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BIOCHEMICAL AND GENETIC ANALYSIS OF SYSTEMIC WOUND SIGNALING IN TOMATO (LYCOPERSICON ESCULENTUM)

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BIOCHEMICAL AND GENETIC ANALYSIS OF SYSTEMIC WOUND SIGNALING IN TOMATO (*LYCOPERSICON ESCULENTUM*)

By

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ABSTRACT

BIOCHEMICAL AND GENETIC ANALYSIS OF SYSTEMIC WOUND SIGNALING IN TOMATO (*LYCOPERSICON ESCULENTUM*)

By

Gyu In Lee

Tomato plants activate the synthesis of defense proteins such as proteinase inhibitors (PIs) in response to insect attack. Systemic accumulation of PIs in wounded plants is mediated by a long-distance signaling pathway that transmits a mobile signal from the wound site to distal undamaged leaves. Previous studies have established that the polypeptide signal systemin, which is derived from a precursor protein called prosystemin, regulates systemic expression of *PI* genes via the jasmonic acid (JA) signaling pathway. However, the precise role of JA and systemin in systemic wound signaling remains unclear. To address this question, two tomato mutants that are compromised in the systemic wound response were characterized.

The *suppressor of prosystemin-mediated responses1* (*spr1*) mutant was blocked in JA biosynthesis and subsequent *PI* gene expression in response to systemin. The systemin-insensitive phenotype of *spr1* plants indicates that the *Spr1* gene product plays a role in linking the perception of systemin to the activation of JA biosynthesis. Reciprocal grafting experiments between wild-type and *spr1* plants demonstrated that this mutant is deficient in the production of the long-distance wound signal in damaged leaves, rather than the perception of that signal in neighboring undamaged leaves. This

result indicates that systemin acts at or near the site of wounding to increase JA synthesis to a level that is required for the systemic response.

The role of JA in systemic wound signaling was investigated using the JL-1 mutant that is defective in both JA biosynthesis and wound-inducible expression of *PI* genes. Gas chromatography-mass spectrometry analysis showed that JL-1 plants are unable to produce JA from its precursor, 12-oxo-phytodienoic acid (OPDA), presumably because of a defect in fatty acid β-oxidation. It was found that OPDA is not an active signal for *PI* expression in tomato. Analysis of reciprocal grafts between wild-type and JL-1 plants showed that JA biosynthesis is required for the generation of the systemic signal in wounded leaves. Taken together with the finding that JA perception is essential for systemic expression of *PI* in undamaged leaves, these results indicate that JA or a derivative of JA is the long-distance signal for systemic *PI* expression.

JA biosynthesis is regulated by substrate availability. Two related cytochrome P450s, allene oxide synthase (AOS) and hydroperoxide lyase (HPL), metabolize a common hydroperoxy fatty acid substrate to JA and volatile C_6 aldehyde, respectively. To study the regulation of JA biosynthesis, genes encoding AOS and HPL were cloned from tomato and expressed in *E. coli*. Wounding induced the expression of the *AOS* gene in both wild-type and JA-deficient mutants, indicating that a JA-independent signaling pathway may contribute to systemic *AOS* expression. These results, together with those obtained from gene expression profiling in *spr1* plants, point to the existence of both systemin/JA-dependent and -independent signaling pathways that regulate distinct sets of wound-inducible target genes in tomato plants.

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Introduction:

Systemic wound signaling in tomato

Introduction

Herbivore attack causes plants to induce several defense responses (Kessler and Baldwin, 2002; Walling, 2000). One of the most extensively studied defense responses is the wound-induced synthesis of defensive proteinase inhibitors (PIs) in tomato (Gatehouse, 2002; Ryan, 2000). Accumulation of PIs in response to herbivory and mechanical wounding is regulated at the level of gene expression (Nelson et al., 1983). Accumulated PI proteins in tomato adversely affect the growth of lepidopteran caterpillars by interfering with digestive enzymes in the gut of the attacking insect (Ryan, 1990).

Wound-induced synthesis of PIs occurs not only in the vicinity of the wound site, but also in undamaged leaves throughout the plant (Green and Ryan, 1972). This systemic response implies the existence of a long-distance signal transduction pathway that connects local injury to the activation of gene expression in distal undamaged tissue. Detachment of the damaged leaf immediately after wounding was shown to abolish systemic PI accumulation, indicating that the mobile signal is synthesized in the damaged leaf and transported to distal undamaged leaves (Green and Ryan, 1972).

Extensive research effort has been focused on the identification of the systemic wound signal. Chemical elicitors of *PI* expression have been isolated from tomato plants and characterized as candidates for the mobile signal (Bishop et al., 1981; Pearce et al., 1991). These studies have shown that a polypeptide signal called systemin, and the plant hormone jasmonic acid (JA), play crucial roles in the systemic wound response (Farmer and Ryan, 1992; McGurl et al., 1992; Pearce et al., 1991). Our current understanding of systemic wound signaling is summarized in this chapter.

Wound-induced gene expression

Over 20 genes have been shown to be expressed systemically in response to wounding of tomato plants. Based on their expression pattern, these genes have been classified into two groups (Ryan, 2000). The first group is the 'early genes' that are induced rapidly and transiently upon wounding. Expression of early genes is detected within 30 min of wounding. Maximal expression occurs about 2 hr after wounding, after which mRNA levels quickly decrease. The group of early genes includes those encoding calcium calmodulin (Bergey and Ryan, 1999), transcription factors (Stankovic et al., 2000; Strassner et al., 2002), and enzymes involved in the biosynthesis of JA (Heitz et al., 1997; Sivasankar et al., 2000; Strassner et al., 2002). Because the levels of cytosolic calcium and endogenous JA rapidly increase in response to wounding and elicitor treatment (Moyen et al., 1998; Parchmann et al., 1997), the induction of genes encoding calcium calmodulin and JA biosynthetic enzymes may represent a positive feedback mechanism to amplify the wound response.

The second group of wound response genes in tomato is referred to as 'late genes'. Transcript levels of these genes increase slowly and steadily in response to wounding. The expression of late genes is detected about 2 hr after wounding, with the highest expression level occurring 8 to 12 hr after wounding. Examples from this group include genes encoding PIs, polyphenol oxidases, leucine aminopeptidase and LE RNase (Chao et al., 1999; Constabel et al., 1995; Lers et al., 1998). Similar to PIs, polyphenol oxidases mediate plant defense against attacking insects by inhibiting insect digestive processes (Constable et al., 1995). LE RNase and proteolytic enzymes such as leucine aminopetidase (Chao et al., 1999; Schaller and Ryan 1996) may play a role in

reprogramming plant metabolism to allow for massive synthesis of PIs and other defensive phytochemicals.

Advances in cDNA microarray technology have enabled investigators to monitor global changes in gene expression in response to wounding. A microarray experiment using 230 tomato cDNAs supports the idea that wound-inducible genes in tomato can be classified according to their temporal expression pattern (Strassner et al., 2002). The sequential expression of these genes indicates that transcription factors encoded by early genes may activate the expression of late genes. Alternatively, proteins encoded by the early genes may amplify the signaling pathway leading to the expression of late genes, which results in maximal production of defensive chemicals. Larger-scale microarray experiments have been performed in Arabidopsis. Mechanical wounding significantly changed the expression level of 657 genes (Cheong et al., 2002). About 20% of these wound-inducible genes encode signal transduction-related proteins such as protein kinases, protein phosphatases, GTP-binding proteins, calcium binding proteins, phosphatidylinositol-related enzymes, JA biosynthetic enzymes and transcription factors. Although it is likely that these gene products are involved in wound signaling, the precise contribution of each of gene remains to be determined. Other wound-inducible genes encode enzymes involved in the phenylpropanoid pathway. Activation of these genes leads to the synthesis of defensive chemicals and the reinforcement of the cell wall (Constabel, 1999; Franke et al., 2002; Peters and Constabel, 2002), which may enhance resistance to pest attack.

Wound signals

A simple bioassay has been used extensively to identify systemic wound signals in tomato plants (Ryan, 1992). This assay involves supplementation of young plants through the cut stem with compounds purified from tomato leaf extract, followed by measurement of PI accumulation using an immunodiffusion assay (Ryan, 1967). Two structurally distinct molecules were found using this bioassay: oligogalacturonic acid (OGA) derived from the cell wall (Bishop et al., 1981), and a polypeptide, systemin (Pearce et al., 1991), In addition, the plant hormone JA and its methyl ester (MeJA) were identified as inducers of *PI* expression (Farmer and Ryan, 1990; Farmer and Ryan, 1992). Studies of these compounds have established a signaling cascade that regulates wound-induced expression of *PI* and other defense-related genes (Figure 1-1; Ryan 2000).

Oligogalacturonic acid

Previously it was reported that a pectin-enriched fraction purified from tomato leaf hydrolysate induced PI synthesis (Bishop et al., 1981). Further purification of the fraction showed that the active component was the oligosaccharide OGA, which is a hydrolysis product of pectin. Therefore, it was speculated that wounding results in the release of OGA from the cell wall. A cDNA encoding a polygalacturonase that degrades pectin to OGA was recently cloned from tomato (Bergey et al., 1999). The activity of this enzyme is up-regulated locally and systemically upon wounding. This finding

supports the idea that OGA is an authentic signal for the activation of wound-inducible genes in tomato. However, genetic evidence to support this hypothesis is lacking.

Although OGA is a potent elicitor of *PI* expression, the mobility of OGA in plant tissue is very limited. Radioactively labeled OGA applied to wound sites was not transported to unwounded tissue (Baydoun and Fry, 1985). Thus, it is unlikely that OGA is the long-distance signal of the systemic wound response.

In addition to its effect on *PI* expression, OGA treatment results in phosphorylation of a plasma membrane protein (Farmer et al., 1989), depolarization of the plasma membrane (Moyen and Johannes, 1996; Thain et al., 1995), a reactive oxygen burst (Stennis et al., 1998), and an increase in endogenous JA levels (Doares et al., 1995b). Among these rapid cellular responses, it was shown that activation of JA biosynthesis is essential for OGA-mediated expression of *PI* genes. OGA failed to induce PI accumulation in plants treated with inhibitors of JA biosynthesis (Doares et al., 1995a), or in plants that are compromised in JA biosynthesis (Howe et al., 1996). These results indicate that OGA activates *PI* expression through the JA signaling pathway.

The oligosaccharide, chitosan, which is derived from fungal cell walls, also induces JA synthesis and PI accumulation in tomato plants (Walker-Simmons and Ryan, 1984). This observation is consistent with the fact that JA regulates defense response against some fungal pathogens of tomato (Diaz et al., 2002). These results indicate that plants use chitosan as a signal to trigger defense response.

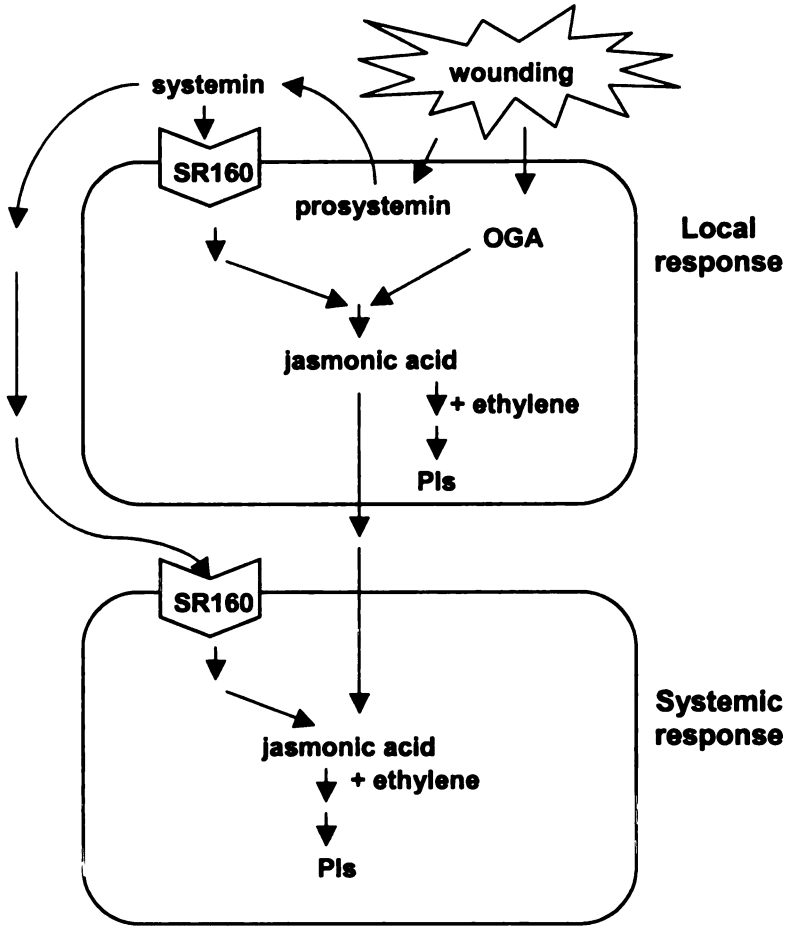

Figure 1-1. The current model of systemic wound signaling. This schematic model is adapted from Ryan (2000). Wounding activates JA biosynthesis via systemin- and OGA-mediated signaling pathways in wounded tissue (local response). Systemin functions as a mobile signal to activate *PI* expression in distal unwounded tissue (systemic response). A recent study indicates that JA, not systemin, is the systemic wound signal (Li et al., 2002). SR160, the systemin receptor.

Systemin

The 18-amino-acid polypeptide systemin was purified from tomato leaf extracts on the basis of its ability to induce PI accumulation when supplied to young tomato plants through the cut stem (Pearce et al., 1991). Synthetic systemin is active in the fmol range, indicating that it is the most potent elicitor of PI accumulation ever found. Systemin is derived from the C-terminal region of a 200 amino acid precursor protein named prosystemin (McGurl et al., 1992). Tomato has a single copy of the *prosystemin* gene, which is expressed at low levels in aerial parts of unwounded plants. Expression of the *prosystemin* gene is elevated locally and systemically within cells of the vascular bundles in response to wounding (Jacinto et al., 1997; McGurl et al., 1992).

Several lines of evidence indicate that systemin plays a critical role in systemic wound signaling. First, systemic *PI* expression was severely reduced in transgenic plants that express the *prosystemin* cDNA in antisense orientation (McGurl et al., 1992). Moreover, these transgenic plants were more susceptible to insect attack than wild-type plants (Orozco-Cardenas et al., 1993). Second, *PI* and other wound-inducible genes were constitutively expressed in transgenic plants (called *35S::prosys*) that overexpress *prosystemin* from the cauliflower mosaic virus (CaMV) 35S promoter (McGurl et al., 1994). In a grafting experiment in which wild-type scions were grafted onto *35S::prosys* transgenic rootstocks, the wild-type scions accumulated significant amounts of PIs in the absence of wounding. This observation indicates that *35S::prosys* transgenic rootstocks produce a systemic wound signal that is transmitted to the wild-type scions. Unlike OGA, systemin may be a mobile signal *in vivo* because radioactively-labeled systemin applied to wound sites moved via the phloem to

unwounded tissue (Narvaez-Vasquez et al., 1995; Pearce et al., 1991). Therefore, systemin was regarded as a strong candidate for the systemic wound signal.

Previous studies demonstrated that systemin requires JA biosynthesis for induction of wound-inducible genes. Endogenous levels of JA rapidly increase in response to systemin (Conconi et al., 1996; Doares et al., 1995b), followed by expression of *PI* genes. Systemin treatment failed to induce *PI* expression in tomato mutant plants that are impaired in JA biosynthesis (Howe et al., 1996). Also, inhibitors of JA biosynthesis abolished systemin-induced PI accumulation (Doares et al., 1995a). These results indicate that systemin, like OGA, acts upstream of JA in the wound signaling pathway (Figure 1-1).

In addition to up-regulating JA synthesis, exogenous systemin activates several rapid cellular events. These events include increased cytosolic calcium levels, inactivation of a proton-ATPase located on plasma membrane, activation of a wound-inducible mitogen-activated protein kinase (MAPK), and membrane depolarization (Felix and Boller, 1995; Moyen and Johannes, 1996; Moyen et al., 1998; Schaller and Oecking, 1999; Stratmann and Ryan, 1997). Pharmacological studies showed that inhibition of the proton-ATPase and changes in calcium ion flux induced *PI* expression (Schaller and Frasson, 2001; Schaller and Oecking, 1999). Thus, calcium mobilization and modulation of proton-ATPase may be early causal events of the wound signaling pathway.

Systemin specifically binds a plasma membrane protein called SR160 (Meindl et al., 1998; Scheer and Ryan, 1999). This 160 kDa protein is a leucine-rich repeat receptor kinase that shows high similarity to the brassinolide receptor, BRI1, of

Arabidopsis (Scheer and Ryan, 2002). Interestingly, the tomato mutant *curl3* is insensitive to brassinolide as a result of a nonsense mutation in the *SR160* gene (Montoya et al., 2002). This finding indicates that SR160 is a dual receptor for systemin and brassinolide. It is currently unclear how the same receptor regulates two distinct signaling pathways.

Orthologues of the *prosystemin* gene have been found in members of the Solanaceae family, such as potato, black nightshade, and bell pepper (Constabel et al., 1998). In tobacco, two polypeptides were found to strongly induce *PI* expression. These polypeptides are processed from the same precursor protein, and do not show sequence similarity to tomato systemin (Pearce et al., 2001). Therefore, it is possible that other plant species use polypeptide signals to regulate the wound response.

Jasmonic acid

The cyclopentanone compound JA is synthesized from linolenic acid by the octadecanoid pathway (Figure 1-2; Wasternack and Hause, 2002). JA biosynthesis is initiated in chloroplasts, where phospholipases release linolenic acid from membrane lipids (Ishiguro et al., 2001). Lipoxygenase (LOX) converts linolenic acid to 13-hydroperoxylinolenic acid (13-HPOT), which is metabolized by various enzymes belonging to the CYP74 family of cytochrome P450s (Howe and Schilmiller, 2002; Howe et al., 2000). Among these, allene oxide synthase (AOS) commits 13-HPOT to the biosynthesis of JA. AOS transforms 13-HPOT to an unstable epoxide intermediate (12, 13-epoxylinolenic acid), which is subsequently transformed by allene oxide cyclase

to 12-oxo-phytodienoic acid (OPDA). The remaining reactions of the octadecanoid pathway take place in peroxisomes (Strassner et al., 2002). The double bond of OPDA is reduced by OPDA reductase (OPR3) to yield 3-oxo-2-(2Z-pentenyl)-cyclopentane-1-octanoic acid (OPC-8). Finally, three rounds of β-oxidation shorten the carboxylate side chain of OPC-8, yielding JA. It remains unknown how OPDA is transported from chloroplasts to peroxisomes.

JA is subject to several metabolic transformations such as methylation, conjugation to amino acid or glucose, adenylation, hydroxylation and sulfonation (Gidda et al., 2003; Hause et al., 2000; Kramell et al., 1997; Seo et al., 2001; Staswick et al., 2002). MeJA, amino acid-conjugated JA, and glucose-conjugated JA have been shown to induce expression of *PI* (Farmer and Ryan, 1990; Kramell et al., 1997; Wasternack et al., 1998). These results indicate that some JA derivatives act as signals for gene expression. In consistent, defective adedylation of JA abolishes JA-inducible gene expression (Staswick et al., 2002). Alteration of the expression of JA-metabolizing enzymes in transgenic Arabidopsis has provided insight into the role of JA metabolism. For example, a specific methyl transferase converts JA to volatile MeJA (Seo et al., 2001). Overexpression of this enzyme in Arabidopsis results in elevated endogenous MeJA levels and enhanced resistance to fungal pathogens. Thus, MeJA seems to be an active signal for defense gene expression.

A wealth of evidence indicates that JA is an essential component of the wound signaling pathway. First, tomato plants produce high levels of PIs in response to JA and MeJA (Farmer and Ryan, 1990; Farmer and Ryan 1992). Second, wounding results in a rapid increase in JA biosynthesis (Parchmann et al., 1997), which is followed by

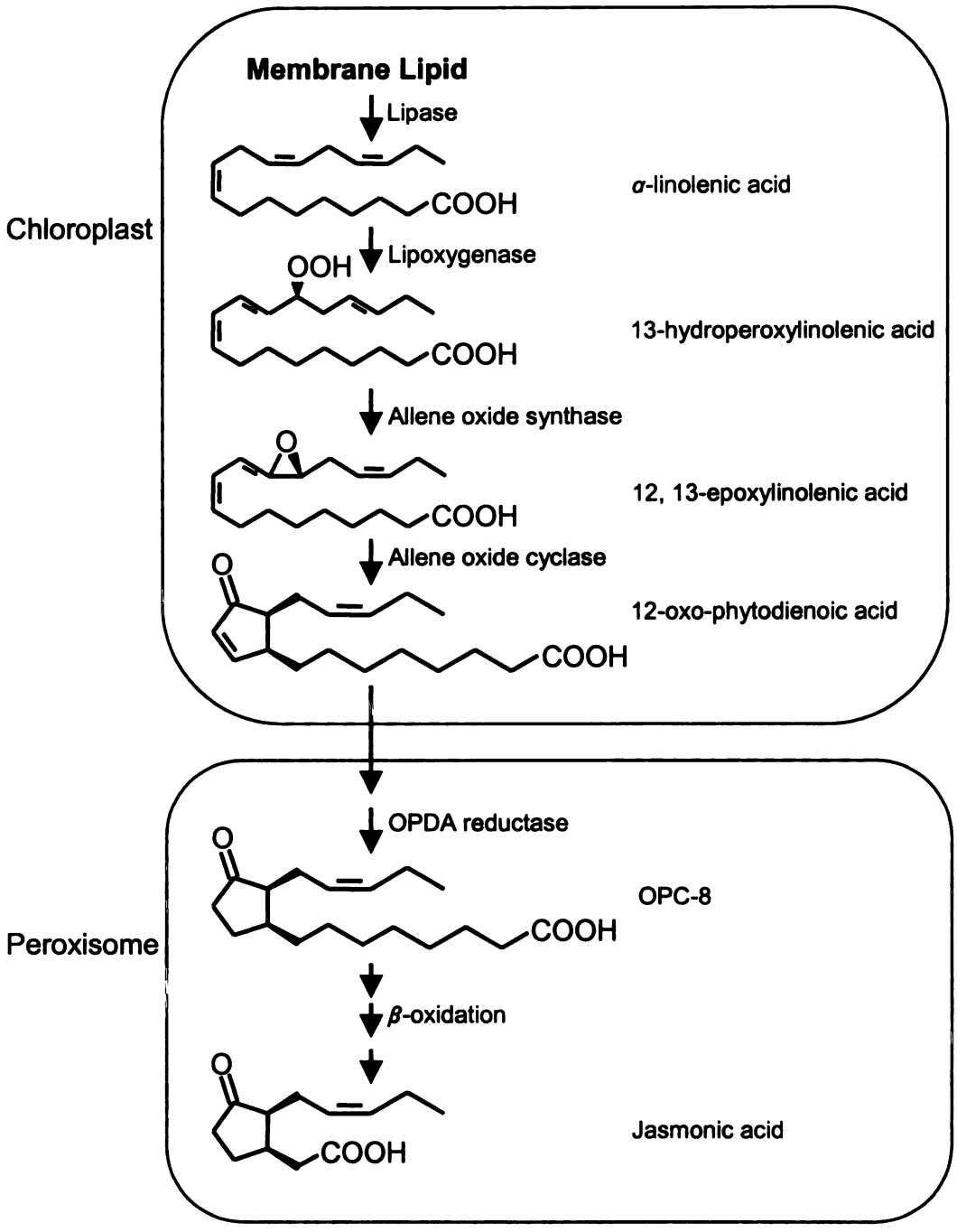

Figure 1-2. The octadecanoid pathway. Linolenic acid is released from chloroplast membranes and converted to OPDA by the sequential action of three enzymes. OPDA is processed to JA in peroxisomes.

PI expression. Finally, wound-induced PI accumulation is blocked in JA-deficient tomato mutants (Howe et al., 1996; Li et al., 2002). It has also been established that wounding rapidly induces systemic expression of several JA biosynthetic genes including *LOX, AOS, AOC,* and *OPR3* (Heitz et al., 1997; Howe et al., 2000; Sivasankar et al., 2000; Stenzel et al., 2003; Strassner et al., 2002). JA biosynthetic enzymes such as AOC and OPR3 are highly enriched in vascular bundles, indicating that JA is mainly synthesized in these tissues (Stenzel et al., 2003; Strassner et al., 2002). Overexpression of *AOS* and *AOC* did not result in increased JA levels or constitutive expression of wound-inducible genes (Laudert et al., 2000; Stenzel et al., 2003). These observations indicate that JA biosynthesis is not regulated by transcriptional control of the genes. Because application of linolenic acid to tomato results in *PI* expression (Farmer and Ryan, 1992; Howe et al., 1996), JA biosynthesis is probably regulated by substrate availability.

Despite extensive knowledge of the effects of JA on gene expression and defense-related processes, our understanding of the signaling events that couple the production of JA to the activation of target genes is still in its infancy. Our current understanding of this problem has been significantly enhanced by the identification and characterization of mutants that are insensitive to JA. For example, an Arabidopsis mutant *coronatine insensitive1* (*coi1*) is unable to express wound-inducible genes in response to JA and MeJA (Feys et al., 1994). The *COI1* gene encodes a protein containing an F-box motif and leucine-rich repeats that functions in ubiquitin-mediated protein degradation (Xie et al., 1998). As a component of ubiquitin ligase complex, F-box proteins act as receptors that recruit regulatory proteins as substrates for ubiquitin-

mediated degradation (Bai et al., 1996). Previous studies demonstrated that the COI1 protein participates in the assembly of a ubiquitin ligase complex that presumably recruits regulatory proteins to the 26S proteosome for degradation (Feng et al., 2003; Xu et al., 2002). A recent study indicates that COI1 mediates degradation of a histone deacetylase that modulates chromosome remodeling (Devoto et al., 2002). These results indicate that COI1 is involved in the removal of negative regulators acting downstream of JA

There is also evidence indicating that a MAPK cascade operates downstream of JA to regulate gene expression. JA failed to activate wound-inducible genes in Arabidopsis plants deficient in MAPK4 activity (Petersen et al., 2000). In addition, a pharmacological study indicated that protein dephosphorylation catalyzed by protein phosphatase 2C is required for the induction of JA-responsive genes in Arabidopsis (Rojo et al., 1998).

Previous studies indicated that OPDA, in addition to serving as an intermediate in JA biosynthesis, is a signal for several plant developmental processes (Koch et al., 1999; Weiler et al., 1993; Weiler et al., 1994). The Arabidopsis *opr3* mutant is unable to convert OPDA to JA due to the loss of OPDA reductase activity (Stintzi et al., 2001). Whereas JA-deficient and JA-insensitive mutants are susceptible to insect attack, *opr3* plants were shown to be as resistant as wild-type plants. Moreover, wild-type and *opr3* plants showed very similar gene expression patterns in response to wounding, and OPDA treatment induced a subset of wound-inducible genes in *opr3* plants. These results demonstrate that OPDA is an active signal for defense responses in Arabidopsis.

Physical signals

Mechanical wounding generates an electrical pulse (Wildon et al., 1992) and changes in xylem tension capable of producing a hydraulic signal (Malone and Alarcon, 1995; Malone et al., 1994). Because these physical signals are rapidly propagated long distance through the whole plant, they were proposed as candidates for the systemic signal for *PI* expression (Malone, 1996; Wildon et al., 1992). To date, however, there is no genetic evidence that an electrical signal is the causative agent of *PI* expression. In the case of hydraulic signals, it was proposed that mass flow of xylem sap might deliver signaling molecules produced at the wound site to undamaged leaves (Rhodes et al., 1999). However, this idea is not consistent with the observation that steam girdling of the petiole, which prevents phloem transport, but not xylem transport abolished systemic *PI* expression in tomato (Nelson et al., 1983). Thus, a causal role of physical signals in wound-induced systemic *PI* expression has not been established.

