmond et al., 2000; Froman et al., 2000), and AtACX4 exhibits short-chain substrate specificity (Hayashi et al., 1999). These ACX isozymes are highly similar at the amino acid sequence level (Hayashi et al., 1999). AtACX1, AtACX2 and AtACX3 have similar K_m values (around 5 μM), similar molecular weights (75 kDa), and function as homodimers *in vivo* (Graham and Eastmond, 2002). Biochemical evidence suggests that acyl-CoA oxidase isozymes in plants have partially overlapping acyl-CoA substrate chain-length specificities (Kirsch et al., 1986). Even though *jl1* plants synthesize very little JA in response to wounding (unpublished data), other ACXs in tomato may metabolize OPC:8-CoA generated in response to exogenous elicitors.

Recently, Narváez-Vásquez and Ryan (2004) presented *in situ* hybridization and immunocytochemical evidence that wound-induced and MeJA-induced prosystemin mRNA and protein are exclusively located in vascular phloem parenchyma cells of minor veins and midribs of leaves, and in the bicollateral phloem bundles of petioles and stems of tomato. Wound-activated AOC accumulation was restricted to companion cells and sieve elements of vascular bundles (Stenzel et al., 2003a). Compartmentalization of prosystemin, which is a positive regulator of JA, and JA biosynthetic enzymes in vascular bundles suggests the JA biosynthesis is also vascular bundle-specific (Hause et al., 2003;

Stenzel et al., 2003a; Narváez-Vásquez and Ryan, 2004). The basal levels of JA and OPDA in untreated tomato leaves are low, indicating that JA biosynthetic enzymes are not a limiting step in JA accumulation. The rapid and transient rise of JA upon wounding suggests that JA generation is substrate dependent and occurs in specific cells within the vascular bundles (Stenzel et al., 2003a). In wounded tomato plants, systemin is proposed to be processed from prosystemin and translocated into the apoplast of the vascular bundles where it initiates a positive amplification loop in which systemin and JA are self-induced as a wave through the plant vasculature to activate local and systemic responses (Stenzel et al., 2003a; Narváez-Vásquez and Ryan, 2004). Due to the defect in ACX1, we propose that *jl1* plants cannot metabolize wound-induced OPC:8-CoA to JA in vascular bundles. In case of feeding exogenous elicitors, intermediates in the JA biosynthesis pathway may be produced non-specifically in all cell types, leading to induction of PI-II. In *jl1* plants, other ACXs may fulfill the role of ACX1 to produce JA in this manner.

However, JA did not accumulate in *jl1* plants in response to applied OPDA (Figure 3.9). These results indicate that ACX1 is required for the biosynthesis of JA in tomato plants, and that other ACXs are not involved in JA biosynthesis in response to applied OPDA. It appears that the expression of *PI-II* in OPDA-treated *jl1* plants is mediated by a signal other than JA, most likely OPDA itself.

Although JA is a physiological signal in the regulation plant defense responses against herbivorous insects and pathogen attack, the possibility that the JA precursor, 12-OPDA, is active without metabolism to JA has been proposed (Weiler et al., 1993; Weiler et al., 1994; Weber et al., 1997; Stintzi et al., 2001). The best evidence for this comes from studies of the Arabidopsis *opr3* mutant (Stintzi and Browse, 2000; Stintzi et al.,

2001), which is defective in OPDA reductase (OPR). Wounded *opr3* leaves do not accumulate detectable bioactive JA, and levels of OPDA in wounded *opr3* leaves are about 50% of that in wounded wild-type leaves. Thus, the *opr3* mutant provides a useful tool to separate the effect of 12-OPDA and JA *in vivo*. In contrast to JA-insensitive *coi1* plants and the *fad3/fad7/fad8* mutant lacking the fatty acid precursors of JA, *opr3* plants exhibited a wild-type level of resistance to the dipteran *Bradysia impatiens* and the fungus *Alternaria brassicicola*. This striking result indicated that resistance of *opr3* plants is mediated by a signal other than JA, the most likely candidate being OPDA (Howe, 2001). cDNA microarray analysis in *opr3* plants showed the wound induction of genes previously known to be JA-dependent, suggesting that OPDA could fulfill some roles of JA *in vivo*. Treating *opr3* plants with exogenous OPDA up-regulated several genes, indicating that this JA precursor can activate gene expression in the absence of JA (Stintzi et al., 2001).