Involvement of other plant hormones and signaling molecules in wound signaling

Ethylene is required for wound-inducible gene expression in tomato. Wounding and systemin treatment activated ethylene biosynthesis. Inhibitors of ethylene perception, such as silver thiosulphate, were shown to prevent the induction of JA biosynthesis and *PI* expression. Also, wounding failed to stimulate PI synthesis in transgenic plants that are deficient in ethylene biosynthesis (O'Donnell et al., 1996). These results demonstrate that ethylene is a positive regulator of wound signaling in tomato. However, ethylene is not an independent signal for wound-inducible gene expression

because ethylene treatment alone does not induce *PI* expression. It was proposed that ethylene and JA work together in a positive feedback loop to amplify wound-induced expression of *PIs* (O'Donnell et al., 1996).

Abscisic acid (ABA) was proposed to be a positive regulator of wound signaling in tomato because ABA-deficient mutants fail to express *PI* in response to wounding (Herde et al., 1996). It is not clear whether ABA alone is a signal for *PI* expression because studies involving ABA treatment gave contradictory results (Birkenmeier and Ryan, 1998; Pena-Cortes et al., 1995). It is unlikely that ABA is the systemic wound signal because ABA-inducible genes were not expressed systemically upon wounding (Birkenmeier and Ryan, 1998).

Hydrogen peroxide accumulates systemically in response to wounding (Orozco-Cardenas and Ryan, 1999). Wounding, systemin, OGA, and JA did not induce PI accumulation when hydrogen peroxide synthesis was suppressed by inhibitors (Orozco-Cardenas et al., 2001). Interestingly, such inhibition did not affect expression of early wound response genes. Consistent with this, application of the hydrogen peroxide-generating enzyme glucose oxidase plus glucose to tomato plants resulted in the induction of late wound response genes such as *PI*, but not early wound response genes. Therefore, hydrogen peroxide appears to act downstream of JA to activate expression of late wound response genes.

Other signals have been shown to inhibit wound signaling in tomato. For example, salicylic acid (SA) treatment suppressed JA biosynthesis and *PI* expression (Doares et al., 1995a). Likewise, nitric oxide (NO) was shown to decrease the level of hydrogen peroxide and PI accumulation (Orozco-Cardenas and Ryan, 2002). Because

SA and NO regulate defense responses to pathogenic bacteria (Durner and Klessig, 1999; McDowell and Dangl, 2000), these results indicate that defense responses against herbivores and bacterial pathogens are mediated by signaling pathways that antagonize one another (Kunkel and Brooks, 2003).

Systemic wound signaling and the long-distance signal

Based on previous studies, Ryan and coworkers proposed a model for systemic wound signaling in tomato (Orcozco-Cardenas et al., 2001; Ryan, 2000). According to this model, wounding activates the processing of prosystemin to systemin by unknown mechanism. Subsequently, systemin is loaded to the phloem and transported to distal undamaged leaves. Upon binding to its receptor, SR160, systemin activates JA biosynthesis in vascular bundle cells where prosystemin and JA-biosynthetic enzymes are located. JA then activates expression of early wound response genes in vascular bundles. In addition, JA induces release of OGA from cell wall by the activation of wound-inducible polygalacturonase, which triggers an oxidative burst. Hydrogen peroxide produced during the oxidative burst diffuses from vascular bundles to mesophyll cells, where *PI* expression is activated. In this model, systemin acts as the long-distance signal for systemic gene expression.

Recent studies using reciprocal grafting techniques have challenged the idea that systemin is the systemic signal for *PI* expression (Li et al., 2002). Analysis of wound-induced systemic *PI* expression in grafts using JA-deficient and JA-insensitive mutants demonstrated that systemic *PI* expression requires JA biosynthesis in wounded leaves but not in distal undamaged leaves. Conversely, JA perception is essential for

recognition of the systemic wound signal in undamaged leaves, but not for the generation of that signal in wounded leaves. The simplest interpretation of these results is that JA or a derivative of JA is the systemic wound signal. Because systemin induces *PI* expression by activation of JA biosynthesis (Doares et al., 1995a; Doares et al., 1995b; Howe et al., 1996), these experiments indicate that systemin is unlikely to act in unwounded tissue as the systemic signal. Since prosystemin and JA biosynthetic enzymes are enriched in vascular bundle cells, systemin and JA might interact synergistically to amplify the production of the systemic wound signal along the vascular bundles (Ryan and Moura, 2002).

Emerging evidence indicates that some systemic wound responses are mediated by a signaling pathway that operates independently of JA and systemin. From example, neither systemin nor JA activates expression of the wound-inducible *glucosyl transferase* gene in tomato (O'Donnell et al., 1998). It was also shown that rapid systemic induction of a wound-inducible MAPK activity is unlikely to involve JA or systemin (Stratmann and Ryan, 1997). Moreover, destruction of phloem transport by steam girdling did not block the systemic induction of the kinase activity. This finding clearly demonstrates that a systemin- and JA-independent signal is involved in this particular systemic wound response.

Systemic wound signaling in Arabidopsis

Arabidopsis has been used as an alternative model system to study the wound responses (Berger, 2002; Leon et al., 2001). In this system, it is clear that JA plays a crucial role in wound-induced systemic expression of various defense-related genes. Wounding

induces JA biosynthesis in damaged leaves, which is followed by local and systemic gene expression (Kubigsteltig et al., 1999; Laudert and Weiler, 1998; Reymond et al., 2000; Titarenko et al., 1997). Because these responses are impaired in JA-deficient and JA-insensitive mutants (Berger et al., 1996; McConn et al., 1997; Park et al., 2002; Reymond et al., 2000; Rojo et al., 1999), JA seems to be an essential component of the systemic wound signaling pathway. Interestingly, the JA-mediated signaling pathway appears to work independently of OGA in Arabidopsis, whereas JA is required for OGA-induced gene expression in tomato (Doares et al., 1995b; Rojo et al., 1999; Titarenko et al., 1997). Furthermore, expression of JA-responsive genes is inhibited in wounded Arabidopsis leaves by OGA and ethylene (Rojo et al., 1999). Therefore, although JA is required for systemic wound responses in both tomato and Arabidopsis, the regulatory mechanism of JA-mediated signaling pathway may depend on the plant species. This idea is consistent with the observation that systemin-mediated signaling is absent in Arabidopsis.

Wound response mutants of tomato

Mutants impaired in the wound response provide valuable tools to elucidate the systemic wound signaling pathway. JL1 and *defenseless1* (*def1*) were identified by screening an ethyl methane sulfonate (EMS)-mutagenized population for plants unable to produce PIs in response to wounding (Lightner et al., 1993). Genetic analysis showed that these mutations are recessive. Since MeJA treatment induced PI accumulation in JL1 and *def1* plants, these mutants likely contain a defect in JA biosynthesis or an upstream step in the wound signaling pathway. Additional characterization of *def1*

plants showed that this mutant is deficient in wound-inducible JA accumulation, and has reduced AOC activity (Howe et al., 1996; Stenzel et al., 2003).

Transgenic plants (*35S::prosys*) that overproduce prosystemin constitutively express defensive proteins such as PIs and polyphenol oxidase (PPO) in the absence of wounding (McGurl et al., 1994). To investigate the systemin-mediated signaling pathway, *35S::prosys* plants were subject to EMS-mutagenesis, and mutant plants that fail to accumulate PPO and PIs were isolated (Howe and Ryan, 1999). Genetic analysis of these mutants showed four complementation groups, named *suppressor of prosystemin-mediated responses* (*spr*) *1-4*. Interestingly, *spr1* plants showed a severe reduction in systemic but not local *PI* expression. Thus, *Spr1* appears to function specifically in the systemic response. In contrast, wounded *spr2* plants did not accumulate PIs in either damaged or undamaged leaves. Recently, it was shown that *Spr2* encodes an omega-3 fatty acid desaturase that is required for the synthesis of linolenic acid, which is the precursor of JA (Li et al., 2003).

Tomato mutants that are insensitive to JA and MeJA were isolated by screening of EMS-mutagenized and fast-neutron mutagenized populations for plants that fail to express *PI* in response to volatile MeJA (Li et al., 2001). Genetic analysis showed that these lines define a single gene named *Jasmonic acid-insensitive1* (*Jai1*). As is predicted from the current model of wound signaling (Figure 1-1), wounding, systemin, and JA treatment are unable to induce *PI* expression in *jai1* plants (Li et al., 2002).

Overview of thesis

Systemic wound responses play important roles in plant defense against insect pests. Since the identification of wound-inducible PIs by Green and Ryan (1972), tomato has been used as a model system for the study of systemic wound signaling. A wealth of evidence indicates that systemin and JA are two essential signals required for the systemic wound response. Recent studies using wound response mutants indicate that JA or a derivative of JA acts as a long-distance signal for the systemic *PI* expression. In addition, other systemic signals appear to be involved in systemic responses that are induced rapidly upon wounding.

The current thesis is focused on understanding the role of systemin and JA in the systemic wound response. Systemin-insensitive *spr1* plants were characterized to investigate how systemin and JA regulate systemic *PI* expression. Chapter 2 describes the characterization of *spr1* and discusses the role of systemin in the systemic wound response. In Chapter 3, the role of JA and its precursor OPDA in systemic wound signaling was examined by characterizing the JL1 wound response mutant. To study the regulation of JA biosynthesis during the wound response, endogenous levels of JA and OPDA were measured in wild-type and mutant plants by gas chromatography-mass spectrometry (GC-MS). These results are presented in Chapter 4, along with a discussion of the regulation of JA biosynthesis. Chapter 5 reports on JA-independent and JA-dependent signaling events that occur during wound-induced root-to-leaf signaling. Chapter 6 presents a study aimed at characterizing two related cytochrome P450 enzymes, AOS and hydroperoxide lyase, that metabolize hydroperoxy fatty acids

to JA and volatile C_6 aldehyde, respectively. In the final chapter, a model of systemic wound signaling is proposed based on the results from thesis study.

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Chapter 2

The tomato mutant *spr1* is defective in systemin perception and the production of a systemic wound signal for defense gene expression

A version of this chapter has been published.

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Introduction

Many plants respond to insect attack and wounding by modulating the expression of genes involved in various defense-related processes. The synthesis and deployment of wound-induced phytochemicals is regulated by signal transduction pathways that operate both locally at the site of wounding and systemically in undamaged leaves throughout the plant (Green and Ryan, 1972; Karban and Baldwin, 1997). Wound-inducible defensive proteinase inhibitors (PIs) in Solanaceous plant species provide an attractive model system in which to study the mechanism of long-distance wound signaling, and several ideas have been proposed regarding the identity of the systemic signal transmitted from wound sites (reviewed by Bowles, 1998; León et al., 2001; Malone, 1996; Ryan, 2000; Walling, 2000). Among the proposed intercellular signals for wound-induced *PI* gene expression are systemin, an 18-amino acid peptide that is produced from cleavage of a larger precursor protein called prosystemin, and the octadecanoid pathway-derived hormone jasmonic acid (JA) (Farmer and Ryan, 1992; Li et al., 2002a; McGurl et al., 1992; Pearce et al., 1991). A wealth of biochemical and genetic evidence indicates that systemin and JA work together in the same signaling pathway to activate expression of *PI* and other defense-related genes (Li et al., 2001; Ryan, 2000).

The systemin/JA signaling pathway is activated upon binding of systemin to a 160-kDa plasma membrane-bound receptor called SR160 (Meindl et al., 1998; Scheer and Ryan, 1999). This receptor was recently identified as a member of the leucine-rich repeat (LRR) receptor kinase family (Scheer and Ryan, 2002). Binding of systemin to the cell surface is associated with several rapid signaling events including increased

cytosolic Ca^{2+} levels, membrane depolarization, inhibition of a plasma membrane proton ATPase, and activation of a MAP kinase activity (Felix and Boller, 1995; Moyen and Johannes, 1996; Moyen et al., 1998; Schaller and Oecking, 1999; Stratmann and Ryan, 1997). The systemin signaling pathway leading to *PI* expression is thought to culminate in activation of a phospholipase that releases linolenic acid, the metabolic precursor of JA, from membrane lipids (Farmer and Ryan, 1992; Narváez-Vásquez et al., 1999). Chitosan oligomers and oligogalacturonides (OGAs) derived from fungal and plant cell walls, respectively, also activate *PI* expression via the octadecanoid pathway (Bishop et al., 1981; Doares et al., 1995; Walker-Simmons and Ryan, 1984). The presence of wound-inducible polygalacturonase activity in tomato leaves (Bergey et al., 1999), together with the relative immobility of OGAs in the plant vascular system (Aldington and Fry, 1996), indicates that these compounds induce *PI* expression at or near the site of wounding. JA synthesized in response to wounding, systemin, and OGAs acts in concert with ethylene (O'Donnell et al., 1996) and hydrogen peroxide (Orozco-Cárdenas et al., 2001) to coordinate the induction of *PI* gene expression. Recent studies in Arabidopsis indicate that JA signaling depends upon assembly of ubiquitin-ligase complexes that presumably target transcriptional repressors of JA-responsive genes for proteolytic degradation (Xie et al., 1998; Xu et al., 2002).

Mutants affected in the synthesis or perception of prosystemin and JA provide useful tools to understand the mechanism of systemic wound signaling. For example, antisense-mediated depletion of prosystemin expression in tomato plants abrogated wound-induced systemic accumulation of PIs, indicating that this gene is essential for a normal systemic wound response (McGurl et al., 1992). To identify genes involved in

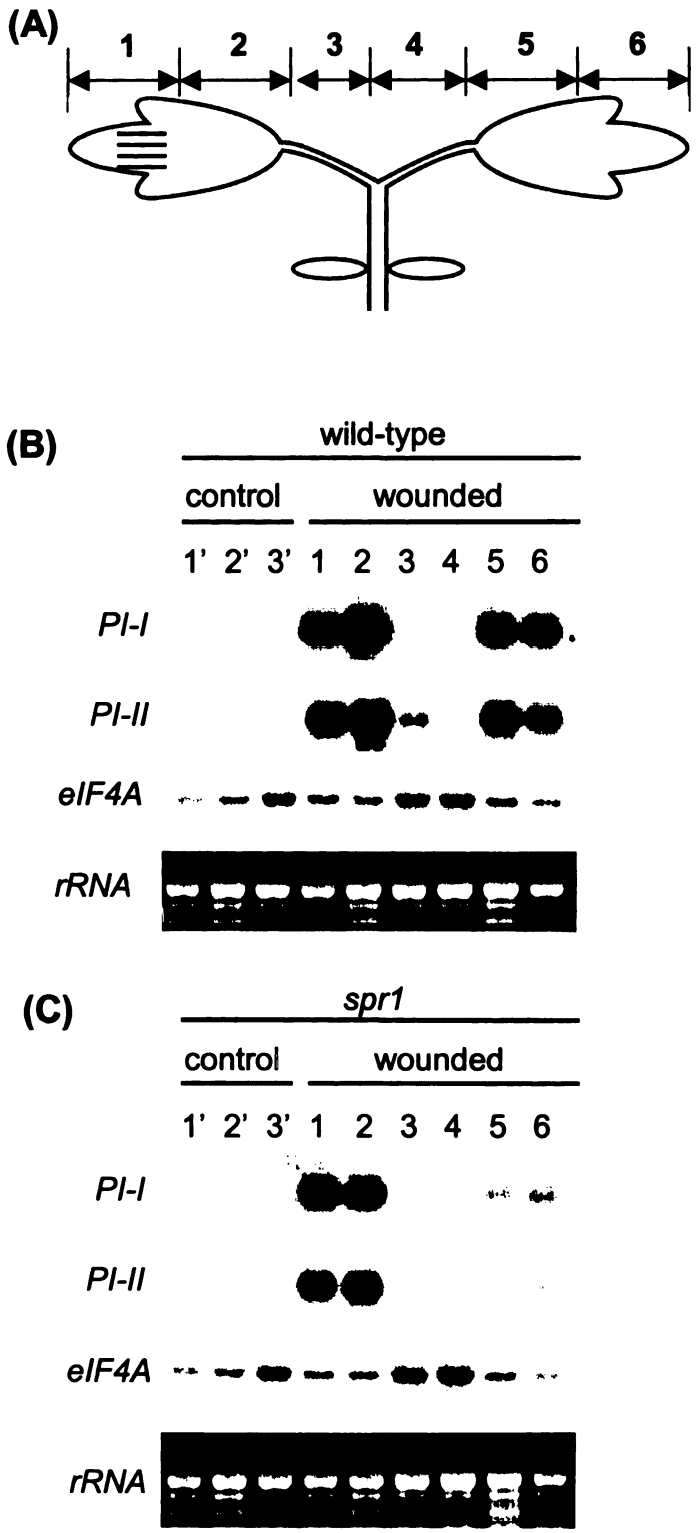
the systemin/JA signaling pathway using a forward genetic approach, we took advantage of a transgenic plant (called *35S::prosys*) that overexpresses *prosystemin* from the CaMV 35S promoter and, as a consequence, constitutively expresses *PI* and other defense-related genes in the absence of wounding (Constabel et al., 1995; McGurl et al., 1994). An ethyl methane sulfonate (EMS)-mutagenized population derived from this line was screened for mutants that are impaired in *35S::prosys*-mediated signaling (Howe and Ryan, 1999). Among several '*spr*' suppressed in prosystemin-mediated responses mutations identified, five independent alleles were shown to define one locus called *Spr1*. Evidence presented herein indicates that *Spr1* is involved in a signaling step that couples systemin perception to activation of the octadecanoid pathway. The results of grafting experiments further indicate that *Spr1* function is required at or near the site of wounding to amplify JA accumulation to a level sufficient to promote long-distance signaling. The existence of a wound response pathway that operates independently of *Spr1* is also described. These results are discussed in the context of the role of systemin in the wound response of tomato plants.

Results

spr1 preferentially affects wound-induced systemic *PI* expression

Recessive mutations in *Spr1* were previously shown to suppress *35S::prosys*-mediated expression of the well-characterized serine *PI* genes, *PI-I* and *PI-II* (Howe and Ryan, 1999). Further characterization of this mutant was conducted using *spr1/spr1* homozygous lines in which the *35S::prosys* transgene was removed by outcrossing (see

Figure 2-1. Spatial pattern of *PI-I* and *PI-II* mRNA accumulation relative to the wound site. A single wound was inflicted at the distal end of the terminal leaflet of the lower leaf of two-leaf-stage plants. Eight hours thereafter, various sections of the leaf blade and petiole were dissected for RNA isolation and analysis. Leaf sections from six plants were pooled for RNA isolation. (A) Schematic drawing illustrating the leaf and petiole sections that were harvested for RNA extraction. Panels in (B) and (C) show the results obtained for analysis of wild-type (WT) and *spr1-1* plants, respectively. Lanes 1 through 6 represent analysis of RNA isolated from the corresponding tissue sections shown in panel in (A). Lanes 1', 2', and 3' represent analysis of RNA isolated from various tissue sections of unwounded plants: 1', pooled tissue from sections 1 and 6; 2', pooled tissue from sections 2 and 5; 3', pooled tissue from sections 3 and 4. RNA gel blots containing 5 μg total RNA were hybridized to probes for *PI-I* and *PI-II*. To facilitate the comparison between WT and *spr1*, RNA blots shown in panels in (B) and (C) were hybridized together in the same containers and exposed to autoradiographic film for the same time. Blots were hybridized to a probe for *eIF4A* as a loading control. Note, however, that *eIF4A* mRNA abundance is greater in petiole tissue relative to leaf lamina. As an additional loading control, a picture of an ethidium bromide-stained gel of the total RNA (rRNA) is shown. The results shown are representative from three independent experiments.


(A)
1
2
3
4
5
6
(B)
wild-type
control
wounded
1' 2' 3' 1 2 3 4 5 6
PI-I
PI-II
eIF4A
rRNA
(C)
spr1
control
wounded
1' 2' 3' 1 2 3 4 5 6
PI-I
PI-II
eIF4A
rRNA

Materials and Methods). To determine the effect of *spr1* on wound-induced local and systemic gene expression, RNA gel blot analysis was used to measure *PI-I* and *PI-II* transcript levels in leaf tissue located at defined distances from a single wound inflicted at the distal end of the lower leaf (Figure 2-1A). As previously observed in wild-type (WT) tomato plants (Howe et al., 1996), *PI* mRNA accumulation in the undamaged section (section 2) of the wounded leaf was significantly greater than that in the damaged section (section 1) of the same leaf (Figure 2-1B). A relatively strong systemic response was observed in the undamaged leaf (sections 5 and 6), whereas WT petioles (sections 3 and 4) showed little or no *PI* expression. In the case of *spr1*, *PI* mRNA accumulation in the damaged section of the wounded leaf was comparable to that in WT (Figure 2-1C). Mutant plants also showed *PI* expression in adjacent unwounded tissue (section 2), albeit at a level lower than in WT. More significantly, however, the steady-state level of *PI* mRNA in the unwounded leaf (sections 5 and 6) of wounded *spr1* plants was <10% of that observed in WT. This result indicates that *spr1* impairs a signaling pathway that mediates or amplifies wound-induced systemic *PI* expression, but contributes less to *PI* expression near the wound site. This interpretation was supported by measurements of *PI-II* protein levels in damaged (local response) and undamaged (systemic response) leaves of wounded plants (data not shown). In six independent experiments involving at least six plants per genotype, the local response of *spr1* plants ranged between 50 and 75% of the WT response. Systemic *PI-II* accumulation in *spr1* plants ranged between 0 and 35% of that in WT plants, with the average response in the mutant being approximately 15% of WT levels. Analysis of plants homozygous for an independent allele of *spr1* (*spr1*-2) gave very similar results;

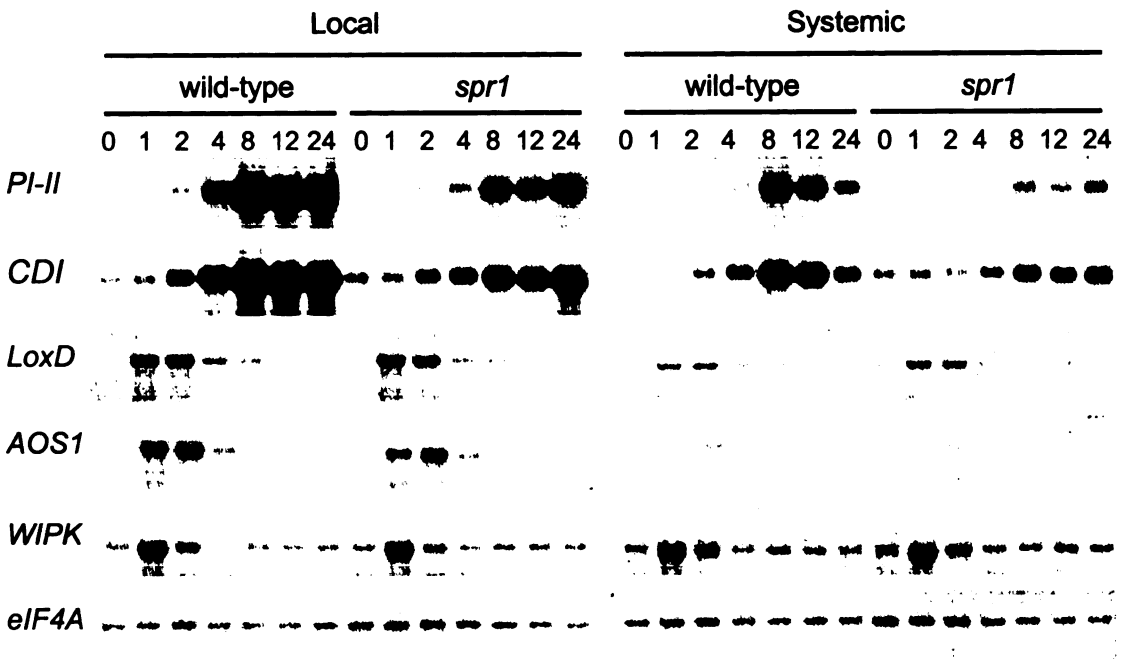


Figure 2-2. Time course of wound-induced gene expression in wild-type (WT) and *spr1* plants. WT and *spr1* plants (15-day-old) were wounded once on the lower leaf with a hemostat. Lower damaged (local response) and upper undamaged (systemic response) leaves were harvested at various times (hours) after wounding for RNA isolation and analysis as described in the legend for Figure 2-1. For each time point, six plants were harvested and pooled for RNA extraction.

wound-induced local and systemic *PI-II* accumulation in *spr1*-2 plants was 51 and 13%, respectively, of WT levels.

RNA gel blot analysis was used to determine the time course of local and systemic expression of various wound-responsive genes in *spr1* plants. Two classes of genes that differ with respect to their timing of wound-induced expression have been described in tomato (Ryan, 2000). Transcripts of so called 'late'-response genes, including *PI-I*, *PI-II*, and *cathepsin D inhibitor* (*CDI*), accumulate to maximum levels 8-12 hr after wounding of WT plants (Figure 2-2). Consistent with the results shown in Figure 1, *spr1* plants were deficient in the magnitude but not the timing of induction of these genes. Genes whose expression is induced rapidly and transiently in response to wounding comprise a second class of 'early'-response genes. Included among this group are genes encoding signaling-related proteins such as lipoxygenase (LoxD), allene oxide synthase1 (AOS1), and a putative mitogen-activated protein kinase (WIPK). Interestingly, wound-induced local and systemic expression of these early genes was not affected in *spr1* plants (Figure 2). These results indicate that *spr1* specifically affects the expression of late-response genes (i.e. *PI* genes).

spr1 plants are impaired in systemin-mediated signaling

To gain additional insight into the wound response phenotype of *spr1*, the capacity of the mutant to respond to various PI-inducing compounds was determined (Figure 2-3). Consistent with the ability of *spr1* to suppress *35S::prosys*-mediated *PI* expression, *spr1* plants did not accumulate PI-II in response to exogenous systemin or its bioactive precursor, prosystemin (Dombrowski et al., 1999). However, the mutant was responsive

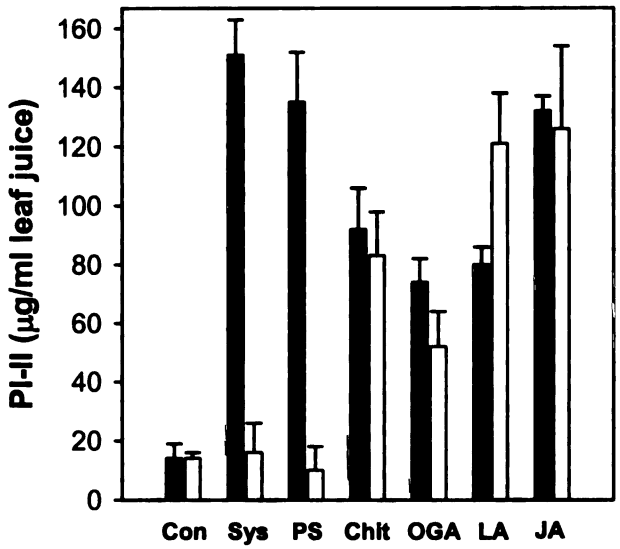


Figure 2-3. PI-II accumulation in wild-type (WT) and *spr1* plants in response to exogenous signaling compounds. WT (filled bar) and *spr1* (open bar) seedlings (15-day-old) were excised at the base of the stem and supplied with 15 mM sodium phosphate buffer (Con), systemin (Sys, 15 nM), recombinant prosystemin (PS, 0.1 µg ml^{-1}), chitosan (Chit,250 µg ml^{-1}), oligogalacturonide (OGA, 250 µg ml^{-1}), linolenic acid (LA, 5 mM), or jasmonic acid (JA, 100 nM). PI-II levels were measured 24 hr after treatment. Data points represent the mean and SD ($n = 6$).

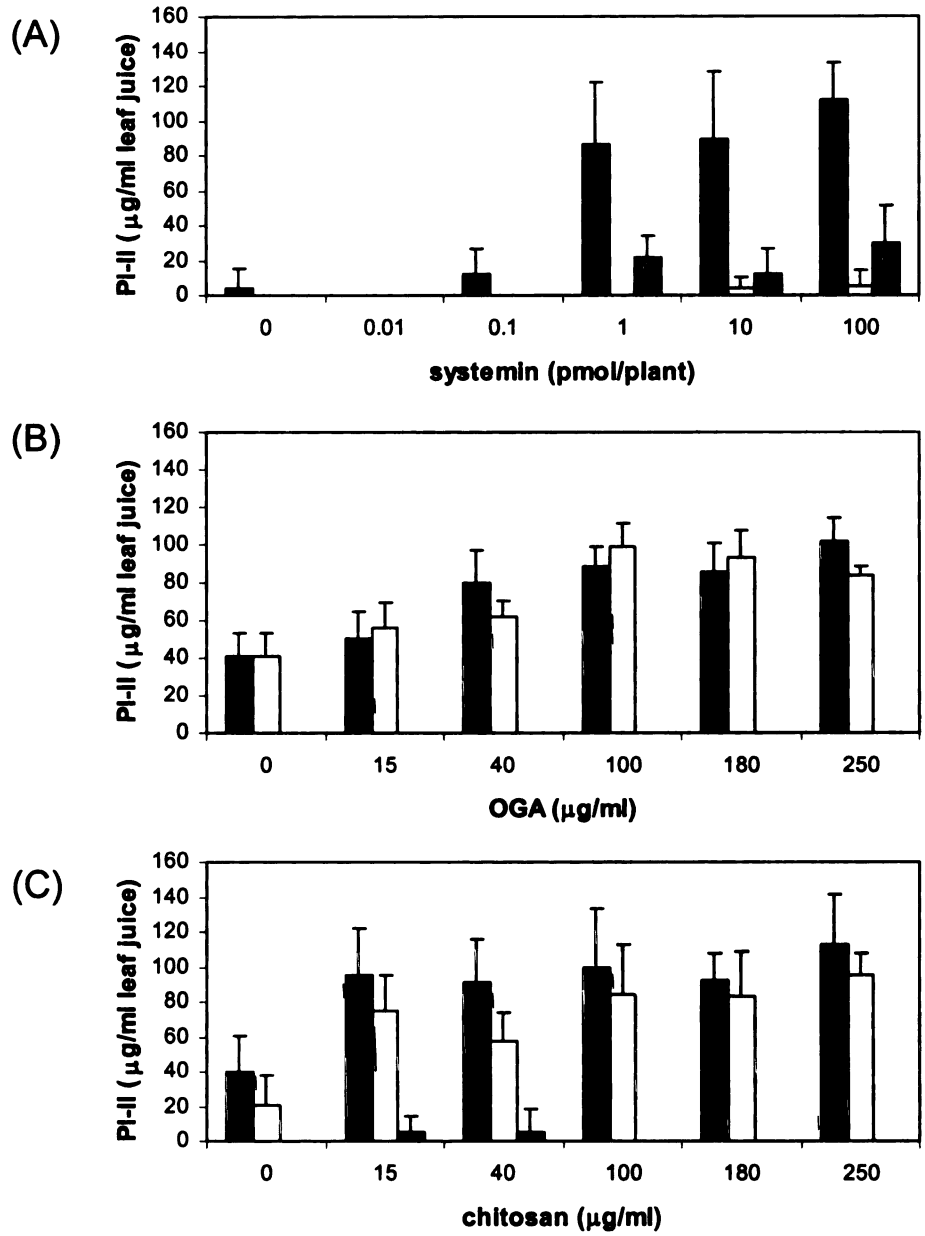
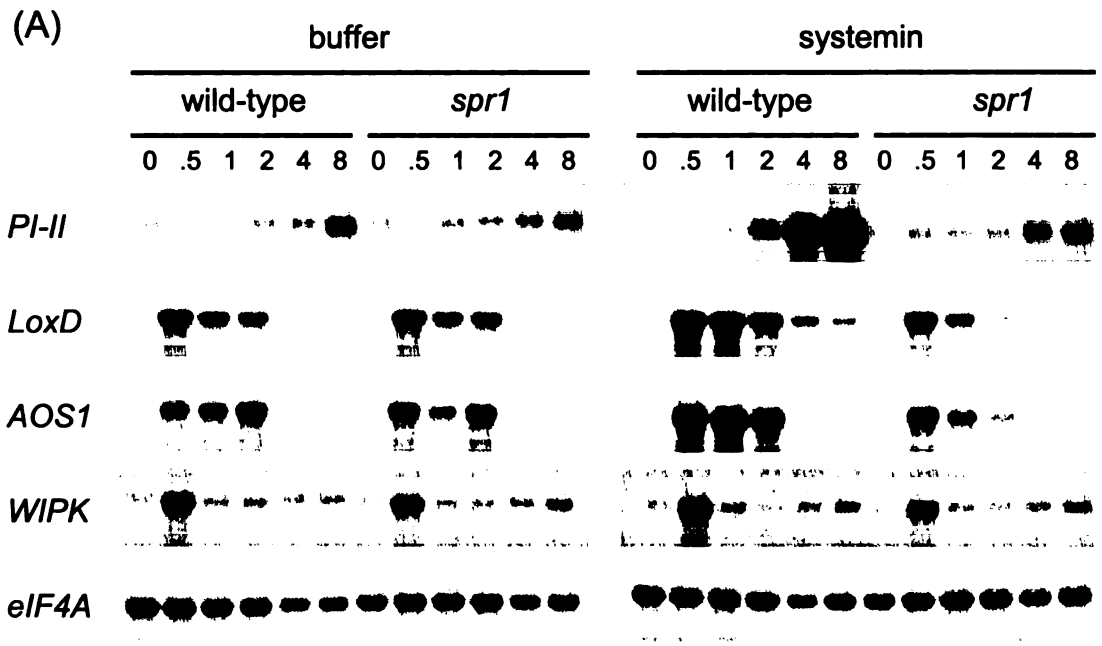


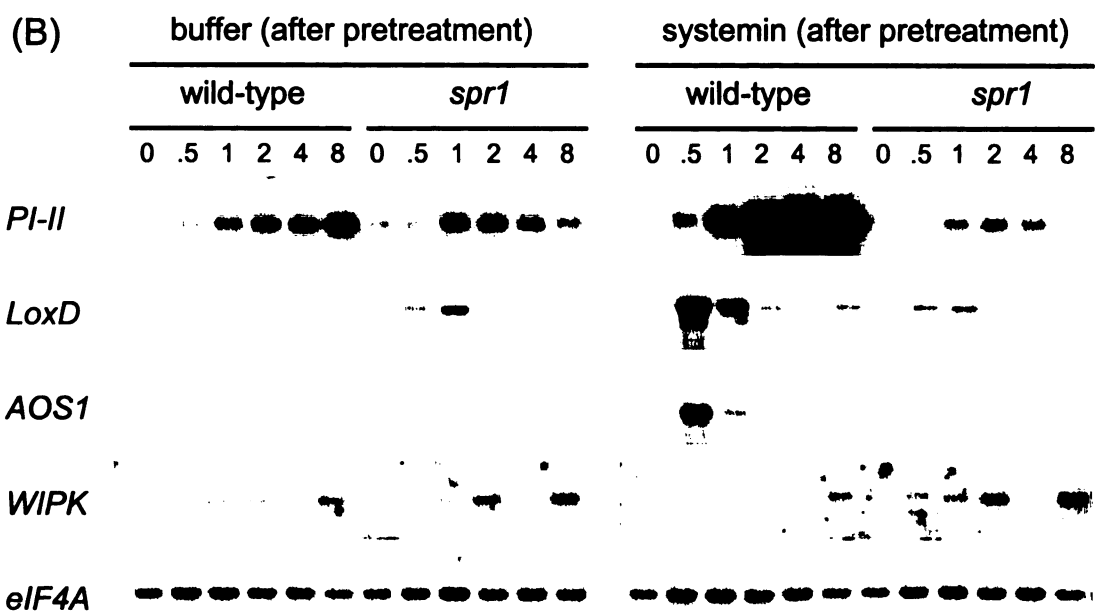
Figure 2-4. Response of *spr1* plants to exogenous systemin, oligogalacturonides (OGA), and chitosan. Two-leaf-stage wild-type (WT) (filled bars), *def1* (gray bars), and *spr1* (open bars) plants were excised at the base of the stem and supplied with either phosphate buffer ('0') or buffered solution containing various concentrations of systemin (A), OGA (B), or chitosan (C). PI-II accumulation in leaves was measured 24 hr after treatment. Values indicate the mean and SD ($n = 6$).

to octadecanoid signaling compounds (linolenic acid and JA) and to the polysaccharide elicitors OGA and chitosan. Because exogenous chitosan, OGA, and systemin activate *PI* expression via the octadecanoid pathway (Doares et al., 1995), these findings indicated that *spr1* affects systemin-mediated signaling at a point upstream of the octadecanoid pathway. To further test this hypothesis, the responsiveness of *spr1* plants to a range of concentrations of systemin, OGA, and chitosan was compared to that of WT (Figure 2-4). Parallel analysis of the JA-deficient *defenseless1* (*def1*) that is impaired in *PI* expression in response to systemin, OGA, and chitosan (Howe et al., 1996) was included as a control. WT plants showed a strong response to systemin concentrations of 1 pmol per plant and greater, whereas *def1* accumulated low levels of *PI-II* in response to high concentrations of systemin, as previously reported (Howe et al., 1996). *spr1* plants failed to accumulate significant levels of *PI-II* (<5% WT levels) in response to all concentrations of systemin tested. As expected, the *spr1* mutant responded normally to a range of concentrations of OGA and chitosan, whereas *def1* plants were unresponsive to these elicitors (Figures 2-4B, C). These results support the idea that *spr1* specifically affects the systemin branch of the wound-response pathway.

RNA gel blot analysis was used to determine the effect of *spr1* on systemin-mediated expression of early and late wound-response genes. Excision of seedlings at the base of the stem resulted in a gradual, low-level increase in *PI-II* mRNA accumulation in leaves of both WT and *spr1* plants (Figure 2-5A). WT plants accumulated high levels of *PI-II* mRNA in response to exogenous systemin, whereas *spr1* showed no response above that observed in the buffer control. This finding is consistent with the inability of *spr1* plants to accumulate PI-II protein in response to

Figure 2-5. Effect of exogenous systemin on gene expression in wild-type (WT) and *spr1* plants. (A) Excised seedlings (2-week-old) were incubated for 45 min in a solution containing 5 pmol systemin, and then transferred to water. At various times (hours) after the beginning of systemin treatment, leaf tissue was harvested for RNA isolation. Leaf tissue from six plants was pooled for each RNA isolation. RNA gel blots containing 5 μg total RNA were hybridized to probes for *PI-II*, *LoxD*, *AOS1*, *WIPK* and, as a loading control, *eIF4A*. (B) Excised seedlings were pre-incubated in water for 4 h and then transferred either to 300 μl phosphate buffer (buffer) or the same volume phosphate buffer containing 5 pmol of systemin ('0'). Following uptake of this solution (approximately 45 min), seedlings were transferred to water. At various times (hours) thereafter, leaves were harvested for RNA isolation.


(A)
buffer
systemin
wild-type
spr1
wild-type
spr1
0 .5 1 2 4 8 0 .5 1 2 4 8 0 .5 1 2 4 8 0 .5 1 2 4 8
PI-II
LoxD
AOS1
WIPK
eIF4A


(B)
buffer (after pretreatment)
systemin (after pretreatment)
wild-type
spr1
wild-type
spr1
0 .5 1 2 4 8 0 .5 1 2 4 8 0 .5 1 2 4 8 0 .5 1 2 4 8
PI-II
LoxD
AOS1
WIPK
eIF4A

systemin (Figure 2-4). Mock treatment (excision and incubation in buffer) of both WT and *spr1* seedlings resulted in rapid and transient expression of three early wound-response genes: *LoxD, AOS1*, and *WIPK*. In WT plants, systemin clearly enhanced the accumulation of *LoxD* and *AOS1* mRNA, as previously reported (Heitz et al., 1997; Sivasankar et al., 2000). However, the expression of these genes in *spr1* was not enhanced by systemin. In contrast to *LoxD* and *AOS1*, the steady-state level of *WIPK* mRNA in both WT and *spr1* plants was not affected by systemin treatment. The analysis of systemin-induced gene expression in WT and mutant plants was complicated by the fact that excision of seedlings at the base of the stem induced significant expression of early-response genes (Figure 2-5A). To determine the effect of systemin in the absence of this excision-induced effect, excised plants were pre-incubated in water for 4 hr (to allow mRNA levels to return to basal level), and then transferred to tubes containing either buffer or systemin (Figure 2-5B). A very low level of *LoxD* and *AOS1* expression was detected in buffer-treated WT and *spr1* plants, presumably as a result of handling (i.e. touching) of plants during the transfer procedure. Transfer of pre-incubated WT plants to a systemin-containing solution resulted in a rapid and transient increase in the steady-state level of *LoxD* and *AOS1* mRNAs, and a more gradual, massive accumulation of *PI-II* transcripts. The level of *WIPK* mRNA in WT plants was unaffected by systemin treatment, indicating that exogenous systemin stimulates expression of some (e.g. *LoxD*, *AOS1*, and *PI-II*) but not all (e.g. *WIPK*) wound-response genes. Treatment of pre-incubated *spr1* plants with systemin did not increase the accumulation of *LoxD*, *AOS1*, *PI-II*, or *WIPK* mRNAs above the level observed in buffer-treated plants. In summary, these results show that

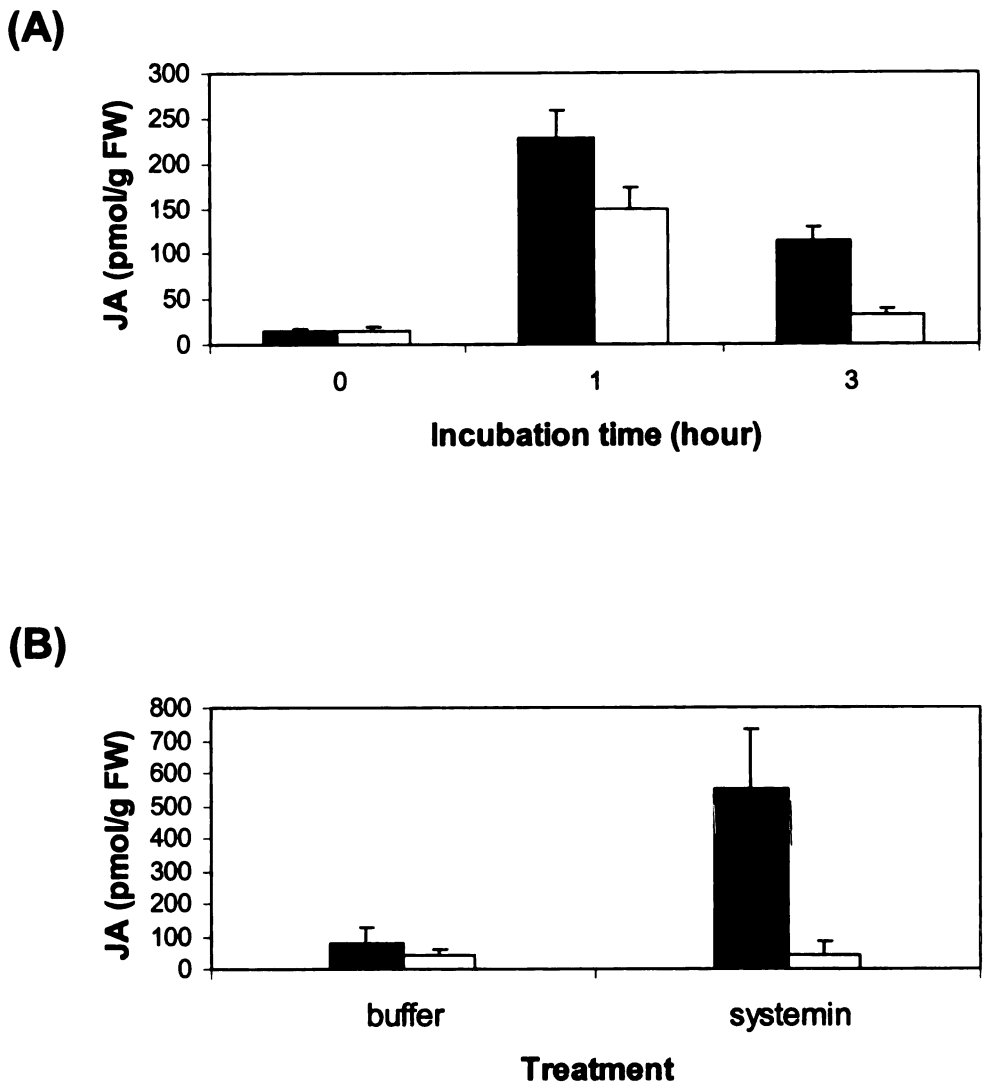


Figure 2-6. Jasmonic acid (JA) accumulation in response to systemin and mechanical wounding. (A) Leaves of 2-week-old wild-type (WT) (filled bar) or *spr1* (open bar) plants were mechanically wounded with a hemostat. At various times after wounding (1 or 3 hr), wounded leaf tissue was harvested for JA extraction. JA was also extracted from leaves of unwounded plants ('0').(B) Two-week-old seedlings were excised at the base of the stem, pre-incubated in water for 4 hr, and then transferred to either buffer unwanted plant ('0') or a buffered solution containing systemin (5 pmol per plant). Leaves were harvested for JA extraction 2 hr 45 min after systemin application. The amounts of JA in plant extracts were quantified by GC-MS. Data represent the mean and SD of three independent experiments.

spr1 impairs systemin-mediated activation of both early- and late-response genes. However, the mutation does not affect the rapid and transient activation of early genes in response to excision or wounding, indicating the existence of an *Spr1*-independent wound signaling pathway.

Effect of *spr1* on wound- and systemin-induced JA accumulation

Because wound- and systemin-induced *PI* gene expression is dependent upon the synthesis and subsequent action of JA, it was of interest to determine the capacity of *spr1* plants to accumulate JA in response to wounding and systemin. JA levels in unwounded leaves of WT and mutant plants were 15.1 ± 1.5 pmol JA/g Fresh Weight (FW) and 14.5 ± 4.6 pmol JA/g FW, respectively (Figure 2-6A). In wounded WT plants, JA levels increased 15- and 7.5-fold, 1 and 3 hr after wounding, respectively. Wounding also increased JA accumulation in *spr1* plants, albeit to levels that were significantly lower ($P < 0.05$) than WT levels. The amount of JA in wounded *spr1* leaves throughout the time course was estimated to be approximately 57% of that in WT leaves. Exogenous systemin induced high levels of JA accumulation in WT, but had no effect in *spr1* (Figure 2-6B). These findings indicate that *Spr1* is necessary for maximal levels of JA accumulation in response to wounding, but is strictly required for systemin-induced JA accumulation.

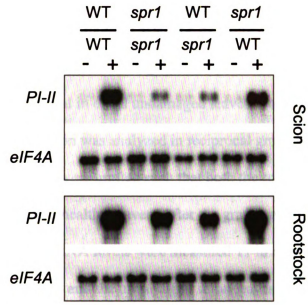


Figure 2-7. Wound-inducible *PI-II* expression in grafts between wild-type (WT) and *spr1* plants. WT and *spr1* plants were grafted in the four combinations indicated. The genotypes listed above and below the horizontal line correspond to the scion and rootstock, respectively. For each graft combination, plants were divided into a control (-) and experimental (+) group consisting of four grafted plants per group. For the experimental group, each leaflet on the rootstock was mechanically wounded with a hemostat. Eleven hours after wounding, leaf tissue was harvested separately from wounded rootstock leaves and undamaged scion leaves (scion) for RNA extraction. The control set of plants received no wounding, other than that inflicted by the grafting procedure itself. Levels of *PI-II* mRNA were analyzed by RNA blot analysis, using an *eIF4A* cDNA probe as a loading control. The results shown are representative of three independent experiments.

spr1 plants are defective in the generation of a systemic wound signal for *PI* expression

The deficiency of wound-induced systemic *PI* expression in *spr1* plants could result from a defect in production of a long-distance wound signal or a defect in the perception of this signal in distal undamaged leaves. To address this question, wound-induced *PI-II* expression was analyzed in reciprocal grafts between WT and *spr1* plants. Four-week-old plants were grafted such that both the rootstock (stock) and the scion contained at least two healthy leaves. After the graft junction healed, stock leaves were wounded and *PI-II* mRNA levels were measured 11 hr after in both the damaged stock leaves (local response) and the undamaged scion leaves (systemic response). Wounding of WT stock leaves resulted in local and systemic accumulation of *PI-II* transcripts to levels well above that observed in unwounded control plants that had also been grafted (Figure 2-7, lanes 1 and 2). This result demonstrates that wounding of WT stock leaves leads to the production of a graft-transmissible signal that is recognized in undamaged scion leaves. Consistent with the pattern of *PI-II* expression in two-leaf-stage *spr1* plants (Figure 2-1), wounded *spr1* stock leaves showed a relatively strong local response and a weak (10% WT) systemic response (Figure 2-7, lanes 3 and 4). Analysis of *spr1*/WT hybrid grafts showed that upon wounding of *spr1* stock leaves, WT scions failed to activate *PI-II* expression to levels greater than that in *spr1* scions that had been grafted to *spr1* stock (Figure 2-7, lanes 5 and 6). In the reciprocal combination, however, *spr1* scions were responsive to a signal emanating from wounded leaves of WT stock (Figure 2-7, lanes 7 and 8). Taken together, these findings indicate that *spr1* impairs wound-induced systemic *PI* expression mainly by blocking the production of

the long-distance wound signal in damaged leaves, rather than the recognition of that signal in systemic, undamaged leaves.

Discussion

spr1 defines a novel class of wound-response mutant

Forward genetic screens have identified two general classes of mutants that are defective in wound-induced systemic *PI* expression (Howe and Ryan, 1999; Li et al., 2001; Lightner et al., 1993). One group includes JA biosynthetic mutants (e.g. *def1* and *spr2*) that are unresponsive to upstream signals (e.g. systemin, OGA, and chitosan) that activate the octadecanoid pathway, but are responsive to exogenous JA. The second group includes JA-insensitive mutants (e.g. *jai1*) that are responsive neither to upstream signals nor to JA. Here, we show that *spr1* differs from existing wound-response mutants in that it is responsive to OGA and chitosan but unresponsive to systemin and its precursor, prosystemin. Consistent with the fact that OGA and chitosan activate PI expression through JA (Doares et al., 1995), *spr1* plants were responsive to exogenous JA and its metabolic precursor, linolenic acid (Figure 2-3). These findings indicate that the octadecanoid pathway and downstream signaling steps leading to *PI* expression are intact in *spr1* plants. The capacity of the mutant to accumulate significant levels of JA (approximately 57% WT levels) in response to wounding supports this idea. Considered collectively, the most straightforward interpretation of the results is that *Spr1* is involved in the perception of systemin or a subsequent systemin-specific signaling event necessary for activation of the octadecanoid pathway. Because systemin

perception occurs at the level of the plasma membrane (Meindl et al., 1998; Scheer and Ryan, 1999) and the initial steps of the octadecanoid pathway occur in the chloroplasts, it is possible that *Spr1* is involved in relaying a signal from the plasma membrane to the chloroplast. Included among the early signaling events induced by systemin are increased cytosolic Ca^{2+} levels, membrane depolarization, inhibition of a plasma membrane proton ATPase, activation of a MAP kinase activity, and activation of a phospholipase A_2 activity (Felix and Boller, 1995; Moyen and Johannes, 1996; Moyen et al., 1998; Narváez-Vásquez et al., 1999; Schaller and Oecking, 1999; Stratmann and Ryan, 1997). The insensitivity of *spr1* plants to both prosystemin and systemin indicates that the mutant is not defective in the synthesis or proteolytic processing of prosystemin. However, the data leave open the possibility that *spr1* impairs the interaction of systemin with SR160, or signaling output of the activated receptor complex. Additional work is needed to distinguish these possibilities.

Spr1-independent wound signaling

It is noteworthy that *spr1* appears to impair wound-induced systemic *PI* expression much more than it affects local *PI* expression. This finding indicates that the signaling pathway for systemic *PI* expression can be uncoupled from the signaling pathway that operates in tissue adjacent to the wound site. This aspect of *spr1* is reminiscent of the wound-response phenotype of prosystemin antisense plants that are compromised in prosystemin production but nevertheless have the capacity to respond to exogenous prosystemin (Dombrowski et al., 1999; McGurl et al., 1992). The robust local wound response of *spr1* and prosystemin antisense plants supports the hypothesis that multiple

signals generated at the wound site activate the octadecanoid pathway in wounded leaves (Doares et al., 1995; Farmer and Ryan, 1992; Ryan, 2000). This interpretation is consistent with the observation that *spr1* plants respond normally to OGAs, and accumulate significant levels of JA in response to wounding (Figures 2-4 and 2-6). Whether OGAs are responsible for the entire pool of wound-induced JA in *spr1* leaves or whether other mechanisms are involved in initiating the octadecanoid pathway remains to be determined.

Genetic analysis of the wound response in tomato plants indicates that the bulk of wound-induced systemic *PI* expression requires the systemin/JA signaling pathway. However, we did observe that *spr1* plants exhibit a low but significant level of wound-induced systemic *PI* expression. This residual signaling activity could reflect incomplete loss of *Spr1* function or, alternatively, a *Spr1*-independent pathway for systemic *PI* expression. Given the complete lack of systemin-induced gene expression in *spr1* plants, however, the latter possibility seems more likely. The existence of a systemin-independent wound-response pathway is clearly supported by the observation that *spr1* plants are not affected in wound- or cut-induced expression of early wound-response genes such as *LoxD* and *AOS1*. The fact that exogenous systemin enhances expression of *LoxD* and *AOS1* in WT plants (Figure 2-5) indicates that wounding and exogenous systemin may regulate the expression of these genes in somewhat different ways. For instance, it is possible that responses to systemin, when supplied through the transpiration stream, do not accurately reflect the wound-induced activity of systemin produced in vascular bundle cells of intact plants (Jacinto et al., 1997; Ryan, 2000). The expression pattern of *WIPK*, which was wound and cut inducible in both WT and *spr1*

plants, provides further evidence for systemin-independent wound signaling. Unlike the *LoxD* and *AOS1* genes, exogenous systemin did not stimulate *WIPK* mRNA accumulation in WT (or *spr1*) plants. Several other wound-induced rapid systemic responses have been described in plants (e.g. O'Donnell et al., 1998; Seo et al., 1995; Stratmann and Ryan, 1997), and may involve physical (e.g. hydraulic) signals propagated through the xylem (Malone, 1996) or the phloem (Rhodes et al., 1999).

Role of systemin in wound signaling

The signaling-related phenotypes of *spr1* plants are fully consistent with a role for prosystemin in regulating wound-induced systemic *PI* expression through the octadecanoid pathway (Farmer and Ryan, 1992; Li et al., 2001; McGurl et al., 1992). What is less clear, however, is how systemin and JA (and other signals) interact to effect wound signaling over long distances. Insight into this question was recently provided by means of grafting experiments showing that JA biosynthesis in rootstock leaves is essential for production of a long-distance signal for *PI* expression, whereas JA biosynthesis in undamaged leaves is not required for PI expression (Li et al., 2002a). Given that prosystemin works through JA, the most straightforward interpretation of these data is that systemin, acting in the rootstock portion of the graft, amplifies the synthesis of JA to levels that are required for long-distance signaling. Support for this model comes from the analysis of systemic *PI* expression in reciprocal grafts between WT and *spr1* (Figure 2-7). These experiments show that *Spr1* function is involved primarily in the production of the long-distance signal, rather than the recognition or processing of that signal in systemic undamaged leaves. The reduced level of wound-

induced JA in *spr1* plants further indicates that systemin may activate the synthesis of a specific pool of JA that is necessary for the systemic response, which is consistent with the lack of systemin-induced JA accumulation in *spr1* (Figure 2-6). Along these lines, two hypotheses have recently been proposed to explain the role of systemin in long-distance *PI* expression (Ryan and Moura, 2002). First, systemin may induce localized production of JA that subsequently exits the wounded leaflet and activates *PI* expression in distal leaves. Alternatively, systemin produced at the wound site may be translocated in the phloem where it activates JA synthesis in vascular tissues of leaves, petioles, and stems of the rootstock. The latter scenario indicates that a positive feedback loop between systemin and JA may amplify and propagate the systemic signal along the vascular system (Ryan, 2000; Ryan and Moura, 2002). Additional insight into the function of *Spr1* and other genes involved in systemic wound signaling may help to distinguish these hypotheses.