Like the Arabidopsis *opr3* mutant, the tomato *jl1* mutant appears to be defective in the metabolism of OPDA to JA (Lee and Howe, unpublished data). However, we found that *jl1* plants are deficient in wound-induced expression of defensive *PI*s (Figure 3.3), and are much more susceptible to hornworm attack than wild-type (Figure 3.6; Table 3.1). This finding indicates that in tomato, OPDA is not a signal for defense against insects. Furthermore, we conclude that JA, but not OPDA, is essential for expression of wound-induced *PI*s.

The expression of *PI-II* in the absence of JA in *jl1* plants when supplied with OPDA leads us to a hypothesis that exogenous OPDA and other JA precursors trigger JA-independent but OPDA-dependent gene expression in *jl1* plants. A model summarizing

the roles of JA and OPDA in the control of gene expression in OPDA treated tomato plants is given in Figure 3.10. We postulate that JA and OPDA play distinct, but maybe complementary roles in the fine-tuning of PI-II expression. In wounded plants, the pathway obviously involves OPDA and JA, and acts through the COI1 complex. JA is required for this pathway. But it is possible that OPDA can activate the *PI-II* expression directly in the absence of JA when exogenous OPDA or other JA precursors are applied to tomato plants through cut stem. OPDA-induced PI-II expression is also COI1-dependent (Li and Howe, unpublished data). In this process, OPDA may bind to the same receptor as JA, based on their structural similarity.

The idea that JA and related octadecanoid compounds regulate different target processes in different plants has been proposed (Li et al., 2003). Mutants of Arabidopsis and tomato have been instrumental in establishing the roles of JA and related compounds both in regulating developmental and defense processes. In this context, the *jl1* mutant provides a valuable genetic resource to further investigate the roles of JA and OPDA in regulating defense responses in tomato.

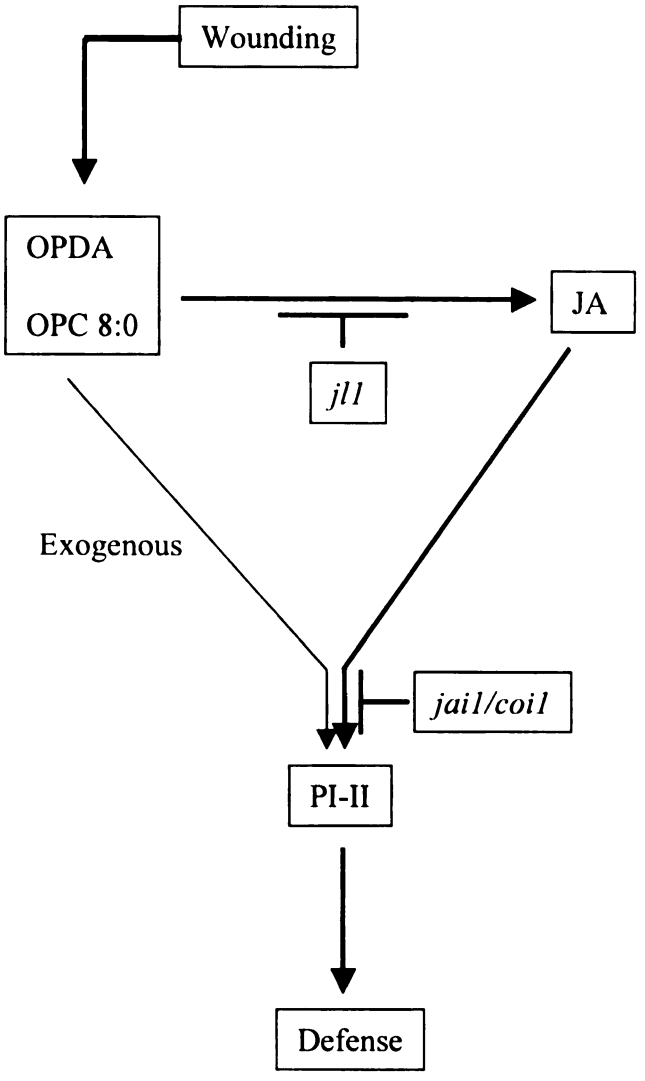


Figure 3.10. Model for the role of JA and OPDA in the fine control of gene expression in tomato leaves.

In wounded plants, the conversion of OPDA to JA is required for the activation of *PI-II* expression. When exogenous OPDA or other JA precursors are supplied to tomato plants through cut stems, OPDA or OPC 8:0 can regulate the expression of PI-II directly in a COI1-dependent pathway.

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