Materials and Methods

Plant material and treatments

Tomato (*Lycopersicon esculentum* Mill cv Castlemart) seedlings were grown in Jiffy peat pots (Hummert International, Earth City, MO) in a growth chamber maintained under 17 hr of light (200 $\mu E\ m^{-2}\ sec^{-1}$) at 28°C and 7 hr of dark at 18°C. Seed for *def1* was collected from a *def1/def1* homozygous line that was back-crossed four times using Castlemart as the recurrent parent. To simplify and standardize the genetic nomenclature, herein we refer to the previously described *spr-1* ^{593F} and *spr-1* ^{961E} alleles (Howe and Ryan, 1999) as *spr1-1* and *spr1-2*, respectively. Lines homozygous

for either of these two alleles were generated as described below. Unless otherwise indicated, *spr1-1* was used for all experiments.

Wounding and chemical elicitor experiments were performed with two-leaf-stage plants (14 to 16-day-old) as previously described (Howe et al., 1996). To assay the responsiveness of mutants to PI-inducing compounds, plants were excised at the base of the stem and placed in 0.5 ml microfuge tubes containing 300 µl of the inducing compound. When >75% of the elicitor solution had been imbibed (approximately 45 min), plants were transferred to glass vials containing 20 ml of water, and incubated in a Lucite box for 24 hr under continuous light. PI-II levels in leaves were measured by radial immunodiffusion assay (Ryan, 1967). Systemin, oligogalacturonic acid (OGA), and recombinant prosystemin (Dombrowski et al., 1999) were obtained from Dr. C.A. Ryan (Washington State University). Chitosan, JA, and linolenic acid were obtained from Sigma. All inducers except linolenic acid were diluted from stock solutions into sodium phosphate buffer (15 mm sodium phosphate, pH 6.5) prior to use. Linolenic acid was diluted into 15 mm sodium phosphate, pH 6.5, containing 0.05% (v/v) ethanol. Control experiments showed that 0.05% ethanol in 15 mm sodium phosphate buffer did not affect the background level of PI expression (data not shown). Grafting experiments were performed as described by Li et al. (2002a).

Genetic analysis

spr1-1 and *spr1-2* were originally isolated as recessive suppressors of *35S::prosys*-mediated responses (Howe and Ryan, 1999). Segregation of *spr1-1* and *spr1-2* from the *35S::prosys* transgene, for the purpose of isolating homozygous *spr1* alleles in an

otherwise WT genetic background, was achieved by crossing mutant lines (homozygous for the *35S::prosys* transgene and either *spr1-1* or *spr1-2*) to WT (cv Castlemart). Plants in the resulting F2 populations were tested for wound-induced PI-II accumulation with the assumption that *spr1* homozygotes would display a significantly reduced systemic response, as is the case for *prosystemin* antisense plants (McGurl et al., 1992). F2 plants showing a deficiency (<10% of WT levels) in the systemic response comprised approximately one-quarter of the population (data not shown) and were selected as putative *spr1* homozygotes. A polymerase chain reaction (PCR)-based assay (Li and Howe, 2001) was used to test these plants for the presence of the *35S::prosys* transgene. Individuals lacking the transgene were brought to the greenhouse for collection of F3 seed. To verify homozygosity of the *spr1* allele, the deficiency in wound-induced systemic *PI-II* expression and insensitivity to exogenous systemin were confirmed in F3 seedlings. Southern blot analysis confirmed the absence of 35S::prosys. Selected *spr1* homozygotes (either *spr1-1* or *spr1-2*) were back-crossed again to WT (cv Castlemart). Seedlings in the resulting F2 populations were scored for wound-induced systemic accumulation of PI-II, as well as for systemin-induced PI-II accumulation. The results were consistent with the expectation that *spr1-1* and *spr1-2* impair both responses and behave as single recessive mutations.

RNA gel blot analysis

RNA was isolated from tomato leaves and analyzed by gel blot hybridization as described by Li et al. (2002b). Gels were run in duplicate, with one set stained with ethidium bromide to check for equal loading of the samples and intactness of the RNA.

DNA probes were isolated and radiolabeled with [^{32}P-α] dCTP as described by Howe et al. (2000). The tomato EST clones, cLEC9C14 and cLET1D13, were used as probes for detection of *AOS1* (Sivasankar et al., 2000) and *WIPK* transcripts, respectively. Hybridization results were visualized by autoradiography using Kodak XAR-5 film and, when appropriate, quantified using a Phosphorimager (Molecular Dynamics). Hybridization signals were normalized to the signal obtained using a cDNA probe (EST clone cLED1D24) for translation initiation factor *eIF4A*. To directly compare transcript levels in WT and *spr1* plants, blots containing RNA from both plant types were hybridized in the same container, washed under the same conditions, and exposed to film for the same length of time.

Measurement of jasmonic acid

Jasmonic acid was extracted from leaves (10 g FW) of two-leaf-stage plants as previously described (Li et al., 2002b). Dihydrojasmonic acid (DHJA) was used as an internal standard for quantification of JA levels by gas chromatography-mass spectrometry (Li et al., 2002a).

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Appendix of Chapter 2

Spr1 does not encode SR160, the systemin receptor

The cloning of *SR160* from *spr1* plants was performed by Mr. Carl Andre as part of his lab rotation project.

The BC1 mapping population used in this study was generated by Dr. Chuanyou Li.

Introduction

The previous chapter describes the putative role of the *Suppressor of prosystemin-mediated responses1* (*Spr1*) gene in systemin-mediated signaling. Systemin failed to induce synthesis of proteinase inhibitors (PIs) in *spr1* plants, whereas other elicitors such as oligogalacturonic acid (OGA) and jasmonic acid (JA) activate normal PI accumulation in the mutant (Figure 2-3 and 2-4). Because both systemin and OGA induce expression of *PIs* by stimulation of JA biosynthesis (Doares et al., 1995a; Doares et al., 1995b; Howe et al., 1996), these results indicate that *spr1* plants are compromised in the perception of systemin or a subsequent systemin-specific signaling event necessary for activation of JA biosynthesis.

Systemin perception is mediated by the systemin receptor, SR160, which is bound to the plasma membrane (Meindl et al., 1998; Scheer and Ryan, 1999). Using a photoaffinify labeling approach, SR160 was purified from cultured cells of the wild tomato species *Lycopersicon peruvianum*. This work led to the isolation of the corresponding cDNA encoding SR160. DNA sequence analysis showed that *SR160* encodes a leucine-rich repeat (LRR) receptor kinase that shows high similarity to the *BRI1* gene that encodes the brassinolide receptor of Arabidopsis (Scheer and Ryan, 2002).

The systemin-insensitivity of *spr1* plants indicates the possibility that *Spr1* encodes SR160. Experiments described here were designed to test this hypothesis. The results demonstrate that the *spr1* mutation does not affect SR160. Thus, it is likely that the *Spr1* gene product acts downstream of SR160 in the wound signaling pathway to stimulate JA biosynthesis.

Results

Cloning of *SR160* from *spr1* plants

To determine whether *spr1* plants carry a mutation in the *SR160* gene, the sequence of *SR160* cloned from *spr1* and its corresponding wild-type *L. esculentum* was compared. Because the *SR160* cDNA was previously isolated from *L. peruvianum* (Scheer and Ryan, 2002), this sequence information was used to design two oligonucleotide primers to clone the *SR160* from *spr1* and wild-type *L. esculentum*. Genomic DNA from these plants was used as a template for PCR to clone *SR160*. Amplified products from these reactions were cloned and sequenced. The results demonstrated that *SR160* genes from both *spr1* plants and wild-type (*L. esculentum*) contain an intronless 3624 bp open reading frame, 62 bp of 5' untranslated region (UTR), and 122 bp 3' UTR. Comparisons between *SR160* from wild-type and *spr1* plants showed no sequence differences (Figure 2-8). Comparison of the deduced amino acid sequence of SR160 from *L. esculentum* to *L. peruvianum* showed sequence variation at 10 amino acid residues (Figure 2-9).

Analysis of linkage between *Spr1* and *SR160* loci

To further investigate whether *Spr1* and *SR160* correspond to the same gene, linkage of the two loci was examined in a BC1 mapping population that segregates for both the *spr1* trait (i.e., systemin insensitivity) and a restriction fragment length polymorphism (RFLP) at the *SR160* locus. The mapping population was generated from a cross

Figure 2-8. Genomic DNA sequence of *SR160* isolated from *L. esculentum*. A PCR-strategy was used to clone *SR160* from *spr1* and wild-type *L. esculentum*. DNA sequence analysis showed no sequence differences in *SR160* between *spr1* plants and *L. esculentum*. Both genes contain an intronless ORF (3624 bp). The initiation and termination codons are boxed. Nucleotide sequences corresponding to the PCR primers are underlined.

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   1 AGAACTCAAGCTATAGATTCAAGAAAATCACCATTTAAGCTATAAAGTTTCAATCTTTGA
  61 AGATGAAAGCTCACAAAACTGTGTTTAACCAACATCCTTTGAGCTTAAACAAGCTTTTCT
 121 TTGTTCTTCTTCTTATCTTTTTTCTTCCACCAGCTTCACCAGCAGCTTCTGTTAATGGTC
 181 TTTATAAAGACTCCCAACAGCTTCTTTCCTTTAAAGCTGCACTCCCACCAACCCCAACTC
 241 TGCTTCAGAACTGGTTGTCATCTACTGACCCTTGTAGTTTCACTGGTGTTTCATGCAAGA
 301 ATTCTAGAGTTTCTTCTATAGATCTCAGTAACACTTTTTTAAGTGTGGATTTCAGTTTGG
 361 TCACTTCTTATTTGCTTCCTCTTTCTAATTTGGAGTCTTTGGTGTTAAAGAATGCTAATC
 421 TTAGTGGTTCTTTAACTTCTGCTGCAAAATCCCAATGTGGGGTTACTTTAGACTCCGTAG
 481 ATCTAGCTGAGAACACAATTTCTGGACCTATTTCTGATATCTCTAGCTTTGGTGTTTGTT
 541 CAAACCTTAAGTCTCTTAATCTTTCTAAGAATTTCTTGGACCCTCCTGGTAAAGAAATGC
 601 TTAACGCTGCAACCTTTAGCCTCCAAGTTCTTGATCTTTCTTACAATAATATCTCAGGGT
 661 TTAACTTGTTTCCATGGGTTTCATCTATGGGGTTTGTTGAACTTGAGTTCTTTTCTCTCA
 721 AGGGTAACAAGCTAGCTGGAAGTATTCCTGAATTAGACTTCAAGAATTTGTCATATTTGG
 781 ATCTTTCTGCAAATAATTTCTCAACTGTTTTTCCTTCATTCAAAGATTGCTCCAATTTGC
 841 AGCACTTGGATTTGTCATCCAACAAGTTTTATGGTGATATTGGTTCTTCACTTTCTTCAT
 901 GTGGGAAGCTCAGTTTTCTCAACCTTACCAATAACCAGTTTGTAGGTTTGGTCCCTAAGC
 961 TACCAAGTGAAAGTCTACAGTATTTGTACTTAAGAGGGAATGATTTTCAGGGTGTGTACC
1021 CAAACCAACTTGCTGATTTGTGCAAAACTGTGGTGGAATTGGACTTGTCATACAATAATT
1081 TCTCAGGCATGGTTCCTGAGAGCCTTGGTGAATGTTCAAGTTTGGAACTTGTTGATATTT
1141 CCTACAATAATTTCTCTGGTAAGTTGCCTGTTGATACTCTCTCCAAGTTGAGTAATATTA
1201 AGACTATGGTCTTATCATTCAACAAATTTGTTGGTGGTTTGCCTGATTCTTTCTCTAATT
1261 TACTGAAATTGGAGACTTTGGATATGAGTTCTAATAATCTCACAGGGGTTATTCCATCTG
1321 GGATTTGCAAAGATCCTATGAATAACTTGAAAGTGCTGTACCTTCAGAATAACTTGTTTA
1381 AAGGCCCTATACCTGACAGTCTAAGCAACTGTTCACAGCTGGTGTCACTTGATCTTAGCT
1441 TTAATTACTTGACTGGGAGTATACCATCTAGTTTGGGGTCATTGTCAAAGCTAAAGGATC
1501 TCATCCTTTGGTTAAATCAGCTTTCAGGGGAAATCCCACAGGAGTTGATGTACTTGCAGG
1561 CTTTGGAGAATTTGATTCTTGATTTTAATGACTTAACTGGACCAATACCTGCAAGTCTTA
1621 GCAACTGTACCAAGTTGAATTGGATTTCATTGTCAAATAACCAATTGAGTGGTGAGATAC
1681 CGGCTTCTCTTGGGCGTTTGTCAAATCTAGCTATTCTTAAGCTTGGAAACAACTCAATCT
1741 CAGGGAATATACCTGCTGAATTGGGTAATTGCCAGAGCTTGATATGGTTGGATCTCAATA
1801 CTAATTTCCTGAATGGATCCATTCCGCCACCTTTGTTCAAGCAATCTGGCAATATTGCAG
1861 TGGCATTACTGACCGGGAAGCGATACGTGTATATCAAGAATGATGGGAGTAAGGAGTGCC
1921 ATGGAGCAGGGAATCTGCTGGAGTTTGGAGGGATTAGACAGGAACAGCTGGATAGAATCT
1981 CAACAAGGCATCCTTGCAATTTCACAAGAGTTTATAGAGGTATCACTCAGCCAACATTTA
2041 ACCACAATGGCTCTATGATATTTCTTGATTTATCTTATAATAAGTTGGAAGGTAGTATCC
2101 CAAAGGAATTAGGGGCAATGTACTATCTGTCTATATTGAATTTGGGGCATAATGATCTGT
2161 CTGGTATGATTCCTCAACAACTTGGAGGCTTGAAGAATGTTGCAATTCTTGATTTGTCAT
2221 ATAATAGGTTCAATGGCACGATCCCGAATTCCCTCACCAGTCTTACATTGCTTGGAGAGA
2281 TTGACCTGTCAAACAATAATCTCAGTGGAATGATTCCTGAATCTGCACCATTTGACACAT
2341 TCCCTGATTATAGGTTTGCGAATAATTCCCTCTGTGGGTATCCTCTCCCCATACCTTGTA
2401 GCTCGGGGCCGAAATCGGATGCAAATCAGCATCAGAAGTCTCACCGCAGACAAGCATCGT
2461 TGGCAGGGAGTGTGGCCATGGGTTTGTTATTTTCCCTCTTTTGTATCTTTGGTTTGATTA
2521 TTGTTGCCATAGAGACGAAGAAGAGGAGGAGGAAGAAGGAGGCTGCTCTTGAAGCTTATA
2581 TGGATGGTCATTCACATTCTGCAACTGCCAACAGTGCCTGGAAGTTTACGAGTGCTCGTG
2641 AGGCGTTAAGCATCAACCTTGCAGCATTTGAGAAGCCTCTCAGGAAGCTCACATTTGCTG
2701 ATCTTCTCGAAGCCACCAATGGTTTCCACAACGACAGTCTTGTAGGCTCTGGTGGTTTTG
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2761 GTGATGTCTACAAAGCTCAGTTGAAGGATGGGAGTGTTGTAGCTATTAAGAAATTGATAC
2821 ACGTCAGTGGACAGGGTGATCGAGAATTCACTGCTGAAATGGAAACCATAGGGAAGATCA
2881 AGCACCGCAACCTTGTCCCTCTTTTGGGCTACTGCAAAGTAGGGGAAGAAAGACTACTGG
2941 TTTATGAATACATGAAGTATGGAAGTCTTGAAGATGTCCTGCATGATCGGAAGAAAATTG
3001 GGATCAAGCTGAATTGGCCTGCAAGAAGGAAAATTGCCATTGGAGCTGCGAGAGGTTTGG
3061 CTTTCCTACACCATAACTGCATTCCACACATCATTCACCGGGACATGAAATCAAGTAATG
3121 TCTTGCTTGATGAAAATTTGGAAGCCAGAGTATCTGATTTCGGAATGGCAAGGTTAATGA
3181 GTGCTATGGACACTCATTTGAGTGTCAGCACTCTTGCCGGCACTCCAGGATACGTACCTC
3241 CTGAATATTACCAAAGCTTTAGATGTTCTACAAAAGGAGACGTTTATAGTTATGGTGTCG
3301 TATTACTTGAGCTTCTAACCGGCAAACAGCCAACAGATTCAGCTGATTTTGGTGACAACA
3361 ATCTTGTCGGATGGGTAAAGCTGCACGCTAAGGGAAAAATAACAGATGTCTTTGACCGGG
3421 AGCTATTGAAAGAGGATGCAAGCATTGAGATTGAACTTCTACAACACTTAAAGGTAGCTT
3481 GTGCTTGCTTAGATGATCGACATTGGAAACGTCCCACAATGATACAAGTTATGGCTATGT
3541 TTAAGGAGATTCAAGCAGGGTCAGGCATGGATTCGACATCGACAATCGGAGCTGATGATG
3601 TTAATTTTAGTGGAGTTGAAGGAGGGATAGAAATGGGGATAAATGGAAGTATAAAAGAAG
3661 GCAATGAGCTGAGCAAACACCTTTGAATGCACTAAATGAAGAGTTTATTGAAAGCTCACA
3721 AATTTTCCAAAATCATCATATGCAAAGTGTAATTTTTAGCCCCCAATTATTGTATGTACC
3781 ACTAGTTCCCATCCATAAAATCTTGTGT
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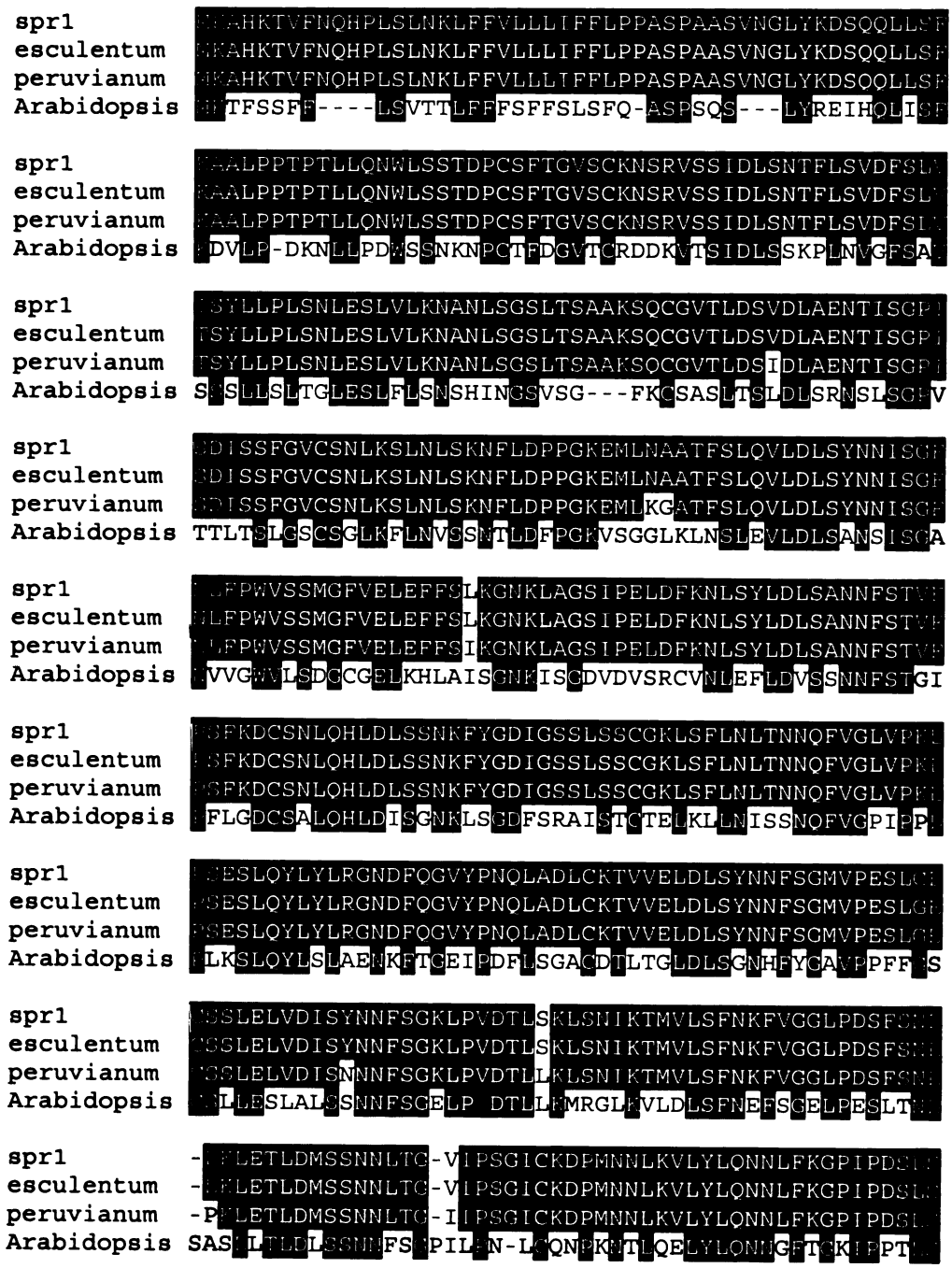
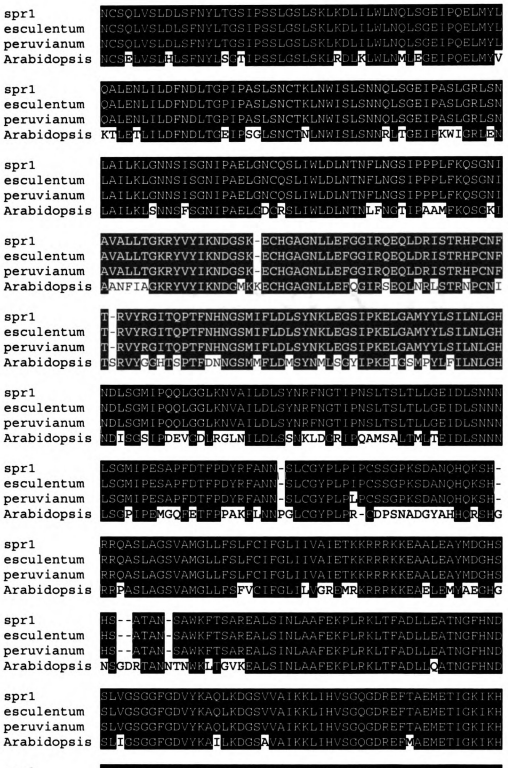
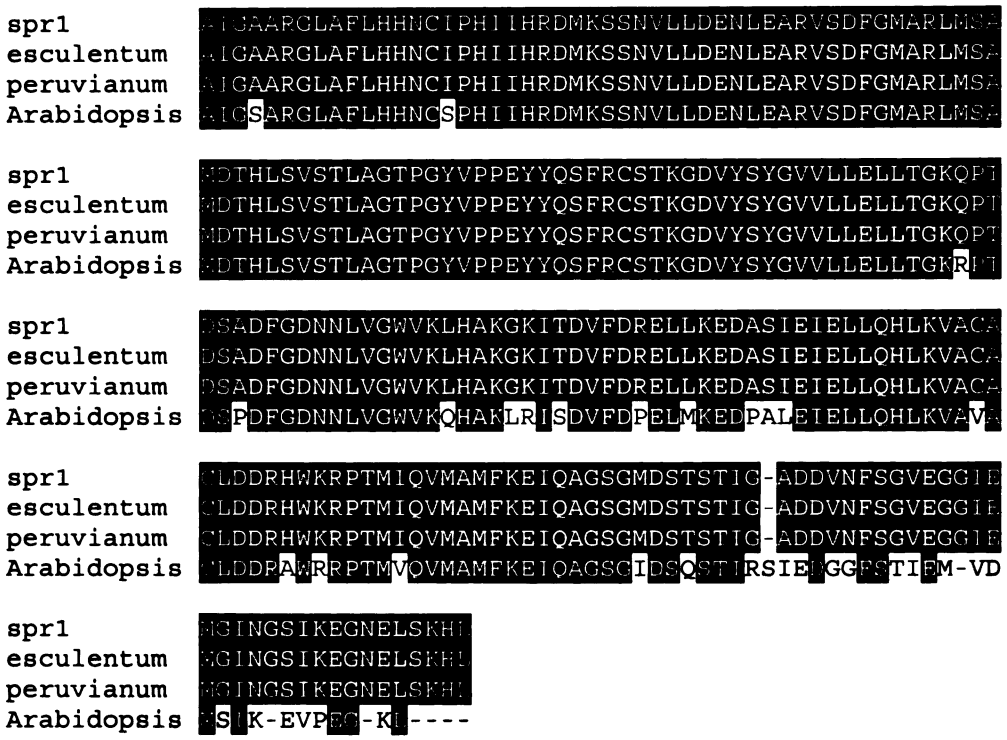
Figure 2-9. Comparison of deduced amino acid sequences of SR160 and BRI1. *SR160* genes were isolated from *spr1* plants, cultivated tomato (*L. esculentum*), and the wild tomato species, *L. peruvianum*. Deduced amino acid sequences of these genes were aligned with that of Arabidopsis brassinolide receptor BRI1, using the Clustal W program available at http://www.ch.embnet.org. Black boxes indicate conserved amino acid residues among all aligned sequences.




spr1
esculentum
peruvianum
Arabidopsis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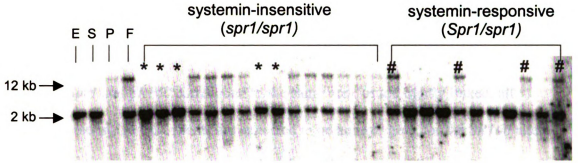

Figure 2-10. Linkage analysis of *SR160* and *Spr1*. To test linkage between *SR160* and *Spr1*, a BC1 mapping population was generated as described in the text. RFLP analysis was used to test for linkage between *SR160* and *Spr1* in this BC1 population. *Xba*I digestion of genomic DNA generated a species-specific RFLP pattern at the *SR160* locus (*L. esculentum*, 2 kb band; *L. pennellii*, 12 kb band). To test whether the *spr1* allele and the *L. esculentum*-specific RFLP cosegregate in the BC1 population, genomic DNA was prepared from 26 BC1 plants for RFLP analysis. The blot was probed with a 2 kb *Eco*RI fragment from the *SR160* DNA. Asterisks show the cosegregation of the *spr1* allele and *L. esculentum*-specific RFLP pattern. The cosegreation of the *Spr1* allele and *L. pennellii*-specific RFLP is noted with #. E, *L. esculentum*; S, the parental *spr1* plant originated from *L. esculentum*; P, *L. pennellii*; F, the F1 plant.

between a homozygous *spr1* plant (*L. esculentum*) and the wild tomato species *L. pennellii* (*Spr1/Spr1*), followed by a backcross of the resulting F1 plant to the parental *spr1* line (*L. esculentum*).

To determine the genotype of individual plants within BC1 population, each BC1 plant was assayed for systemin-induced PI accumulation. Systemin-insensitive BC1 plants do not accumulate PIs in response to systemin, and thus are homozygous for *spr1*. In contrast, systemin-responsive BC1 plants must be heterozygous; they contain an *spr1* allele inherited from *L. esculentum* and an *Spr1* allele transmitted from *L. pennellii*.

If *Spr1* and *SR160* correspond to the same gene or are tightly linked, all systemin-insensitive BC1 plants are expected to be homozygous for the *SR160* allele transmitted from *L. esculentum* ($SR160^{esc}$ /$SR160^{esc}$). Conversely, systemin-responsive BC1 plants should have one allele of *SR160* inherited from *L. pennellii* ($SR160^{pen}$) and another allele transmitted from *L. esculentum* ($SR160^{esc}$). To test this, the genotype ($SR160^{esc}$ /$SR160^{esc}$ or $SR160^{pen}$ /$SR160^{esc}$) of the *SR160* locus for each BC1 plant was determined using RFLP analysis that distinguishes the $SR160^{pen}$ and $SR160^{esc}$ alleles (Figure 2-10). Among 26 BC1 plants examined, only 9 plants showed a match between the systemin-sensitivity phenotype and the expected RFLP pattern of *SR160* (Figure 2-10; lanes indicated with * or #). These results indicate that the *Spr1* and *SR160* loci are assorting independently of one another. Therefore, *Spr1* does not encode the systemin receptor, SR160.

Expression of *SR160* in *spr1* plants

Linkage analysis demonstrated that *Spr1* and *SR160* are different genes. However, this result does not rule out the possibility that the *spr1* mutation may affect the expression of *SR160*. To test this possibility, RNA gel blot analysis was used to determine the steady state level of *SR160* mRNA in *spr1* and wild-type plants (Figure 2-11). Both wild-type and *spr1* plants accumulated comparable levels of *SR160* mRNA in leaves. Wounding did not significantly change the basal expression levels. Taken together with DNA sequence analysis of *SR160*, these results indicate that *spr1* plants are not compromised in *SR160* or its expression.

Discussion

I tested the hypothesis that the systemin-insensitive phenotype of *spr1* plants results from a defect in systemin receptor the SR160. Several lines of evidence disproved this hypothesis. First, linkage analysis demonstrated that *Spr1* does not encode SR160. Second, DNA sequence analysis showed that the *SR160* gene in *spr1* plants does not harbor a mutation in the coding region. Furthermore, *spr1* plants showed normal expression of *SR160*. These results demonstrate that the *spr1* mutation is not related to *SR160*. The *Spr1* gene product may, therefore, act downstream of SR160 to activate JA biosynthesis in systemin-mediated signaling. Alternatively, the *Spr1* product may assist in the binding of systemin to SR160. Precedence for this hypothesis comes from the observation that the Arabidopsis BRI1 associated receptor kinase 1 (BAK1) mediates brassinolide binding by forming a heterodimer with BRI1 (Nam and Li, 2002).

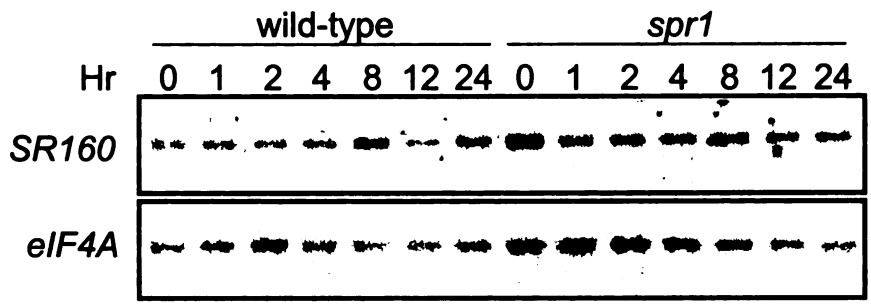

Figure 2-11. Expression of *SR160* in wounded wild-type and *spr1* plants. Two-leaf-stage *spr1* and wild-type plants were wounded once on each leaf with a hemostat. Wounded leaves were harvested at indicated time (hour) after wounding for preparation of RNA. For each time point, six plants were harvested and pooled for RNA extraction. RNA gel-blot analysis was performed using a 2 kb *Eco*RI fragment of *SR160* gene as a probe. A duplicated blot was probed with cDNA encoded eIF4A as a RNA loading control.

The tomato genome contains a single copy of *SR160*, which shows high similarity to Arabidopsis *BRI1* gene that encodes the brassinolide receptor (Scheer and Ryan, 2002). Interestingly, a recent study indicates that SR160 acts as the brassinolide receptor in tomato (Montoya et al., 2002). It was found that brassinolide-insensitive *curl3* plants have a nonsense mutation in *SR160*. This finding indicates that SR160 could be a dual ligand receptor of systemin and brassinolide. Additional insight into the dual role of SR160 could be obtained by investigating systemin binding and the systemic wound response in *curl3* plants that lack a functional SR160. Because brassinolide regulates plant growth, *curl3* plants display a dwarf phenotype (Montoya et al., 2002). If brassinolide and systemin compete for binding to SR160, it is expected that increased levels of systemin may interfere with brassinolide action. In mammals, the peptide hormone oxytocin and steroid hormone progesterone compete for the same receptor (Grazzini et al., 1998). Recent *in vitro* experiments indicate that brassinolide does not inhibit the binding of systemin to SR160 (Scheer and Ryan, 2002). Therefore, the two ligands seem not to compete for binding to SR160 in tomato plants. It remains to be determined how SR160 regulates two distinct signaling pathways.

Systemin binding to the receptor activates several rapid responses in tomato cells, including increased cytosolic Ca^{2+} levels, depolarization of the plasma membrane, inactivation of a proton-ATPase associated with the plasma membrane, and activation of a mitogen-activated protein kinase (MAPK) activity (Felix and Boller, 1995; Moyen and Johannes, 1996; Moyen et al., 1998; Schaller and Oecking, 1999; Stratmann and Ryan, 1997). It is currently unclear how the activated SR160 promotes these effects and

how these changes are related to increased JA synthesis. Molecular cloning of *Spr1* could provide a clue to address these questions.

Materials and Methods

Plant material

Plant growth conditions were the same as described in Chapter 2. The mapping population for linkage analysis was generated from a cross between *L. esculentum* carrying the homozygous *spr1* mutation and wild-type *L. pennellii*. One of the resulting F1 plants was backcrossed to the parental homozygous *spr1* plant to produce the BC1 population. Individual BC1 plants were tested for systemin-induced PI-II accumulation as described in Chapter 2. Briefly, two-leaf-stage plants were excised at the base of the stem and supplied with systemin dissolved in phosphate buffer (5 pmol systemin/plant). Accumulation of PI-II in leaves was measured 24 hr later using a radioimmunodiffusion assay. Each plant was transferred to a glass vial containing distilled water and incubated in the growth chamber to promote rooting. Plants were transferred to soil after roots were established (approximately 7 days). For genotyping, DNA was isolated by leaf tissue of 6-week-old plants.

To examine the expression of *SR160*, two-leaf-stage plants were wounded once on the lower leaf with a hemostat. Leaves were harvested at various times after wounding for extraction of total RNA.

Cloning of *SR160*

A PCR technique was used to clone *SR160* from *spr1* and wild-type *L. esculentum*. Two oligonucleotide primers were designed using the sequence of *SR160* cloned from *L. peruvianum*: 5'-AGAACTCAAGCTATAGA-3' and 5'-ACACAAGATTTTATGGATGGGA-3'. These primers correspond to the 5' and 3' UTRs, respectively. Genomic DNA was prepared from *spr1*and wild-type *L. esculentum* to prepare template in PCR, following the method described in Chapter 6. PCR was performed with Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) following the manufacturer's instruction. The amplified products were subcloned into *Eco*RV site of pBluescript SK (-) plasmid (Stratagene, La Jolla, CA) and sequenced (Genomics Technology Support Facility, Michigan State University, East Lansing, MI).

Nucleic acid hybridization

To find a RFLP for *SR160*, genomic DNA was prepared from *L. esculentum and L. pennellii,* and digested with several restriction enzymes. The reaction products were run on 0.8% agarose gel and transferred to Hybond membrane (Amersham, Piscataway, NJ). To prepare the radiolabeled probe, a 2 kb *Eco*RI fragment of *SR160* was labeled with [^{32}P-α] dCTP. The DNA gel-blot analysis was performed following the method describe in Chapter 6. The result showed that *Xba*I digestion generated an RFLP for *SR160.*

Total RNA was extracted from leaves of *spr1* and wild-type plants following the method described in Chapter 6. The RNA gel-blot was hybridized to the 2 kb *Eco*RI fragment of *SR160*, following the method described in Chapter 2.

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Chapter 3

The wound response mutant JL1 is defective in the conversion of 12-oxo-phytodienoic acid to jasmonic acid

Introduction

The plant hormone jasmonic acid (JA) is synthesized from linolenic acid via the octadecanoid pathway. The pathway is divided into two parts by the subcellular location of the enzymes involved. The first part of the octadecanoid pathway occurs in chloroplasts where the release of linolenic acid from plastid membranes leads to the synthesis of 12-oxo-phytodienoic acid (OPDA). The second part takes place in peroxisomes where OPDA, presumably transported from chloroplasts, is processed to JA (Figure 3-1; Turner et al., 2002; Wasternack and Hause, 2002).

Several lines of evidence demonstrate that JA regulates the wound response of tomato plants. First, application of JA or its methyl ester (MeJA) triggers the accumulation of wound-inducible proteins such as proteinase inhibitors (PIs; Farmer and Ryan, 1992; Farmer et al., 1992). Second, the endogenous level of JA increases rapidly upon wounding, which is followed by expression of *PIs* (Conconi et al., 1996). Third, the expression of *PIs* is abolished by both chemical inhibitors and mutations that block the octadecanoid pathway (Doares et al., 1995; Howe et al., 1996). Recent studies show that JA is a component of the long-distance signaling pathway that relays signals produced in the wound site to systemic expression of *PIs* in undamaged leaves (Li et al., 2002).

Analysis of wound response mutants has expanded our knowledge of the role of JA in the wound response. The JL1 and JL5 (renamed *defenseless1*; *def1*) mutant lines of tomato were isolated from an ethyl methanesulfonate (EMS)-mutagenized population by screening for plants that are unable to accumulate PIs in response to wounding (Lightner et al., 1993). Exogenous MeJA restored the production of PIs in both mutants,

indicating that they are defective in the biosynthesis of JA. Genetic analysis demonstrated that the mutations defined by JL1 and *def1* are non-allelic and recessive. Further study on *def1* plants showed that this mutant is deficient in the accumulation of JA upon wounding (Howe et al., 1996). The *Def1* gene appears to be involved in the regulation of allene oxide cyclase (AOC) that catalyzes the conversion of an unstable allene oxide to OPDA (Stenzel et al., 2003).

The biochemical defect responsible for the wound response phenotype of JL1 remains to be established. Because JL1 plants synthesize PIs in response to exogenous MeJA, it is likely that the mutant is compromised either in the octadecanoid pathway or the activation of the pathway in response to wounding. To address this issue, the JL1 mutant was further characterized. Measurement of JA and OPDA levels in JL1 plants indicate that the mutant is compromised in the conversion of OPDA to JA. This hypothesis was supported by analysis of the response of JL1 plants to exogenous OPDA and JA. The retarded growth phenotype of JL1 seedlings further indicated that JL1 plants are affected in a β-oxidation step that is required for the conversion of OPDA to JA. The results of reciprocal grafting experiments indicate that JL1 plants are deficient in the production of the systemic wound signal, but are able to recognize the signal in unwounded leaves. Because JL1 plants accumulate OPDA but not JA, these results indicate that JA, not OPDA, is an essential component of the transmissible wound signal for *PI* expression.

Results

JL1 plants are deficient in JA accumulation

JL1 plants do not accumulate detectable levels of PI-II protein in response to wounding (Table 3-1). To test whether the absence of PI-II accumulation is because of a defect in JA synthesis, gas chromatography-mass spectrometry (GC-MS) was used to measure JA and OPDA levels in wild-type and JL1 plants. Wild-type plants accumulated 14 ± 1 pmol JA/g FW (fresh weight) in unwounded leaves (Figure 3-2). This basal level increased to 274 ± 18 pmol JA/ g FW 1 hr after wounding. In contrast, unwounded JL1 plants produced significantly lower levels of JA (2 ± 3 pmol/g FW) than wild-type (student's t-test, $P < 0.05$). Furthermore, wounding did not elevate the level of JA (4 ± 5 pmol JA/g FW) in the mutant. These results indicated that JL1 plants are impaired in JA biosynthesis.

Quantification of OPDA showed that JL1 plants accumulated OPDA to levels comparable to those in wild-type plants. Wild-type plants accumulated 597 ± 41 pmol OPDA/g FW in unwounded leaves, which increased to 752 ± 19 pmol OPDA/g FW 1 hr after wounding. OPDA levels in JL1 plants were 413 ± 205 pmol/g FW in unwounded leaves and 578 ± 134 pmol/g FW in wounded leaves. Therefore, JL1 plants synthesize OPDA but appear to be defective in its conversion to JA.

Exogenous OPDA does not activate the expression of *PI-II* in JL1 plants

To further test the hypothesis that JL1 plants are unable to convert OPDA to JA, various amounts of OPDA and JA were applied to JL1 and wild-type plants through the cut stem, and the accumulation of PI-II was measured 24 hr after treatment. JL1 and wild-

type plants accumulated comparable amounts of PI-II in response to JA (Figure 3-3). This finding is consistent with the previous observation that volatile MeJA induced the synthesis of PIs in JL1 plants (Lightner et al., 1993). In wild-type plants, OPDA acted as a potent elicitor of PI-II accumulation (Figure 3-3). In contrast, exogenous OPDA failed to induce PI- II production in JL1 plants. These results indicate that conversion of OPDA to JA is necessary for expression of *PI-II* in wild-type plants, and that the JL1 mutant is impaired in this metabolic process.

JL1 plants fail to generate a systemic wound signal for *PI-II* expression

The inability of JL1 plants to synthesize PIs in response to wounding (Table 3-1; Lightner et al., 1993) could result from a failure to produce or perceive a systemic wound signal. To address this question, reciprocal grafting experiments were performed with JL1 and wild-type plants. Mechanical wounds were inflicted to all leaves of the rootstock, and the expression of *PI-II* was monitored in both the wounded rootstock and the undamaged scion leaves (Figure 3-4). Control experiments showed that the grafting procedure alone resulted in a moderate expression of *PI-II* in both rootstock and scion leaves of grafted wild-type plants (Figure 3-4, lane 1). However, wounding of rootstock leaves resulted in a significant increase of *PI-II* expression in the scion, indicating that the wounded rootstock produced a long-distance signal that was transmitted to the undamaged scion (Figure 3-4, lane 2). Grafted JL1 control plants did not show detectable local or systemic expression of *PI-II* (Figure 3-4, lane 3), consistent with the absence of PI-II in wounded JL1 seedlings (Table 3-1). In response to wounding, weak

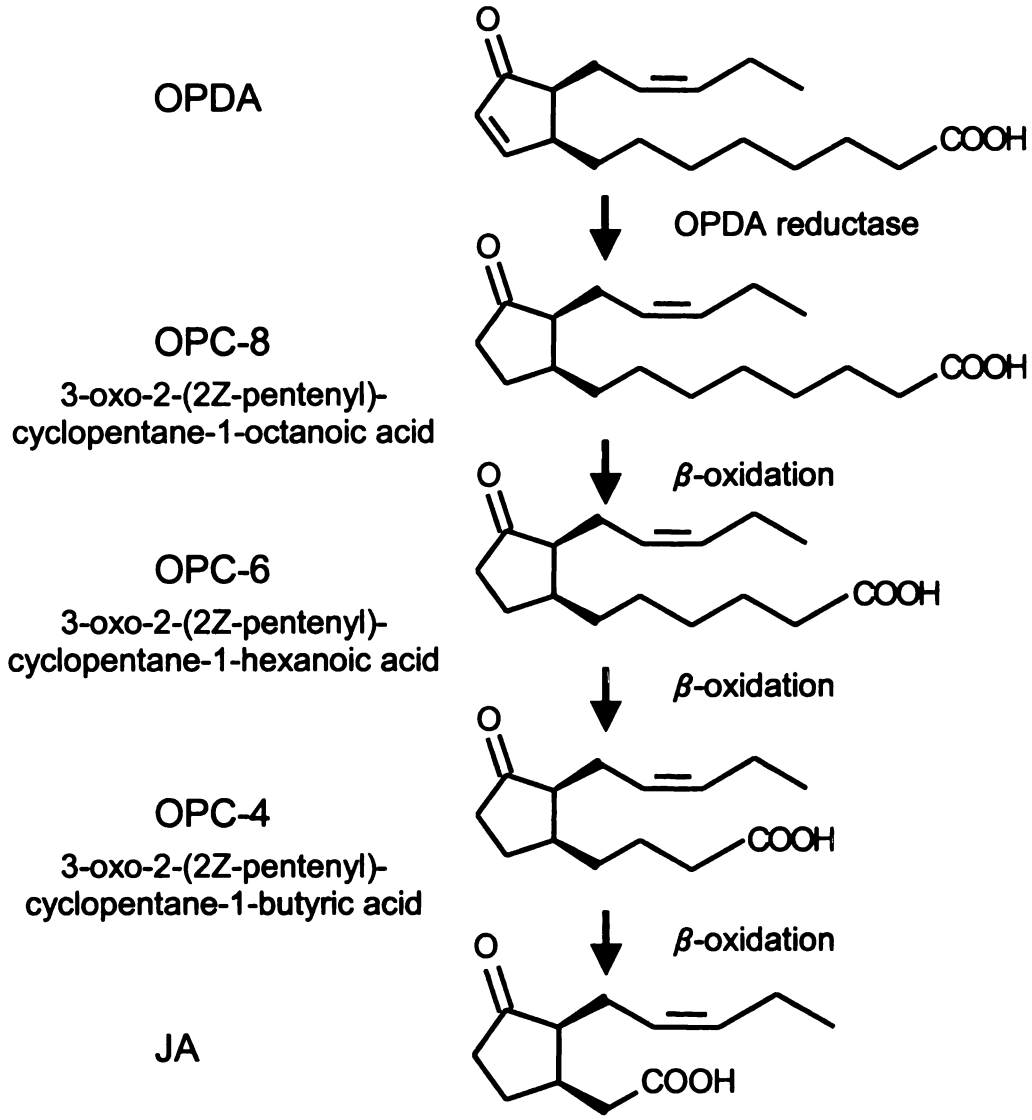


Figure 3-1. Conversion of OPDA to JA. OPDA is synthesized in chloroplasts and converted to JA in peroxisomes by consecutive reactions of OPDA reductase and three rounds of β-oxidation.

genotype	PI-II levels (μg/ml leaf juice)	
	unwounded control	wounded
wild-type	N.D.	68.5 ± 14.1
JL1	N.D.	N.D.

Table 3-1. Wound response of wild-type and JL1 plants. Leaves of two-week-old wild-type and JL1 plants were wounded with a hemostat. The accumulation of PI-II was measured in leaves harvested 24 hr after wounding. As a negative control, PI-II levels were also measured in leaves of unwounded plants. The data present mean and standard deviation from the measurement of three plants for each treatment. N.D. indicates that the accumulation of PI-II was not detected.

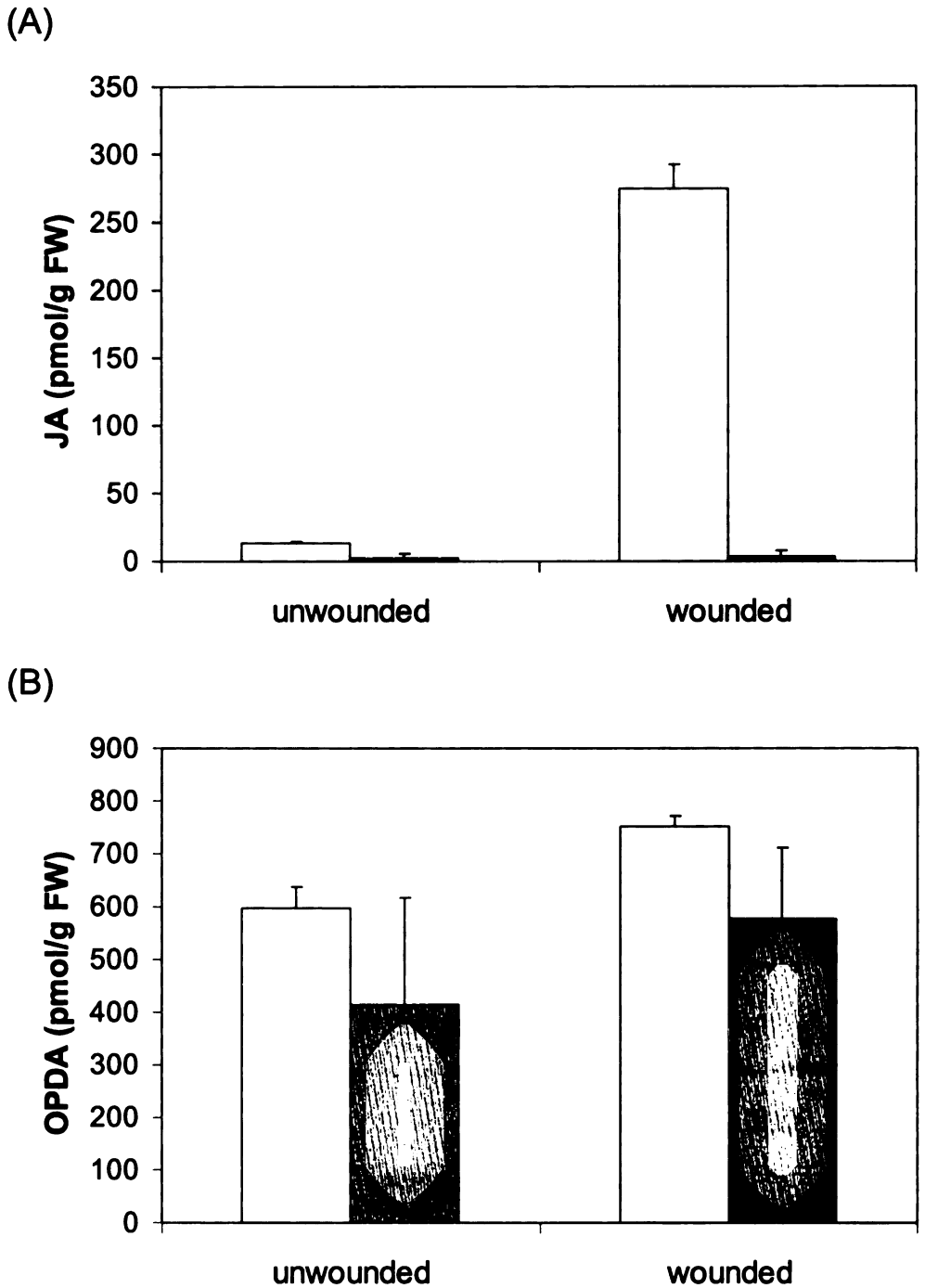
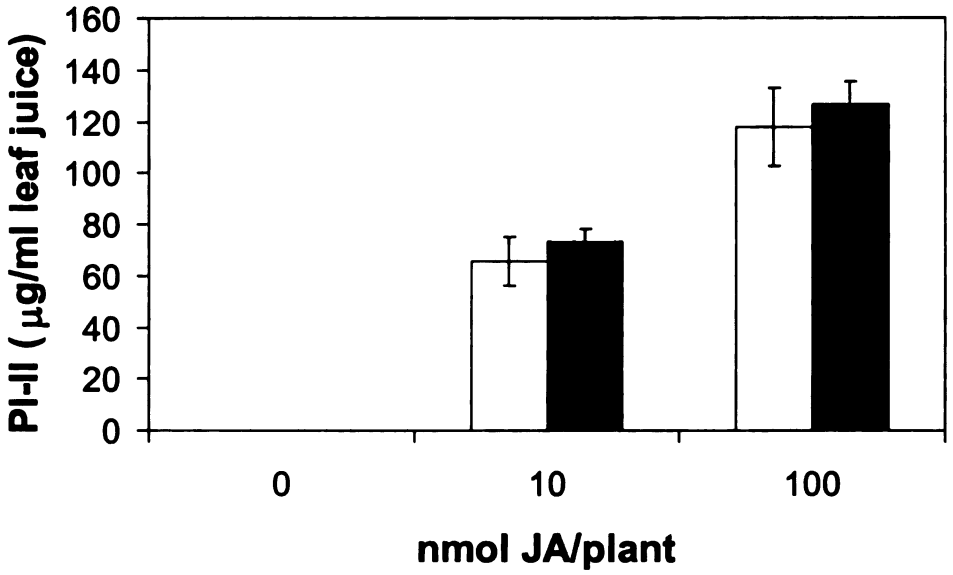


Figure 3-2. Accumulation of JA and OPDA in wild-type and JL1 plants in response to wounding. Leaves of 2-week-old wild-type (open bar) and JL1 (filled bar) plants were wounded with a hemostat. Leaves were harvested for extraction of JA/OPDA 1 hr after wounding. JA/OPDA was also extracted from leaves of unwounded control plants. JA (A) and OPDA (B) levels were measured by GC-MS analysis. Data represent mean and SD of three independent experiments.

(A)



(B)

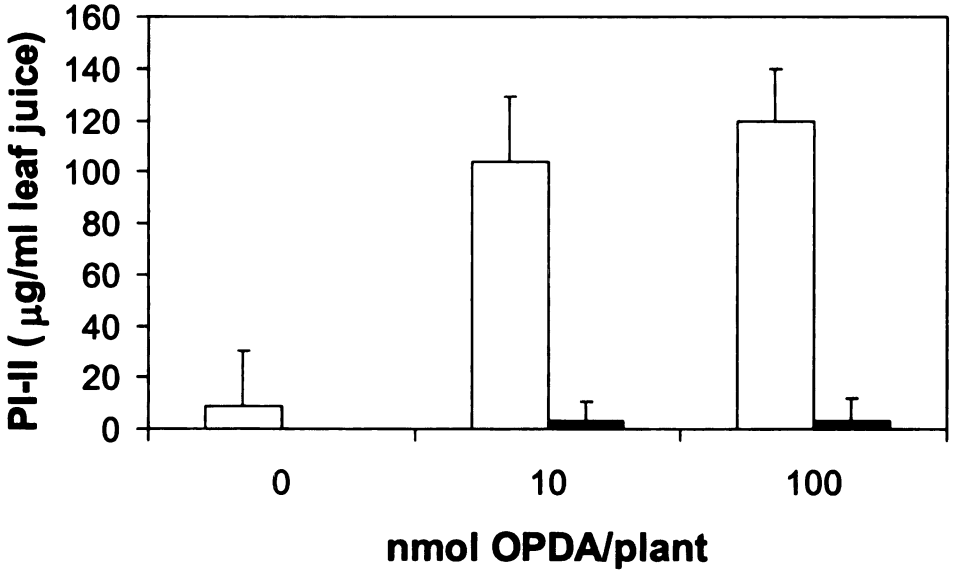


Figure 3-3. Effect of exogenous JA and OPDA on the accumulation of PI in wild-type and JL-1 plants. Two-week-old wild-type (open bar) and JL-1 (filled bar) plants were supplied through the cut stem with phosphate buffer (pH 6.5) containing various amounts of JA (A) and OPDA (B). Plants were assayed for PI-II accumulation in leaves 24 hr after treatment. Data represent the mean and standard deviation of 6 plants.

expression of *PI-II* was observed in the damaged JL1 rootstock but not in undamaged scion leaves (Figure 3-4, lane 4). In hybrid grafts between wild-type and JL1, wounding of JL1 rootstock leaves did not significantly increase the expression of *PI-II* in the wild-type scion (Figure 3-4, lane 5 and 6). Thus, the wounded JL1 rootstock is defective in the production of the long-distance signal for systemic *PI-II* expression. In the reciprocal combination (Figure 3-4, lane 7 and 8), wounding of wild-type rootstock leaves resulted in strong induction of *PI-II* expression in the JL1 scion. This result indicates that JL1 plants are able to respond to the long-distance signal that is produced and transmitted from the damaged wild-type rootstock. Taken together with the deficiency of JA in JL1 plants, these results indicate that the conversion of OPDA to JA is required for the generation of a systemic wound signal in damaged tissue.

Growth of JL1 seedlings is retarded

Defects of JL1 plants were further investigated. In addition to JA deficiency, JL1 plants displayed retarded growth at the seedling stage. To compare the growth rate between JL1 and wild-type plants, the root growth of seedlings was measured. To synchronize the seed germination, both JL1 and wild-type seeds were germinated on filter paper saturated with distilled water. Germinating seeds having a protruding radicle of the same size (about 2 mm) were then transferred to new filter paper saturated with distilled water. These seedlings were incubated for four days in darkness, and then root length was measured. The result shows that root length of JL1 seedlings was 42% of

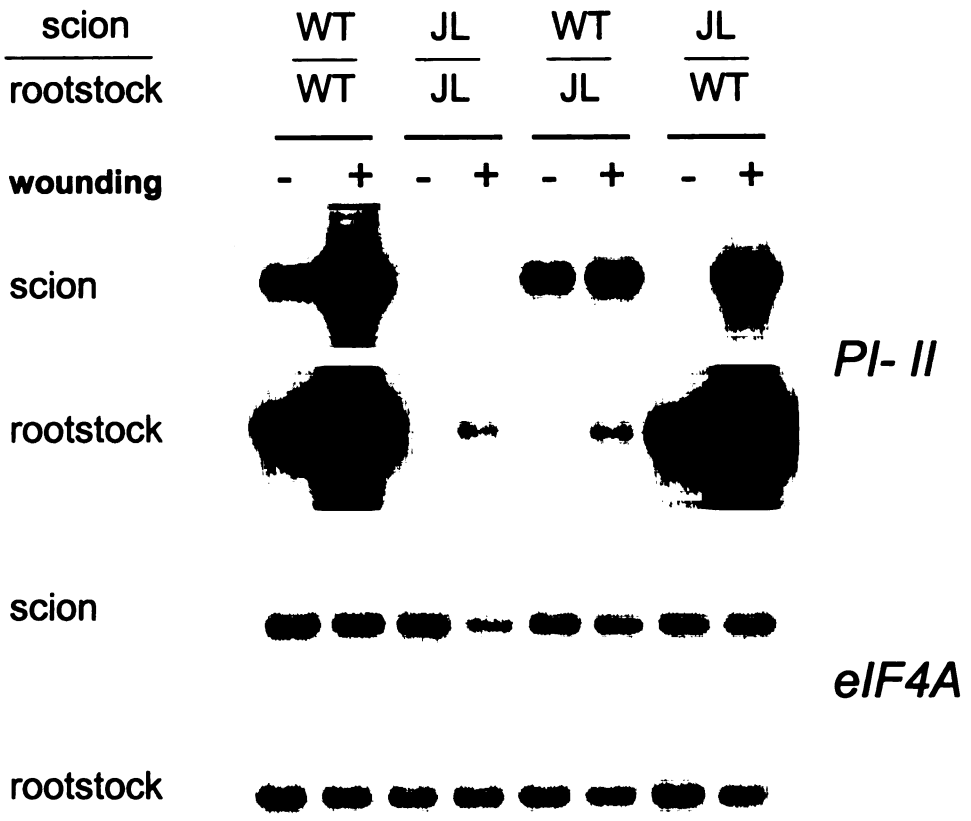


Figure 3-4. Wound-inducible *PI-II* expression in grafts between JL1 and wild-type plants. JL1(JL) and wild-type (WT) plants were grafted in the four combinations indicated. The genotypes listed above and below the horizontal line correspond to the scion and rootstock, respectively. For each graft combination, plants were divided into a negative unwounded control (-) and wounded (+) group consisting of four grafted plants per group. For wound treatment, each leaflet on the rootstock was mechanically injured with a hemostat. Eleven hours after wounding, leaf tissue was harvested separately from wounded rootstock leaves and undamaged scion leaves (scion) for RNA extraction. Levels of *PI-II* mRNA were analyzed by RNA gel blot analysis, using an *eIF4A* cDNA probe as a loading control.

that of wild-type (Figure 3-5). This finding indicates that root growth of JL1 seedlings is significantly retarded relative to wild-type. However, growth retardation was not observed in adult JL1 plants that developed fully expanded leaves (data not shown).

Discussion

JL1 plants are unable to convert OPDA to JA

The results of the present study indicate that the defective wound response of JL1 plants results from a deficiency in JA accumulation. Three lines of evidence indicate that JL1 plants have a defect in the conversion of OPDA to JA. First, JL1 leaves contained less than 2% of the level of JA observed in wild-type leaves (Figure 3-2A). This is the most severe JA deficiency among known wound response mutants of tomato (Howe et al., 1996; Lee and Howe, 2003; Li et al., 2002; Table 4-1, Chapter 4). Second, the OPDA level of JL1 plants was comparable to that of wild-type (Figure 3-2B). In contrast, other wound response mutants such as *spr2* and *def1* are deficient in both of OPDA and JA (Table 4-2, Chapter 4; Stenzel et al., 2003). This result indicates that the mutation in JL1 affects a step downstream of OPDA in the octadecanoid pathway. Finally, exogenous JA, but not OPDA, induced the accumulation of PI-II in JL1 plants (Figure 3-3). Because OPDA is a precursor of JA, these results indicate that JL1 plants are unable to metabolize OPDA to JA.

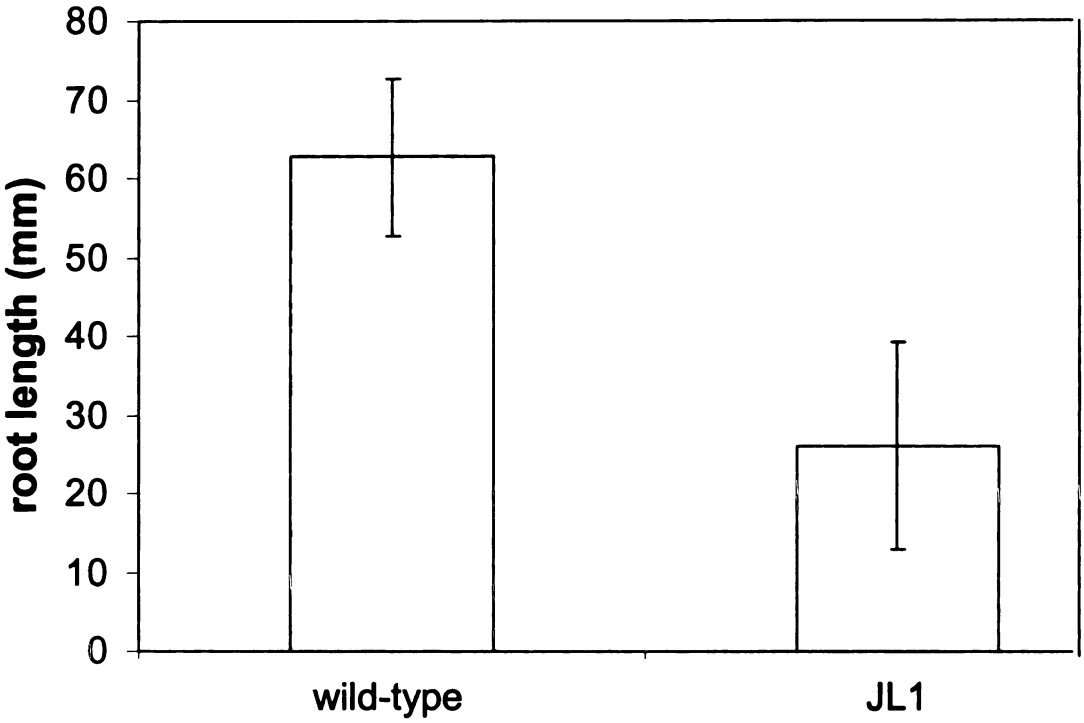


Figure 3-5. Comparison of root length between wild-type and JL1 seedlings. Germinated seeds at the same stage of development (2 mm radicle) were transferred to 3-MM filter paper saturated with distilled water, and incubated at room temperature in darkness. Root length was measured after 4-day incubation. Data represent the mean and standard deviation of 10 plants.

The conversion of OPDA to JA is accomplished by consecutive reactions involving the transport of OPDA from chloroplasts to peroxisomes, reduction of the cyclopentenone ring of OPDA by OPDA reductase, and the removal of 6 carbon units from the carboxylate side chain by three rounds of β-oxidation (Figure 3-1; Wasternack and Hause, 2002). Virtually nothing is known about the mechanism involved in transport of OPDA to peroxisomes. Previous studies in Arabidopsis indicated that substrates for β-oxidation are imported into the peroxisome by an ATP-binding cassette transporter called Pxa1/Ped3/COMATOSE, which is located on the peroxisomal membrane (Footitt et al., 2002; Hayashi et al., 2002; Zolman et al., 2001b). It is unlikely that the JL1 mutant is impaired in OPDA transport because exogenous OPDA failed to induce the accumulation of PI-II. Rather, it is more likely that JL1 plants are defective in the conversion of OPDA to JA in peroxisomes.

OPDA reductase genes (*OPRs*) comprise a small gene family in Arabidopsis and tomato. However, only *OPR3* is involved in the biosynthesis of JA (Sanders et al., 2001; Schaller and Weiler 1997; Schaller et al., 2000; Strassner et al., 2002). The product of *OPR3* is located in peroxisomes (Strassner et al., 2002), and the absence of OPR3 in Arabidopsis results in JA deficiency and male sterility (Sanders et al., 2001; Stintzi et al., 2001). Mapping experiments indicate that the mutation responsible for the wound response phenotype of JL1 does not correspond to the *OPR3* locus of tomato (C. Li and G.A. Howe, unpublished result)

JL1 shows growth retardation at the seedling stage (Figure 3-5). A similar phenotype has not observed in the *opr3* mutant of Arabidopsis or other JA-deficient mutants of tomato (Howe and Ryan, 1999; Howe et al., 1996; Sanders et al., 2001). The

defect in JA biosynthesis and retarded seedling growth of JL1 indicates that this mutant is compromised in β-oxidation. In addition to its role in JA biosynthesis, β-oxidation also mediates degradation of fatty acids by the sequential removal of two carbon units in the form of acetyl–coenzyme A (CoA; Graham and Eastmond, 2002). In germinating seeds, storage lipids are metabolized by β-oxidation with subsequent synthesis of glucose. Thus, impeded seedling growth is often observed in mutants defective in β-oxidation (Hayashi et al., 1998; Zolman et al., 2000). In such mutants, the defect in vegetative growth is typically alleviated after the transition to photoautotrophic growth (Hayashi et al., 1998; Hayashi et al., 2002; Zolman et al., 2001b). This phenotype was observed in JL1 plants; growth retardation was not observed in JL1 plants that had developed more than four leaves (data not shown). Therefore, it is less likely that the defective growth of JL1 seedlings results from a secondary mutation that adversely affects general aspects of plant development. Instead, the JL1 mutant appears to be compromised in β-oxidation, which inhibits both JA biosynthesis and seedling growth.

β-oxidation in plants occurs in peroxisomes and involves the sequential action of three enzymes: first, acyl-CoA oxidase; second, a multifunctional protein possessing L-3-hydroxyacyl-CoA hydrolyase, L-3-hydroxyacyl-dehydrogenase, D-3-hydroxyacyl-CoA epimerase, and 3, 2-enoyl-CoA isomerase activities; third, 3-ketoacyl-CoA thiolase (Eastmond et al., 2000; Germain et al., 2001; Richmond and Bleecker, 1999). Substrates for β-oxidation are first activated by acyl-CoA synthetase (Fulda et al., 2002). Enzymes involved in the β-oxidation cycle are encoded by small gene families in Arabidopsis, which results in the partial overlap of the substrate specificity among isozymes (Eastmond and Graham, 2002; Eastmond et al., 2000; Germain et al., 2001;

Hayashi et al., 1998; Hayashi et al., 1999; Hooks et al., 1999; Richmond and Bleecker, 1999; Shockey et al., 2002). Additional work will be necessary to provide direct evidence for a defect in β-oxidation in JL1 plants.

OPDA is not a wound signal for *PI* expression in tomato

Previous studies have shown that OPDA, in the absence of the conversion to JA, is a signaling molecule that regulates several aspects of plant development. For example, OPDA was more effective than JA in inducing tendril coiling of Cucurbitaceae plants (Weiler et al., 1993; Weiler et al., 1994). Furthermore, exogenous OPDA and JA treatments resulted in distinct patterns of volatile emission in lima bean (Koch et al., 1999). The study of the *opr3* mutant demonstrated that OPDA is a signal for defense responses in Arabidopsis (Stintzi et al., 2001). In spite of being unable to convert OPDA to JA, *opr3* plants were as resistant to insect and fungal pathogens as were wild-type plants. The resistant phenotype of *opr3* plants contrasts the susceptible phenotype of other JA biosynthetic mutants of Arabidopsis that do not synthesize OPDA, or mutants that are insensitive to JA. cDNA microarray experiments further showed that wild-type and *opr3* plants expressed defense-related genes in a similar manner upon wounding. These results indicate that OPDA functions as a defense signal in Arabidopsis.

In contrast to the situation in Arabidopsis, the results presented in this chapter indicate that OPDA is not an active signal for the expression of wound-inducible *PIs* in tomato. JL1 plants failed to express *PIs* upon wounding or application of OPDA, whereas JA treatment restored PI-II accumulation in JL1 plants (Figure 3-3). Therefore,

the conversion of OPDA to JA is essential for *PI* expression in tomato. This finding is consistent with a previous study demonstrating that β-oxidation is required for expression of *PIs* in tomato (Miersch and Wasternack, 2000). In this study, I examined *PI-II* expression in response to JA analogues containing a carboxylate side chain of differing length. *PI-II* expression was induced by analogues carrying an even-number of carbons in the side chain (i. e., OPC-8, OPC-6, and OPC-4; Figure 3-1). In contrast, JA analogues containing an odd-number of carbons in the side chain, which cannot be converted to JA by β-oxidation, were inactive. These results indicated that β-oxidation of OPC-8 is necessary for *PI-II* expression in tomato.

Increasing evidence indicates that JA biosynthesis is essential for the systemic wound response of tomato. Reciprocal grafting experiments using JA-deficient *spr2* plants showed that JA biosynthesis is required for the production of the transmissible wound signal (Li et al., 2002). Because *spr2* plants lack both OPDA and JA because of the loss of an ω-3 fatty acid desaturase (Li et al., 2003; Table 4-2, Chapter 4), these results do not rule out the possibility that OPDA is a mobile signal for the systemic wound response. Grafting experiments performed with JL1 plants, which accumulate OPDA but not JA, showed that this mutant failed to generate a systemic wound signal in damaged leaves, but nevertheless was able to perceive that signal in unwounded leaves. These results indicate that JA rather than OPDA is the a mobile signal for systemic *PI* expression

Materials and Methods

Plant material

Seeds of JL1 were collected from a JL1 homozygous line that was back-crossed two times using *Lycopersicon esculentum* Mill cv Castlemart as the recurrent parent (Lightner et al., 1993). Wounding was performed on two-week-old plants as described in Chapter 2. Grafting experiments were performed following the method described by Li et al. (2002)

Treatment of JA and OPDA

(±)-JA and OPDA were purchased from Sigma (St. Louis, MO) and Cayman Chemical (Ann Arbor, MI), respectively. Two-week-old plants were excised at the base of the stem with a razor blade and immediately placed into 0.5 ml plastic tubes containing various amounts of elicitors diluted in 300 μl of 15 mM sodium phosphate solution (pH 6.5). Because JA and OPDA were originally dissolved in ethanol, the mock control solution contained 0.1 % (vol/vol) ethanol. Excised plants were allowed to imbibe the solution during a 50 min incubation period, and then transferred to glass vials containing distilled water. PI-II levels in leaves are measured 24 hr after treatment as described in Chapter 2.

RNA gel blot analysis

RNA extraction from leaves and RNA hybridization experiments were performed as described in Chapter 2.

Root length measurement

Seeds were surface sterilized as described (Zolman et al., 2000). To synchronize the seed germination, both JL1 and wild-type seeds were germinated on filter paper saturated with distilled water. Germinating seeds with a radicle of about 2 mm length were transferred to two layers of 3-MM filter paper soaked with distilled water. The transferred seedlings were incubated for four days in darkness at room temperature. Root growth was measured with a ruler using overhead projector to enlarge the image of seedlings.

Analysis of JA and OPDA

Quantification of JA and OPDA was performed by GC-MS analysis as described in Chapter 4.

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Chapter 4

Quantification of jasmonic acid and 12-oxo-phytodienoic acid in tomato plants by gas chromatography-mass spectrometry

Introduction

Jasmonic acid (JA) and its methyl ester (MeJA) regulate plant defense responses against insects and pathogens (Cohen et al., 1993; Farmer and Ryan, 1992; Howe et al., 1996; McConn et al., 1997; Penninckx et al., 1996; Seo et al., 2001), and also play an important role in reproductive organ development in plants (McConn and Browse, 1996; Li et al., 2001; Sanders et al., 2000; Stintzi and Browse, 2000). JA is synthesized from linolenic acid by the octadecanoid pathway (Turner et al., 2002; Wasternack and Hause, 2002). The synthesis of JA is initiated in chloroplasts where linolenic acid is released from plastid membranes and converted to 12-oxo-phytodienoic acid (OPDA) by the consecutive action of lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC). The remaining biosynthetic steps occur in peroxisomes, where OPDA is processed to JA by OPDA reductase (OPR3) and three rounds of β-oxidation. Interestingly, OPDA functions as a regulatory signal for defense and development, in the absence of its metabolic conversion to JA (Stelmach et al., 1998; Stintzi et al., 2001; Weiler et al., 1993). These derivatives of linolenic acid, OPDA, JA and MeJA, are well-known examples of oxylipins that are linear or cyclic oxidation products derived from the catabolism of fatty acids (Howe and Schilmiller, 2002).

Precise quantitative analysis of JA and OPDA is crucial for the study of the biosynthesis and function of these molecules (Creelman and Mullet, 1995). Quantification of JA and OPDA requires the use of appropriate internal standards and an analytical method for the detection. Because internal standards are used to correct for the loss of each oxylipin during extraction, it is important that the internal standard has the same or very similar physiochemical properties of the endogenous compound being

analyzed. Two types of internal standards have been used for quantification of JA and OPDA. The first type is isotope-labeled standards such as deuterium- and/or ^{13}C-labeled JA (Baldwin et al., 1994; Creelman and Mullet, 1995; Creelman et al., 1992; Kramell et al., 2000; Laudert and Weiler, 1998) and OPDA (Kramell et al., 2000; Parchmann et al., 1997; Stelmach et al., 1998). Alternatively, 9, 10-dihydrojasmonic acid (DHJA; Gundlach et al., 1992; Parchmann et al., 1997; Weber et al., 1997; Wilbert et al., 1998) and tetrahydro-OPDA (H4OPDA; Weber et al., 1997) have been used as internal standards for quantification of JA and OPDA, respectively (Figure 4-1).

Two detection methods have been used to quantify JA and OPDA. The first method is an enzyme-linked immunosorbent assay (ELISA) that relies on a monoclonal antibody raised against MeJA (Albrecht et al., 1993). To avoid possible cross-reaction of the antibody with JA-related molecules, the JA fraction was purified from plant extracts using high-performance liquid chromatography (HPLC) and methylated prior to ELISA testing. Although this method has been used to measure endogenous JA levels in several plants including tomato (Albrecht et al., 1993; Conconi et al., 1996; Doares et al., 1995; Howe et al., 1996; Weiler et al., 1993), quantification of OPDA is not possible with this method. The second method, gas chromatography (GC)-mass spectrometry (MS), provides highly reliable identification and quantification of JA and related oxylipins. GC-MS analysis has been successfully used for the monitoring of JA levels in several plant species including soybean, broad bean, Arabidopsis, apple, tobacco, potato, and barley (Baldwin et al., 1994; Creelman et al., 1992; Fan et al., 1997; Kramell et al., 1997; McConn et al., 1997; Mueller and Brodschelm, 1994; Wang et al., 1999; Weber et al., 1997). In contrast to the ELISA method, GC-MS analysis

enables simultaneous quantification of JA, OPDA, and other oxylipins (Kramell et al., 2000; Vollenweider et al., 2000; Weber et al., 1997).

Wound-induction of proteinase inhibitors (PIs) in tomato has been widely used as a model system to study wound signaling in plants (Green and Ryan, 1972; Ryan, 2000). Several wound response mutants of tomato have been isolated with genetic screens for plants that lack accumulation of PIs in response to wounding (Howe and Ryan, 1999; Li et al., 2001; Lightner et al., 1993). Quantification of JA and OPDA in these mutants is an essential step in investigating the molecular and biochemical basis of these mutations.

Previously, GC-MS analysis was used to measure the amount of JA and OPDA in tomato leaves (Parchmann et al., 1997). This published protocol relied on extensive purification of JA and OPDA prior to GC-MS analysis, which reduced the recovery of these compounds. The purpose of the experiments described in this chapter was to optimize and simplify this procedure using a solid-phase extraction method that was originally developed for oxylipin analysis in Arabidopsis (Weber et al., 1997). The results indicated that this modified method can be used to efficiently quantify JA and OPDA in tomato tissues. The method was used to measure the levels of JA and OPDA in various wound response mutants. The results obtained are discussed in the context of the regulation of JA synthesis.

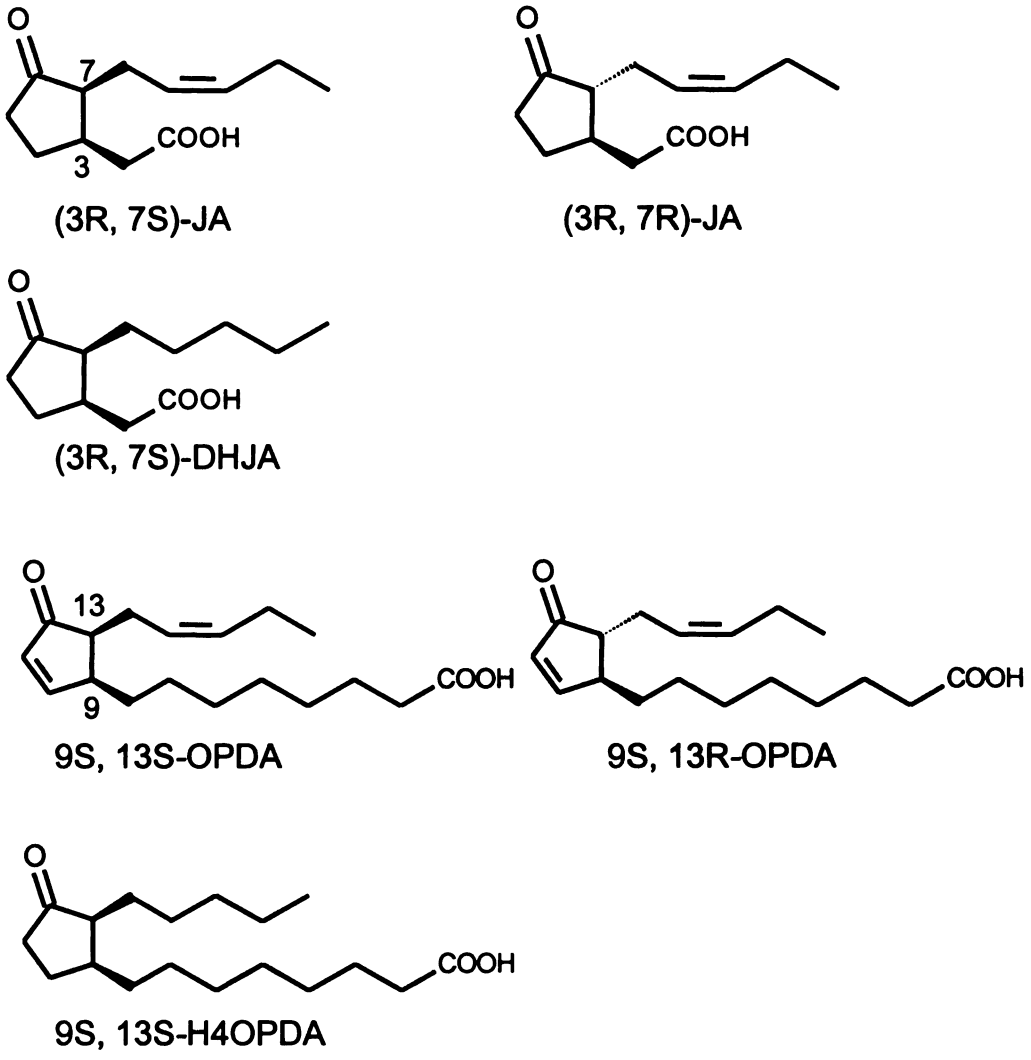


Figure 4-1. Structure of JA, OPDA and their internal standards. The octadecanoid pathway yields only the 3R, 7S-isomer of JA, which is derived from its precursor 9S, 13S-OPDA (Laudert et al., 1997). During extraction and analysis by GC, 9S, 13S-OPDA and 3R, 7S-JA epimerize to 9S, 13R-OPDA, and 3R, 7R-JA, respectively (Mueller and Brodschelm, 1994). JA and OPDA were converted to DHJA and H4OPDA, respectively, for use as internal standards.

Results

Preparation of internal standards and determination of GC-MS parameters

Because JA and OPDA are commercially available, these molecules were used to prepare the corresponding internal standards. JA has two chiral centers at the C-3 and C-7 positions (Figure 4-1), which yield four possible stereoisomers (Creelman and Mullet, 1997): 3R, 7S-JA; 3S, 7R-JA; 3R, 7R-JA; and 3S, 7S- JA [also called (+)-7-iso-JA, (-)-7-iso-JA, (-)-JA, and (+)-JA, respectively]. Chemically synthesized JA [(±)-JA] is a racemic mixture of these stereoisomers. OPDA also has two chiral centers at the C-9 and C-13 positions. Commercially available OPDA [(±)-OPDA] consists of a mixture of 9S, 13R-OPDA and 9S, 13S-OPDA (Figure 4-1). The internal standards DHJA and H4OPDA were prepared from (±)-JA and (±)-OPDA, respectively, by the reduction of double bonds (Weber et al., 1997; Wilbert et al., 1998). Endogenous DHJA and H4OPDA were not detected in the GC-MS analysis of tomato leaf extracts (data not shown), indicating that these compounds would be suitable as internal standards.

(±)-JA, (±)-OPDA, and the corresponding internal standards were used to establish GC-MS conditions. To increase the sensitivity of detection (Fans et al., 1997), these molecules were methylated prior to injection into the GC. These methyl esters were readily separable by GC, and more determined by MS (positive-ion detection mode). Molecules were ionized in MS by the electron impact (EI) method to enhance the fragmentation of the molecules. Two temperature programs were used for the GC-MS analysis. The first program was optimized for quantification of JA (Figure 4-2). The

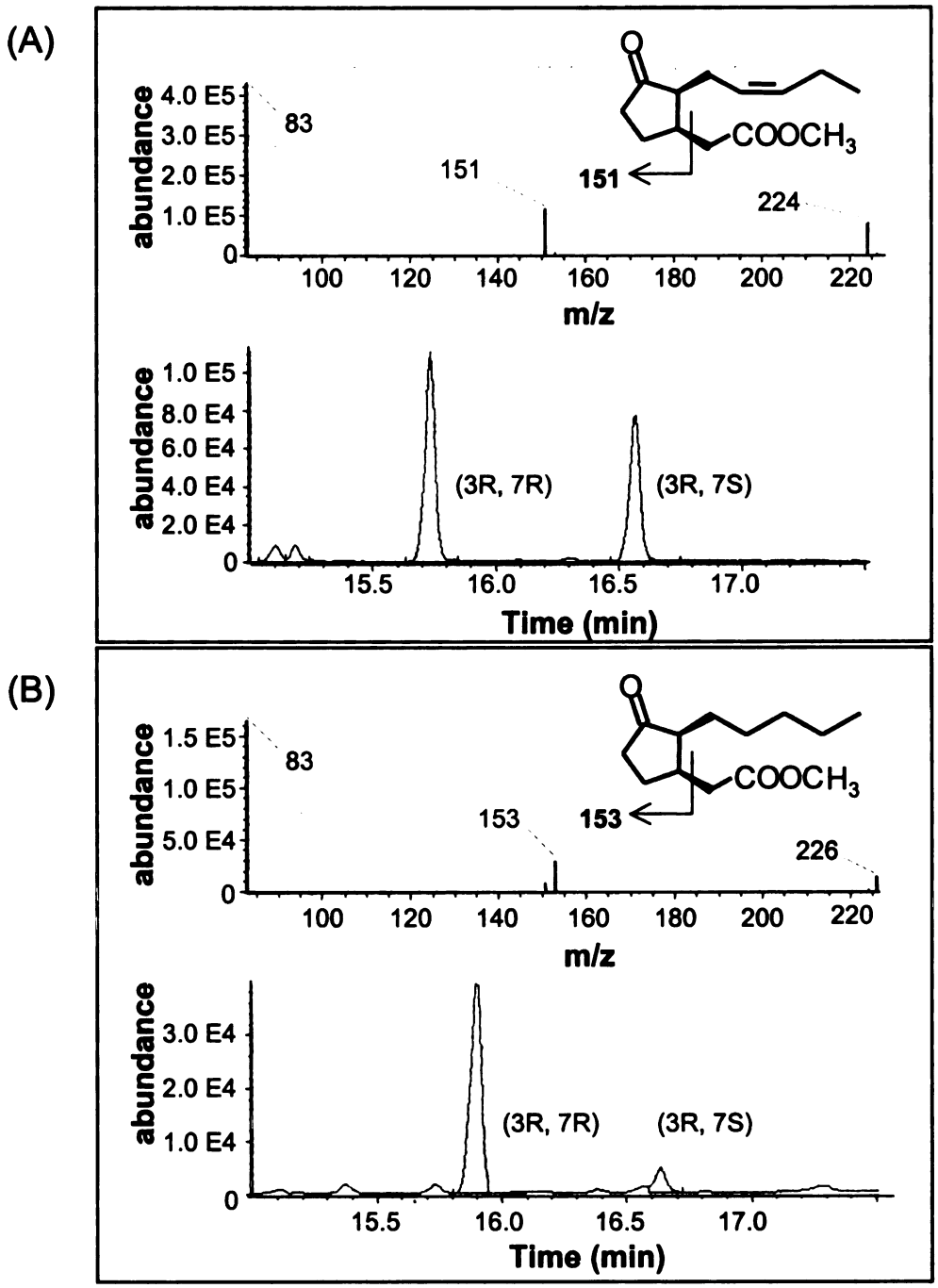


Figure 4-2. Mass spectrum and chromatogram of MeJA and MeDHJA. JA was extracted from wounded leaves of wild-type plants and methylated prior to GC-MS anaylsis. For quantification of endogenous JA levels, ions of m/z = 224 and 226 were monitored for MeJA (A) and MeDHJA (B), respectively. Ions of m/z = 83, 151, 153 were used for the identification of each peak in the chromatogram. The configuration of each stereoisomer is indicated in the chromatogram. GC-MS was run with the temperature program optimized for JA quantification.

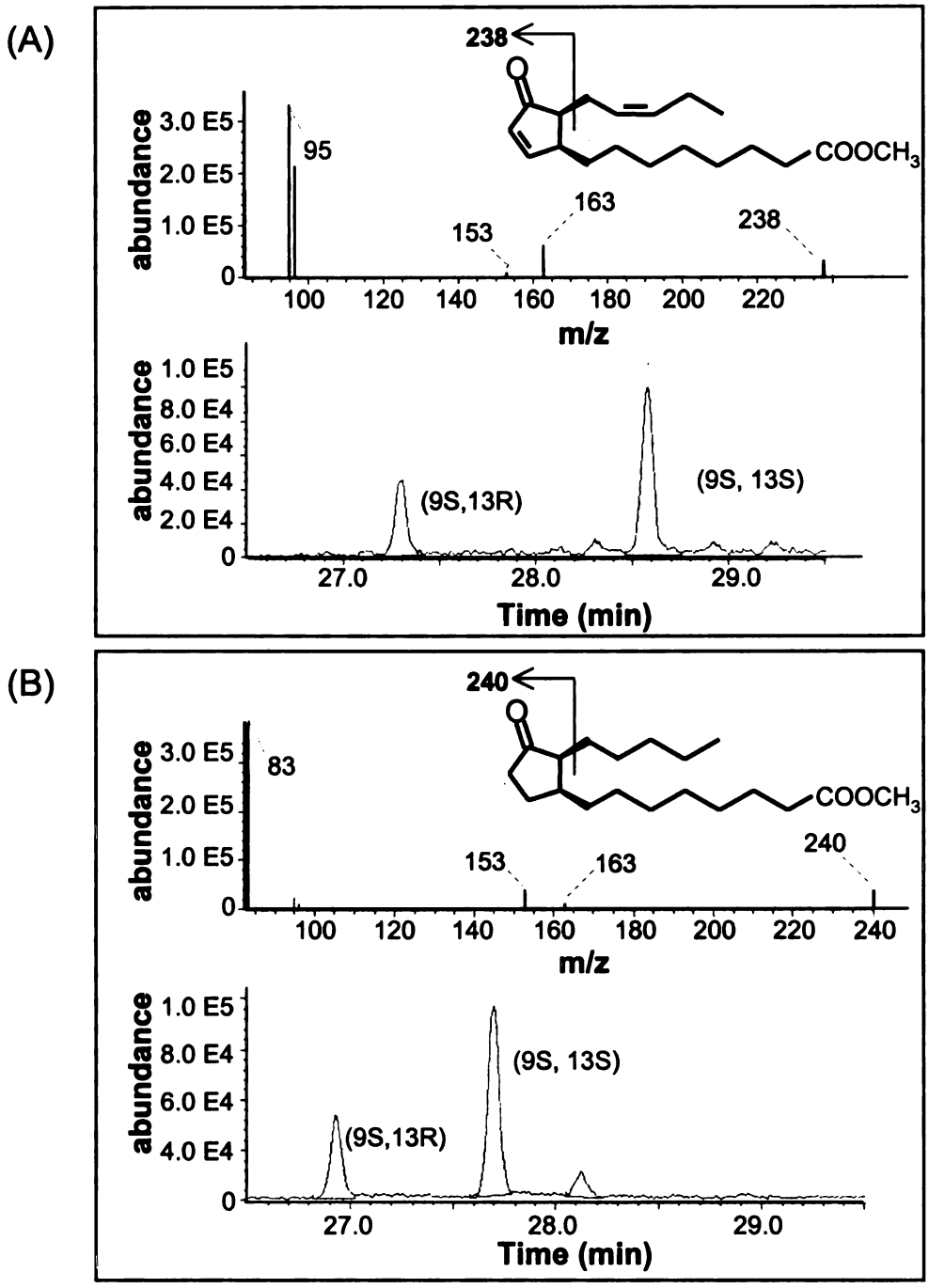


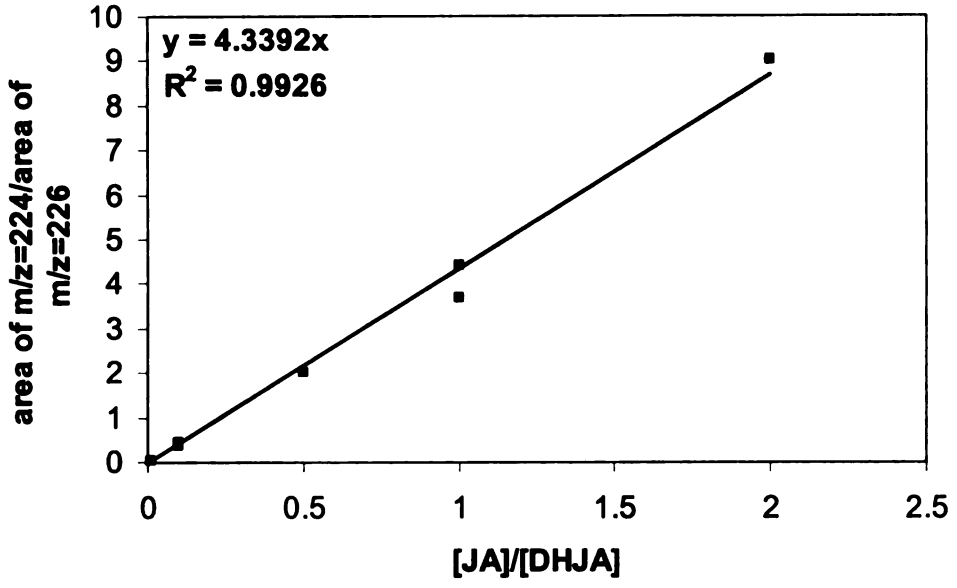
Figure 4-3. Mass spectrum and chromatogram of MeOPDA and MeDHJA. OPDA and JA were extracted from wounded leaves of wild-type plants. For quantification of endogenous OPDA, ions of m/z = 238 and 240 were monitored for MeOPDA (A) and MeH4OPDA (B), respectively. The configuration of each stereoisomer is indicated in the chromatogram. Ions of m/z = 83, 95, 96, 153, 163 were used for identification of each peak.

second program has a longer running time, which is necessary to quantify JA and OPDA simultaneously in the same plant extract. The latter program permits the separation and detection of all peaks corresponding to MeJA, MeOPDA (OPDA-methyl ester), MeDHJA (DHJA-methyl ester), and MeH4OPDA (H4OPDA-methyl ester; Figure 4-3). For quantification of JA and OPDA, only a representative ion of each molecule was counted in the single-ion monitoring (SI) mode. Molecular ions were monitored for MeJA [mass-to-charge ratio (m/z) = 224] and MeDHJA (m/z = 226). Because the molecular ions of MeOPDA (m/z = 306) and MeH4OPDA (m/z = 310) were not sufficiently abundant for detection in SI mode, ions with m/z = 238 and 240 were monitored (Figure 4-3).

Extraction and quantification of endogenous JA and OPDA

To minimize unintended loss or modification of JA and OPDA during sample preparation, it was desirable to simplify the extensive purification procedure described by Parchmann et al. (1997). This was accomplished by a modification of the solid-phase extraction method originally developed for oxylipin analysis in Arabidopsis (Weber et al., 1997). Because this method does not require tedious purification steps such as preparatory HPLC, multiple samples could be prepared at the same time. Both JA and OPDA were extracted from tomato tissue with methanol and then partially purified on a C_{18} column. Known amounts of internal standards were added to the methanol extracts prior to the C_{18} column step. JA and OPDA recovered from the column were methylated with diazomethane, which converts endogenous JA and OPDA to their corresponding methyl esters. Therefore, the MeJA peak in the chromatogram represents

(A)



(B)

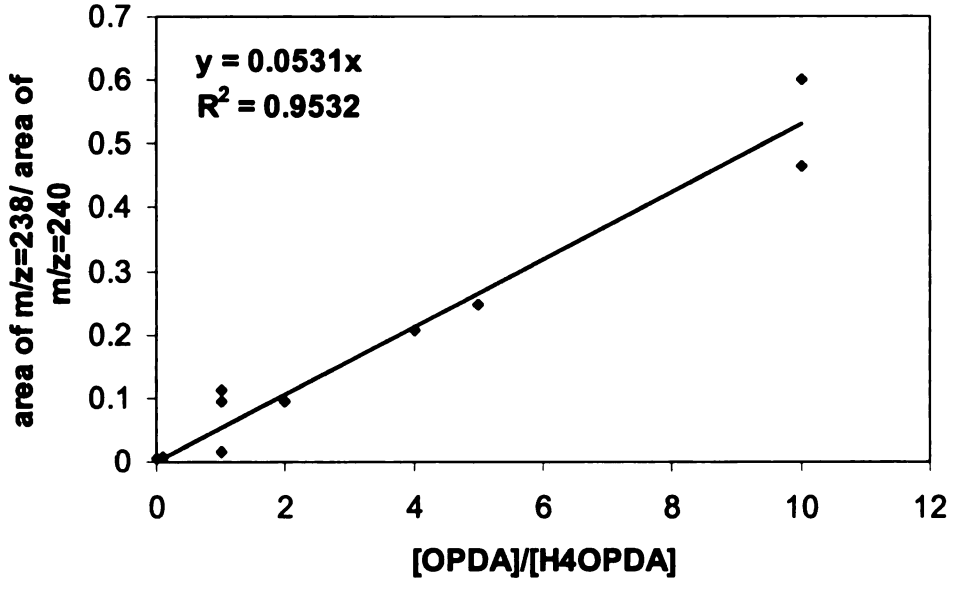


Figure 4-4. Standard curves for quantification of JA and OPDA. The standard curve of JA (A) was constructed using known amounts of JA and DHJA as described (Wilbert et al., 1998). Similarly, the standard curve of OPDA (B) was obtained using mixtures of OPDA and H4OPDA. The slope of each standard curve was used to calculate the endogenous level of JA and OPDA.

the sum total of endogenous JA and MeJA. For simplicity, the amount of endogenous JA was calculated from the MeJA peak in the chromatogram without further distinction between JA and MeJA. Endogenous MeOPDA was not detected in tomato leaves (data not shown).

Two stereoisomers of JA (3R, 7R-JA and 3R, 7S-JA) and OPDA (9S, 13R-OPDA and 9S, 13S-OPDA) were detected in extracts from tomato leaves (Figure 4-2; Figure 4-3). The peak area of the two stereoisomers was added to calculate the level of JA or OPDA. The amount of endogenous JA and OPDA was calculated from the integrated peak areas using a standard curve constructed as follows. Various amounts of JA and DHJA were mixed, methylated, and analyzed by GC-MS to obtain the standard curve for the quantification of endogenous JA in tomato (Figure 4-4). The area under each representative ion peak was measured from the chromatogram, and the ratios of these areas were plotted against the concentration ratios of JA and DHJA, as described previously (Wilbert et al., 1998). Similarly, the standard curve for OPDA was obtained using mixtures of OPDA and H4OPDA (Figure 4-4).

A new standard curve was constructed for each independent experiment. The resulting slope of the curve gave very consistent values among different experiments. The standard curve for JA was linear in the range of 0.5 to 25 nmol, with a correlation coefficient of 0.99. The standard curve for OPDA was linear in the range of 5 to 200 nmol with a correlation coefficient of 0.95. The recovery rate of internal standards was in the range of 60 to 90% in most measurements. Results obtained from experiments in which the recovery rate was less than 50% were discarded to avoid possible errors in the quantification.

genotype	pmol JA/g FW tissue			
	leaf (hour after wounding)			flower
	0 hr	1 hr	3 hr	
WT (cm)	12 ± 1 (n=3)	262 ± 41 (n=3)	151 ± 26 (n=3)	1485 ± 290 (n=3)
spr2	3 ± 1 (n=3)	22 ± 9 (n=3)	7 ± 1 (n=3)	265 ± 36 (n=3)
WT (mt)	9 (n=2)	675 (n=2)	N.D.	555 ± 58 (n=3)
jai1-1	15 (n=3)	493 (n=2)	N.D.	85 ± 34 (n=3)
WT (cm)	16 (n=1)	245 (n=1)	136 (n=1)	N.D.
jai1-3	15 (n=1)	130 (n=1)	53 (n=1)	N.D.

Table 4-1. Summary of JA levels in wound response mutants of tomato. JA levels were compared between wild-type (WT) and wound response mutants. Whereas *spr2* and *jai1-3* plants originated in *L. esculentum* cv Castlemart (cm), *jai1-1* plants were originated in a semi-dwarf cultivar, *L. esculentum* cv Micro-Tom (mt). Therefore, JA levels of each mutant were compared to the appropriate wild-type cultivar. Wound-induced levels of JA were compared between WT and wound response mutants. Leaves of 2-week-old plants were wounded with a hemostat and harvested at the indicated time after wounding for JA extraction. As a negative control, leaves were harvested from unwounded plants (0 hr). JA levels in flowers were compared between wild-type and wound response mutants. Flowers were harvested from one month-old plants grown in the greenhouse. The number of independent measurements is indicated in parentheses. The mean of two independent measurements or the mean ± standard deviation from three independent experiments is presented. N.D. indicates that the measurement was not performed.

unit: pmol/g FW

oxylipin	genotype	0 hr	1 hr	3 hr
JA	WT	15 (n=2)	241 (n=2)	120 (n=2)
	spr1	13 (n=2)	162 (n=2)	37 (n=2)
	spr2	5 (n=1)	11 (n=1)	N.D.
OPDA	WT	644 (n=2)	741 (n=2)	1307 (n=2)
	spr1	301 (n=2)	499 (n=2)	556 (n=2)
	spr2	133 (n=1)	179 (n=1)	N.D.

Table 4-2. Wound-inducible JA and OPDA levels in wound response mutants. Leaves of wild-type (WT), *spr1*, and *spr2* plants were wounded and harvested for the extraction of oxylipins as described in the legend of Table 4-1. Levels of JA and OPDA were simultaneously measured to examine the effect of these mutations on the biosynthesis of JA. Except for *spr2*, each value represents the mean of two independent measurements. N.D., not determined.

Quantification of JA and OPDA from wound response mutants of tomato

In wild-type plants (*L. esculentum* cv Castlemart), the JA level rapidly increased within one hour after wounding (Table 4-1). In contrast, wound response mutants showed reduced levels of JA. For example, the *suppressor of prosystemin-mediated responses2* (*spr2*; Howe and Ryan, 1999) mutant accumulated extremely low levels of JA over all time points. The OPDA level was also reduced in *spr2* plants (Table 4-2).

The semi-dwarf tomato cultivar, Micro-Tom, has been shown to be a useful model system for analysis of the wound response (Howe et al., 2000; Meissner et al., 1997). The basal level of JA in unwounded Micro-Tom leaves was comparable to that in Castlemart plants. However, Micro-Tom plants accumulated greater amounts of JA in response to wounding. These results indicate that the genetic background of the cultivar can affect the capacity for JA biosynthesis.

Both wounding and exogenous MeJA failed to induce the accumulation of PIs in *jasmonic acid-insensitive1* (*jai1*) plants (Li et al., 2001). A deletion null allele, *jai1-1*, was isolated in a genetic screen of fast-neutron mutagenized Micro-Tom plants. Within 1 hr after wounding, the JA level in *jai1-1* plants increased to 73% of the level observed in wild-type plants. A similar reduction of JA biosynthesis was found for the EMS-induced *jai1-3* allele (originally named *spr5*) that was isolated in the Castlemart background. Upon wounding, JA levels in *jai1-3* plants were approximately 50% of that observed in wild-type plants. These results indicate that insensitivity to JA prevents the maximal synthesis of JA in response to wounding.

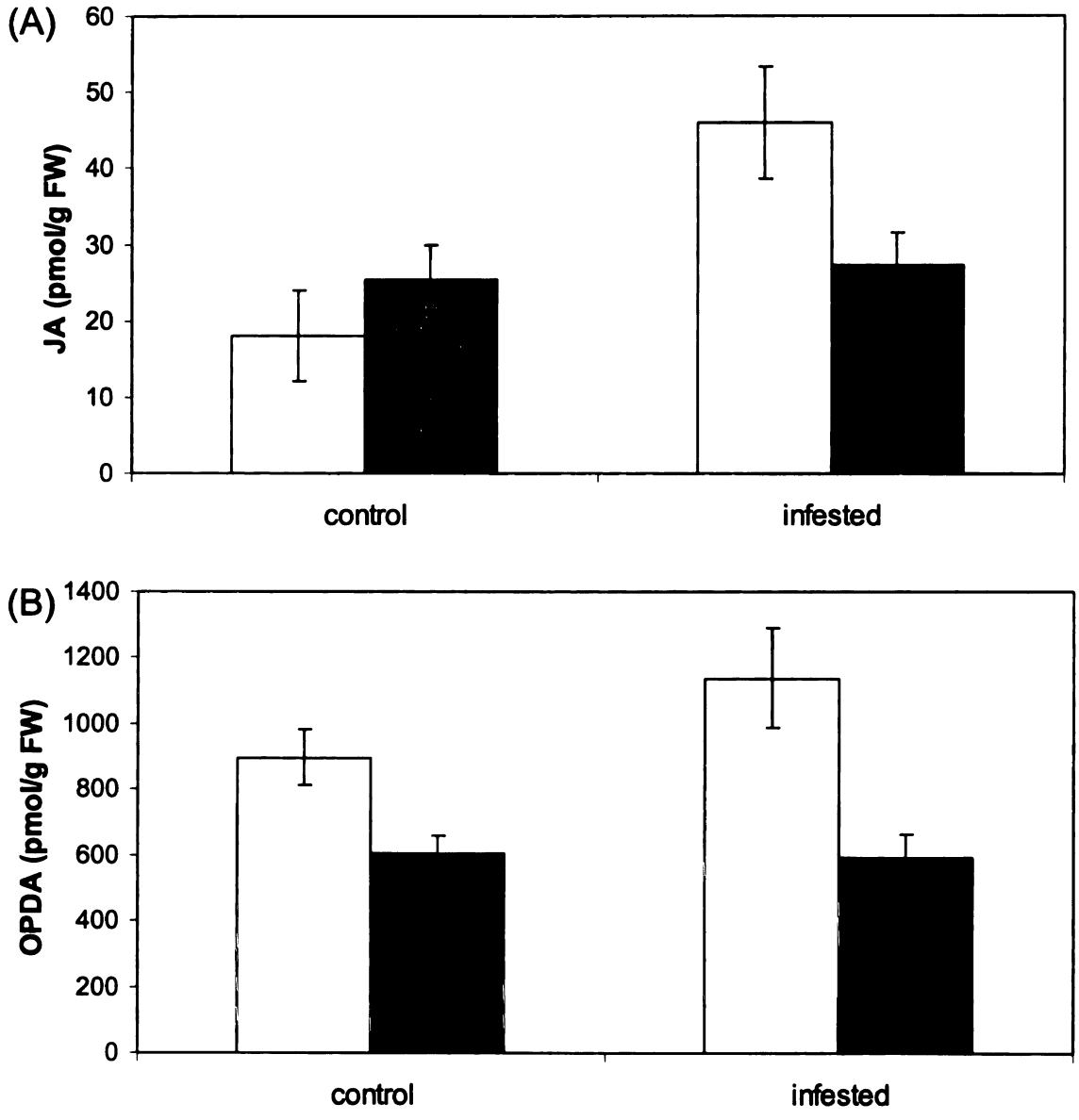


Figure 4-5. Accumulation of JA and OPDA in wild-type and *defl* plants challenged with spider mites. Sixteen-day-old wild-type (open bar) and *defl* (filled bar) plants were infested by spider mites as described in Materials and Methods. Two days after the challenge, leaflets showing visible symptom of damage (infested) were harvested for the quantification of JA (A) and OPDA (B). JA and OPDA were also quantified from leaves of untreated control plants (control). Values indicate the mean and standard deviation of three independent experiments.

The amount of JA in tomato flowers was higher than that in leaves (Table 4-1). Micro-Tom flowers appeared to contain less than half the amount of JA found in Castlemart flowers. Both *spr2* and *jai1-1* showed reduced JA levels in flowers. The JA level in *spr2* flowers was only 18% of the level observed in Castlemart flowers, whereas *jai1-1* flowers accumulated less than 15 % of the level of JA observed in Micro-Tom flowers.

Simultaneous monitoring of JA and OPDA levels showed that these oxylipins displayed different kinetics of accumulation (Table 4-2). The JA level increased transiently within 1 hr of wounding whereas the level of OPDA steadily increased up to 3 hr after wounding. This finding indicated that an increase in OPDA accumulation does not necessarily result in a proportional increase in JA.

Consistent with a previous report (Lee and Howe, 2003), the basal level of JA in the *suppressor of prosystemin-mediated responses1* (*spr1*) mutant was normal. One hr after wounding, this level increased to 65% of the level in wild-type. However, the basal level of OPDA was significantly reduced in *spr1* plants compared to wild-type (Table 4-2). These results also indicate that the synthesis of OPDA is not proportional to that of JA. The wound-induced accumulation of OPDA was impaired in *spr1* plants as well.

Changes in the levels of JA and OPDA were also observed in tomato plants challenged with spider mites (Figure 4-5). This arachnid herbivore pierces epidermal cells and sucks out the cellular contents of the underlying mesophyll cells (Lange and Bronson, 1981). In infested leaves of wild-type plants, a moderate increase in JA was observed (Figure 4-5). Although the OPDA level increased upon challenge of wild-type

plants (student's t-test, $P < 0.05$), the induction of JA and OPDA was not equivalent. The basal level of JA was comparable between wild-type and *defenseless1* (*def1*) plants, but the basal level of OPDA was significantly reduced in *def1* plants compared to wild-type. Neither JA nor OPDA levels increased in response to herbivore attack of *def1* plants ($P < 0.05$). This result is consistent with the previous observation that *def1* plants are defective in the induction of JA synthesis in response to wounding (Howe et al., 1996).

Discussion

Efficient quantification of JA and OPDA is important for the study of wound signaling because the induction of JA synthesis is one of the earliest events in the signaling cascade that results in expression of wound-inducible genes (Doares et al., 1995; Farmer and Ryan, 1992). Accordingly, this study was intended to establish a reliable and simple procedure to measure endogenous JA and OPDA in tomato tissues. Recent studies have shown the use of GC-MS to measure levels of JA and OPDA in flowers (Hause et al., 2000) and leaves (Stenzel et al., 2003; Strassner et al., 2002) of tomato plants. Despite minor differences in the methods used, the results are comparable to those presented here.

A disadvantage of the method used in this study is that simultaneous quantification of endogenous JA and MeJA is not possible owing to methylation of the plant extract prior to GC-MS analysis. Alternative derivatization methods (for example, silylation of the hydroxy group in the carboxylate side chains of JA and OPDA with trimethylsilyl reagents) may be helpful to circumvent this problem.

Regulation of JA biosynthesis in tomato

The results summarized in Table 4-1 show that endogenous JA levels change in response to wounding, the particular tissue type under study, and the genetic background of the cultivar. These factors have been reported to regulate the activity of enzymes involved in JA biosynthesis (Hause et al., 2000; Lauder and Weiler, 1998; Strassner et al., 2002).

In leaf tissue, JA levels seem to be regulated by substrate availability. Previous studies have shown that application of linolenic acid to leaves activates the expression of JA-inducible genes in tomato (Farmer and Ryan, 1992). Furthermore, linolenic acid and JA showed a similar accumulation pattern in tomato leaves in response to wounding (Conconi et al., 1996). Transgenic Arabidopsis plants that overexpress *AOS* did not have altered basal levels of JA in leaves, but showed stronger induction of JA synthesis in response to wounding (Laudert et al., 2000). These results indicate that the biosynthesis of JA in leaves is limited by the release of linolenic acid from membranes.

The results of this study indicate that the conversion of OPDA to JA is another rate-limiting step in JA biosynthesis (Table 4-2; Figure 4-5). The level of OPDA was significantly higher than that of JA in unwounded control plants. Three hr after wounding, the level of OPDA continued to increase, whereas the JA content almost returned to the basal level. These results indicate that increased OPDA levels do not necessarily lead to a proportional increase in JA levels. The differential accumulation of OPDA and JA was also observed in Arabidopsis (Laudert and Weiler, 1998; Weber et al., 1997) and potato (Weber et al., 1997).

Wound response mutants showed reduced levels of JA and OPDA compared to wild-type plants (Table 4-1; Table 4-2; Figure 4-5). These observations provide clues to understanding the signaling defect in these mutants. For example, the product of *Spr2* is expected to be involved in OPDA synthesis, because both JA and OPDA were severely reduced in *spr2* plants (Table 4-2). This speculation was confirmed by the recent discovery that *Spr*2 encodes a fatty acid desaturase catalyzing the synthesis of linolenic acid (Li et al., 2003). Therefore, the deficiency in linolenic acid blocks the production of both OPDA and JA in *spr2* plants.

Both *spr1* and *def1* plants accumulated less OPDA than did wild-type plants in response to wounding. These observations are consistent with recent findings that *Spr1* is a positive regulator of JA biosynthesis (Lee and Howe, 2003), and that the activity of AOC is reduced in *def1* plants (Stenzel et al., 2003). JA-insensitive *jai1-1* and *jai1-3* plants showed reduced levels of JA in wounded leaves. These results indicate that JA biosynthesis may be regulated by a positive feedback mechanism.

Materials and Methods

Preparation of internal standards

DHJA and H4OPDA were prepared by PtO_2-catalyzed hydrogenation of (±)-JA (Sigma, St. Louis, MO) and OPDA (Cayman Chemical, Ann Arbor, MI) as described (Weber et al., 1997). The authenticity of the standards, as well as the absence of endogenous DHJA/H4OPDA in tomato leaf extracts, was verified by GC-MS.

Extraction of JA and OPDA from tomato tissues

Plants containing two fully-expanded leaves and an emerging third leaf were wounded with a hemostat on each leaflet. Three to five g FW of leaves were used for quantification of JA. JA extraction was performed following the procedure described by Weber et al. (1997) with modifications. Harvested leaves were frozen in liquid nitrogen and ground to a fine powder with a chilled mortar and pestle. The tissue was dissolved in 28 ml methanol containing 500 ng DHJA and 500 ng H4OPDA as internal standards and then homogenized with a Polytron for 1 min at 4°C. The homogenate was incubated for 2 hr at 4°C with shaking, diluted with 12 ml ice-cold water, and then centrifuged at 3,500 × *g*. The resulting supernatant was recovered and the pH adjusted to 8.0 with NH_4OH. This solution was centrifuged again at 3,500 x g, and the supernatant was passed through a tC_{18}-SepPak cartridge (Waters Corporation, Milford, MA) that was preconditioned with 70% (vol/vol) methanol and collected in a new glass vials. The cartridge was washed with 7 ml of 75% (vol/vol) methanol. Eluates from both the sample and the wash steps were combined and adjusted to pH 4.0 with 10% (vol/vol) formic acid. This solution was diluted with 160 ml ice-cold water and then loaded on the same tC_{18}-SepPak column that was washed sequentially with methanol, diethylether, methanol, and water immediately after the elution with 75% (vol/vol) methanol. After washing the column with 7 ml of 15% (vol/vol) ethanol and 7 ml water, the JA fraction was eluted with 10 ml diethylether. The eluate was partially dried over anhydrous Na_2SO_4 and then dried completely under a stream of nitrogen gas at 35°C. The dried paste was dissolved in 0.5 ml methanol and subjected to methylation by the addition of diazomethane dissolved in 0.5 ml diethylether. This mixture was dried under nitrogen gas, resuspended in 20 µl hexane, and injected into the GC. Diazomethane was prepared

prior to use: 0.2 g N-nitroso-methylurea (Sigma, St. Louis, MO) was dissolved in a mixture of 2 ml ice-cold diethylether and 600 μl of 40 % (vol/vol) KOH solution.

GC-MS analysis

The amount of JA/MeJA in leaf extracts was quantified by GC-MS by using a Hewlett-Packard GC 5890 equipped with a Hewlett-Packard 5970 mass detector. The GC was fitted with a DB-5 column (30 m × 0.25 mm i.d., J&W Scientific, Folsom, CA) and run with a temperature gradient of 100°C for 1 min, 100°C to 170°C at 5°C/min, 170°C for 2.5 min, and 170°C to 250°C at 20°C/min. GC-MS analysis was performed in the SI mode with monitoring of ions specific for MeJA (*m/z* = 224) and MeDHJA (*m/z* = 226). Molecules were ionized by electron impact in MS. For quantification of JA/MeJA, a standard curve was generated from samples in which MeJA and MeDHJA were mixed in known ratios. Because peaks corresponding to the 3R, 7S and 3R, 7R isomers of endogenous JA/MeJA were detected, the areas of the two peaks were combined.

For simultaneous analysis of JA and OPDA, a modified temperature program was used for GC: 100°C for 1 min, 100°C to 160°C at 20°C/min, 160°C to 238°C at 3°C/min, 238°C to 250°C at 30 °C/min. For the quantification, GC-MS was run in SI mode while monitoring ions for MeJA (*m/z* = 224), MeDHJA (*m/z* = 226), MeOPDA (m/z = 238) and MeH4OPDA (m/z = 240). Retention times of these molecules were: 9.57 min for 3S, 7S-MeJA; 9.88 min for 3R, 7R-MeJA; 9.97 min for 3R, 7R-MeDHJA;10.51 min for 3R, 7S-MeJA; 10.54 min for 3R, 7S-MeDHJA; 26.93 min for 9S, 13R-MeH4OPDA (also called *cis*-MeH4OPDA); 27.30 min for 9S, 13R-MeOPDA; 27.70 min for 9S, 13S-MeH4OPDA (also called *trans*-MeH4OPDA); and 28.60 min for

9S, 13S-MeOPDA. Standard curves for OPDA were constructed using a mixture containing known amounts of OPDA and H4OPDA. The detection limit of MeJA (0.4 nmol) and MeOPDA (0.2 nmol) was determined by injection of known amounts of these molecules.

Plant material

Seeds of *spr1-1, spr2, def1* and *jai1-1* were collected from each homozygous line after at least two backcrosses using Castlemart cv (for *spr1-1, spr2,* and *def1*) or Micro-Tom cv (for *jai1-1)* as the recurrent parent. Leaves were harvested from two-week-old plants for extraction of oxylipins. For the analysis of *jai1-3* plants, the second and the third youngest leaves were harvested from 5-leaf-stage plants. Wounding was inflicted on each leaflet with a hemostat. Flowers were harvested in both open and closed stages from plants grown in the greenhouse. Growth conditions are described in Chapter 2.

Spider mite treatment

Two-spotted spider mite (*Tetranychus urticae* Koch) treatment was performed as described (Li et al., 2002). Spider mite eggs were hatched on leaves of lima bean (*Phaseolus lunatus* cv Fordhook) plants and allowed to grow to a population density of approximately 50 adult spider mites per fully expanded leaf. Infested leaves were cut and placed onto the upper surface of leaves of 16-day-old wild-type and *def1* plants. Two days later, tomato leaflets showing the visible symptom of damage were harvested for JA/OPDA extraction.

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Chapter 5

Analysis of wound-induced root-to-leaf signaling in tomato plants

Introduction

Tomato plants express many genes systemically in response to wounding. Previous studies have shown that the polypeptide systemin and its precursor protein prosystemin play crucial roles in the systemic wound response in tomato (Ryan, 2000). Exogenous systemin induces the accumulation of defense proteins such as Proteinase Inhibitors (PIs; Pearce et al., 1991). Systemic accumulation of PIs in response to wounding is severely reduced in transgenic plants that constitutively express a prosystemin cDNA in the antisense orientation (McGurl et al., 1992). Systemin activates the biosynthesis of jasmonic acid (JA), which results in the expression of *PIs* in tomato (Doares et al., 1995a; Howe et al., 1996). These observations, together with the mobility of systemin applied at wound sites in the phloem (Pearce et al., 1991), led to the hypothesis that systemin is a mobile signal that couples local injury to systemic gene expression (Ryan, 2000).

Results from recent studies do not support the proposed role of systemin as the long-distance signal for systemic gene expression. Grafting experiments performed with various wound response mutants of tomato indicate that JA, not systemin, is the long-distance signal for systemic *PI* expression (Chapter 2; Li et al., 2002). Moreover, it was shown that the systemin-insensitive *suppressor of prosystemin-mediated responses1* (*spr1*) mutant is compromised in the generation but not the perception of the systemic signal for *PI* expression (Chapter 2). This finding indicates that systemin is not the long-distance signal. JA-deficient tomato mutants such as JL1 (Chapter 3), *defenseless1* (*def1*; Howe et al., 1996; Li et al., 2002), and *spr2* (Li et al., 2002) are also defective in the generation of the systemic wound signal. However, these plants are able to perceive

the systemic wound signal transmitted from wild-type rootstock leaves. In contrast, the perception of the systemic wound signal was blocked in *jasmonic acid-insensitive1* (*jai1*) plants that fail to express *PI* in response to JA (Li et al., 2002). Therefore, JA or a derivative of JA appears to be synthesized in damaged leaves and transported to undamaged tissue to induce expression of *PIs*.

Increasing evidence indicates that not all systemic wound responses in tomato are regulated by JA and systemin. For example, wound-induced activation of a mitogen-activated protein kinase (MAPK) is mediated by a rapid, JA-independent signal (Stratmann and Ryan, 1997). It was also reported that wound-induced systemic expression of a glucosyl transferase gene occurs independently of JA and systemin (O'Donnell et al., 1998). Finally, the observation that a subset of wound-inducible genes is systemically expressed in *spr1* plants (Figure 2-2 and 2-5, Chapter 2) points to the existence of a systemin-independent signaling pathway.

Similar to systemic wound signaling in leaves, a long-distance signaling pathway appears to transmit a wound signal between roots and leaves. For example, mechanical wounding to roots induced *PI* expression in leaves of potato plants (Dammann et al., 1997). Conversely, leaf damage activates expression of a fatty acid desaturase gene in roots of Arabidopsis (Nishiuchi et al., 1997; 1999). At present, very little is known about the signaling pathway that mediates this root-to-shoot systemic wound response. The purpose of this study was to investigate the genetic basis of wound-induced root-to-leaf signaling in tomato. This question was addressed by analyzing the systemic expression of various wound-inducible genes in wild-type and mutant plants that are defective in the systemin/JA-dependent signaling pathway. The

results show that a JA-dependent signaling regulates the systemic expression of a subset of wound-inducible genes during root-to-leaf signaling, whereas a JA-independent pathway is involved in the controlling the systemic expression of a different set of genes.

Results and Discussion

In potato plants, damage to roots can induce gene expression in leaves (Dammann et al., 1997). To determine whether this is also the case in tomato plants, PI-II levels in leaves were measured in two-leaf-stage tomato plants after wounding of roots. Mechanical crushing of roots with a hemostat resulted in accumulation of PI-II in undamaged leaves (Figure 5-1). Although the level of PI accumulation was less than that observed in wounded leaves, this finding indicates that a systemic signal travels between roots and aerial parts of the plant.

To investigate whether root-to-shoot signaling depends on systemin and JA, systemic accumulation of PI-II was examined in systemin-insensitive *spr1* and JA-deficient *def1* plants. The results showed that root-damaged *spr1* plants accumulated normal levels of PI-II in leaves. Thus, systemin action appears not to be required for this systemic response. This observation contrasts the role of systemin in a leaf-to-leaf systemic signaling (Chapter 2). A role for systemin in leaf-to-leaf but not root-to-leaf systemic signaling is consistent with the previous report that prosystemin is expressed in leaves but not roots (McGurl et al., 1992).

In contrast to *spr1* plants, root damage failed to induce systemic PI-II accumulation in *def1*. The lack of response in *def1* plants could result from a defect in

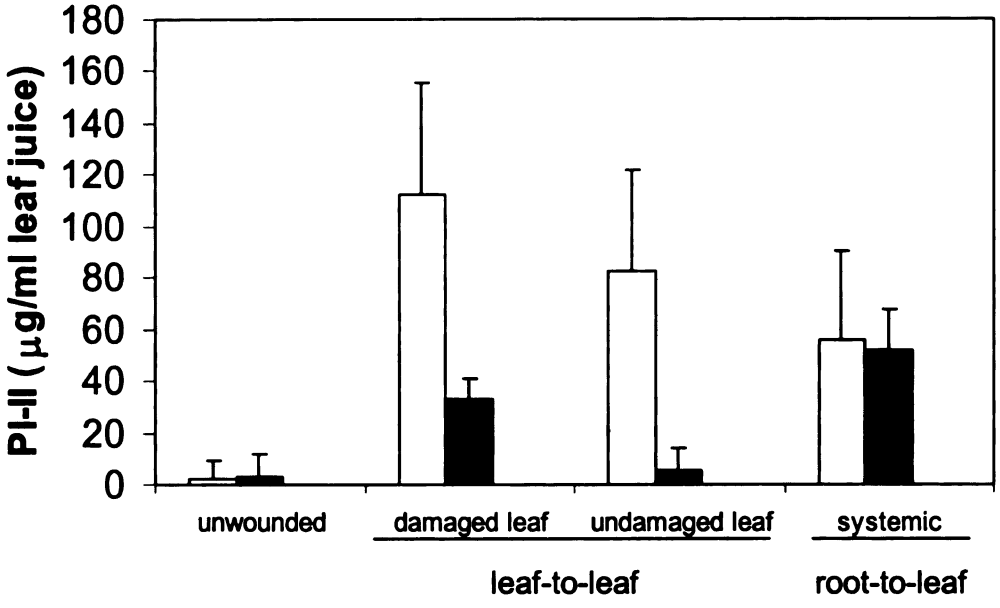

Figure 5-1. Systemic induction of PI-II in leaves in response to wounding of roots. The accumulation of PI-II in leaves was compared among wild-type (open bar), *spr1* (gray bar), and *def1* (*def1* leaves did not accumulate detectable amounts of PI-II) plants. Mechanical wounding was inflicted to roots of two-leaf-stage plants, and leaves were harvested 24 hr later for measurement of PI-II (systemic). As a negative control, PI-II was measured in leaves harvested from unwounded plants (unwounded). For comparison to leaf-to-leaf systemic signaling, a single wound was inflicted on a lower leaf of two-leaf-stage plants, and PI-II levels were measured separately in damaged lower leaves (damaged leaf) and undamaged upper leaves (undamaged leaf) 24 hr later. Data represent the mean and standard deviation of six plants.

the production of the systemic wound signal in wounded roots, or from a defect in the recognition of that signal in unwounded leaves. Previous grafting experiments showed that leaf-to-leaf wound signaling requires JA biosynthesis in wounded leaves, not in undamaged leaves (Chapter 3; Li et al., 2002). Because PI synthesis in leaves is mediated by the action of JA (Doares et al., 1995a; Farmer and Ryan, 1992), these results indicate that JA or a derivative of JA is synthesized in wounded leaves and transported to undamaged leaves (Li et al., 2002). Therefore, the lack of PI production in *def1* leaves in response to root damage indicates that *def1* roots are defective in wound-inducible JA biosynthesis and the generation of a systemic wound signal.

RNA gel-blot analysis was used to determine whether root damage results in activation of gene expression in unwounded leaves. In these experiment, cDNA probes for two groups of wound-inducible genes were employed (Figure 5-2). The first group of these genes is the so-called 'early' wound response genes, which shows rapid and transient induction in response to wounding of leaves (Ryan, 2000; Figure 2-2). Early genes include *lipoxygenase D* (*Lox D*) involved in JA biosynthesis and a *wound-inducible MAPK* (*WIPK*) homologue (Seo et al., 1999). The second group is 'late' wound response genes, which are expressed slowly but steadily upon wounding (Ryan, 2002; Figure 2-2, Chapter 2). *PIs* are representative of this group.

Root damage activated strong expression of *PI-II* in wild-type leaves, similar to the pattern observed in response to leaf wounding or stem excision (Figure 2-5, Chapter 2). Wounding of wild-type roots also resulted in rapid and transient systemic expression of *LoxD* and *WIPK*. The general pattern of systemic expression of early and late wound response genes in *spr1* plants was similar to wild-type plants, although differences in

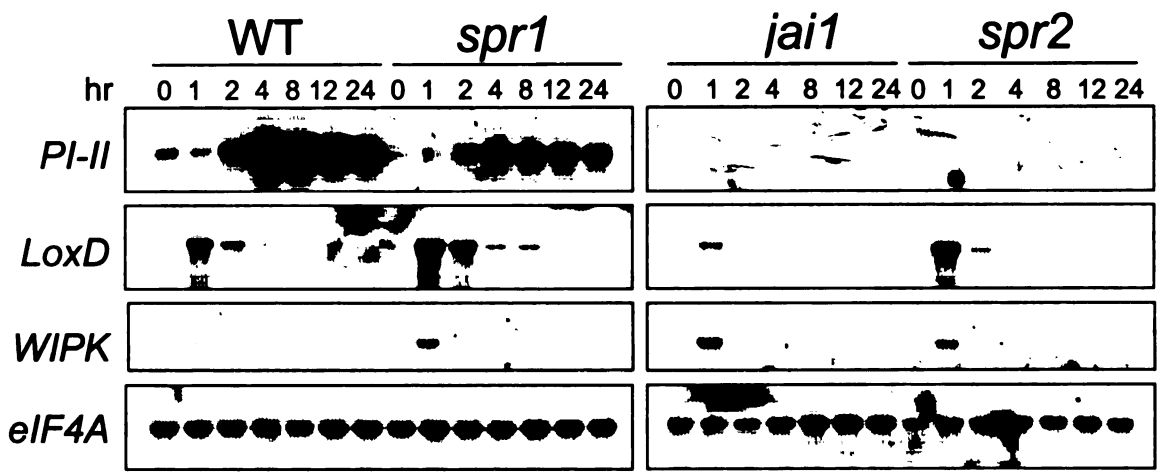

Figure 5-2. Systemic expression of wound-inducible genes in response to root damage. Mechanical wounding was applied to roots of two-week-old plants with a hemostat. Leaves were harvested from six plants at each time point and pooled for RNA extraction. To facilitate direct comparison of transcript levels in wild-type (WT) and mutant plants, blots containing RNA from the four genotypes were hybridized in the same container and washed under the same conditions. Then X-ray film was exposed to the blots for the same length of time.

the magnitude of the response were noted. For example, the expression of *PI-II* in *spr1* leaves was significantly lower than in wild-type plants. Because the level of PI-II protein in leaves of root-damaged *spr1* plants was similar to that of wild-type plants (Figure 5-1), it appears that mRNA levels do not accurately reflect PI protein levels. These phenomena have previously been observed in wounded tomato leaves (Howe et al., 1996).

Systemic *PI-II* expression was abolished in leaves of JA-insensitive *jai1* plants, indicating that a functional JA-dependent signaling pathway is required for this systemic wound response. Consistent with the absence of PI-II in *def1* plants (Figure 5-1), JA-deficient *spr2* plants also failed to express *PI-II* in leaves upon root damage. This result demonstrates that JA biosynthesis is required for systemic *PI* expression. Interestingly, a wound-inducible MAPK homologue (*WIPK*) was uniformly expressed in all genotypes in response to root damage. Therefore, expression of the *WIPK* appears to be mediated by a JA-independent signaling pathway. JA-independent wound signaling was previously proposed to account for the expression of an *LE RNase* gene, a *glucosyl transferase* gene, and activation of a MAPK activity in tomato leaves (Figure 6-8, Chapter 6; O'Donnell et al., 1998; Stratmann and Ryan, 1997).

Unlike *WIPK*, *LoxD* expression in *jai1* plants was less abundant than in *spr1*, *spr2*, and wild-type plants. This result indicates that the JA-dependent signaling pathway is required for maximal induction of *LoxD*. The residual expression of *LoxD* is unlikely due to 'leakiness' of the *jai1* mutation because *PI-II* expression was not detected in *jai1* plants. Furthermore, this allele of *jai1* (*jai1-1*) corresponds to a fast-neutron-induced deletion in the *Jai1* gene (Li et al., 2002). The expression of *LoxD* in

wild-type plants therefore appears to be regulated by both JA-independent and JA-dependent signaling pathways. This conclusion is consistent with the previous finding that *LoxD* expression is regulated by a systemin-independent pathway (Figure 2-5, Chapter 2).

In Arabidopsis, a JA-independent pathway regulates local wound response through the action of oligogalacturonic acid (OGA) released from cell walls (Rojo et al., 1999). It is unlikely that JA-independent signaling in tomato is mediated by OGA because JA biosynthesis is required for OGA-induced gene expression (Doares et al., 1995b). Previous studies indicated that the JA-independent signal could be rapidly transmitted through the xylem (Malone et al., 1995; Rhodes et al., 1999; Stratmann and Ryan, 1997). It may be informative to examine the expression of *WIPK* using steam-girdled plants in which the xylem but not the phloem is intact. This experiment would provide insight into the question of whether the JA-independent signal for *WIPK* expression is phloem- or xylem-borne.

Materials and Methods

Plant material

Seeds of *spr1-1, spr2, def1,* and *jai1-1* were collected from each homozygous line after at least two backcrosses, using Castlemart cv as the recurrent parent. As a result, *jai1-1* plants used in this study displayed normal growth compared to Castlemart cv. Plants were grown in peat pots as described in Chapter 2. Root damage was applied on two-leaf-stage plants by pinching the peat pot 7 times with a hemostat. The PI-II level was measured as described in Chapter 2.

RNA gel-blot analysis

Tomato EST clones cLET1D13 and cLED1D24 were used as probes to detect *WIPK* and *eIF4A* mRNA, respectively. Total RNA was harvested and hybridized following the methods described in Chapter 2.

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Chapter 6

Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase

A version of this chapter has been published.

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Data presented in Figure 6-4 were obtained by Dr. Aya Itoh.

Data presented in Figure 6-6 were obtained by Dr. Amy DeRocher.

Data presented in Figure 6-7 were obtained by Dr. Lei Li.

Data presented in Figure 6-8 were obtained by Dr. Gregg Howe.

Introduction

Fatty acid hydroperoxides produced by 13-lipoxygenases are important intermediates in the oxylipin pathway of fatty acid oxygenation in plants. In one branch of oxylipin metabolism often referred to as the octadecanoid pathway, allene oxide synthase (AOS) commits 13S-hydroperoxy-9(Z),11(E),15(Z)-octadecatrienoic acid (13-HPOT) to the formation of jasmonic acid (JA) and related cyclopenta(e)nones (Creelman and Mullet, 1997; Figure 6-1). Products of the AOS pathway are essential signals for plant defense against pest attack (Staswick and Lehman, 1999), mechanical responses (Weiler et al., 1993), and some developmental processes (McConn and Browse, 1996). An alternative pathway for 13-HPOT metabolism is initiated by fatty acid hydroperoxide lyase (HPL; Figure 6-1). Short chain aldehyde products of HPL, together with their corresponding reduced alcohols, are important volatile constituents of the characteristic odor of fruits, vegetables, and green leaves (Gardner, 1991; Hatanaka, 1993). C_6 aldehydes produced by HPL are also reported to act as phytoalexins against protozoa, bacteria, and fungi (for review, see Blée, 1998), and may be signals for gene regulation (Bate and Rothstein, 1998). The C_{12} oxo-acid product of HPL is the precursor of the previously identified 'wound signal' known as traumatin (Zimmerman and Coudron, 1979). 13-HPOT is metabolized by other plant enzymes including lipoxygenase (Salch et al., 1995), peroxygenase (Blée et al., 1993), and divinyl ether synthase (Grechkin et al., 1995; Hamberg, 1998), and may be subject to degradation by non-specific alkyl hydroperoxide reductases (Baier and Dietz, 1999).

AOS and HPL comprise an unusual class of cytochrome (Cyt) P450s that is specialized for the rearrangement of fatty acid hydroperoxides. Unlike typical P450

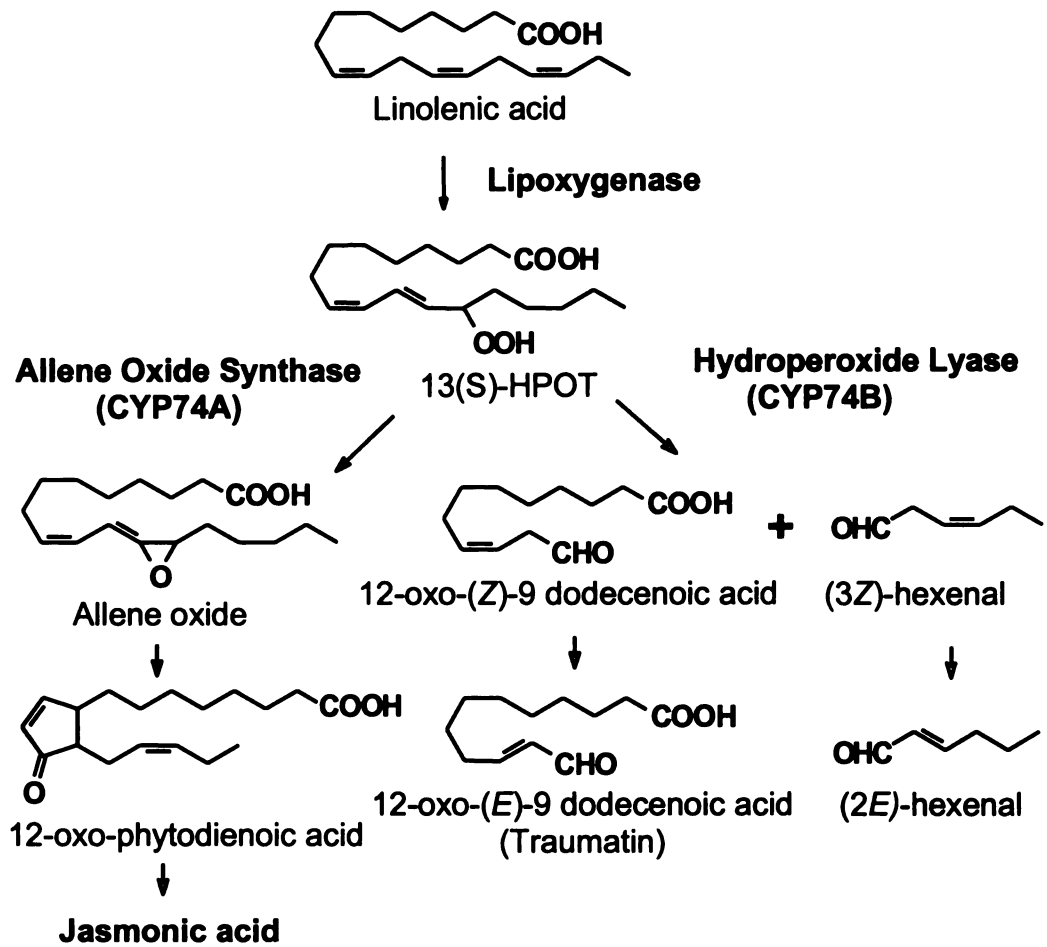


Figure 6-1. The octadecanoid pathway. Cyt P450-dependent metabolism of 13-HPOT. AOS (CYP74A) commits 13-HPOT to the production of JA and related cyclopenta(e) nones. In the absence of allene oxide cyclase (AOC), the epoxide product of AOS undergoes spontaneous hydrolysis to α- and γ-ketols and racemic 12-OPDA. HPL (CYP74B) cleaves 13-HPOT to produce C_6 and C_{12} products that are further metabolized as shown.

monoxygenases, AOS and HPL demonstrate low affinity for carbon monoxide and do not require O_2 or NADPH-dependent Cyt P450 reductase for their activity (Song and Brash, 1991; Shibata et al., 1995a, 1995b). Identification of cDNA sequences encoding AOS and HPL has provided additional insight into the relationship between these two enzymes, and their divergence from classical P450s (Song et al., 1993; Pan et al., 1995; Laudert et al., 1996; Matsui et al., 1996; Bate et al., 1998). Based on the amino acid sequence identity between AOS and HPL (approximately 38%), the two enzymes are classified as subfamilies CYP74A and CYP74B, respectively, within the CYP74 family of P450s (Nelson, 1999).

The importance of oxylipins as signals for plant stress responses has prompted interest in understanding the mechanisms by which their synthesis is regulated (Creelman and Mullet, 1997; Farmer et al., 1998). JA accumulation, for example, is stimulated by mechanical wounding and herbivory (Creelman et al., 1992; Blechert et al., 1995; Conconi et al., 1996), pathogen attack (Penninckx et al., 1996), treatment with elicitors (Gundlach et al., 1992; Doares et al., 1995), and water or nutrient deprivation (Creelman and Mullet, 1995; Lehmann et al., 1995). Similarly, mechanical injury and some plant-pathogen interactions lead to the production of HPL products (Hatanaka et al., 1987; Gardner, 1991; Croft et al., 1993). Formation of AOS- and HPL-derived oxylipins is controlled in large part by the availability of hydroperoxide substrates that are generated from lipase/acyl hydrolase-mediated release of fatty acids from membrane lipids, followed by lipoxygenase-catalyzed conversion to 9- and 13-hydroperoxides (Galliard et al., 1977; Hatanaka, 1993; Mueller et al., 1993; Narváez-Vásquez et al., 1999). Nonenzymatic lipid peroxidation, such as that associated with the initial stages

of plant-pest interactions, may also contribute to the pool of hydroperoxides available to AOS and HPL (Gardner, 1989; Hammond-Kosack and Jones, 1996).

In addition to substrate availability, fatty acid hydroperoxide metabolism may also be influenced by the spatial and temporal expression of enzymes that utilize these substrates. For example, the localization of both AOS and HPL to the chloroplast (Vick and Zimmerman, 1987; Song et al., 1993; Blée and Joyard, 1996; Laudert et al., 1996; Bate et al., 1998; Froehlich et al., 1999) indicates that these enzymes utilize a common pool of hydroperoxide substrates. Recent studies indicate that *AOS* expression is positively regulated by wounding, as well as by terminal products of the AOS pathway (Laudert and Weiler, 1998). These results, together with transgenic studies showing that AOS is a rate-limiting step in JA biosynthesis (Harms et al., 1995), indicate that up-regulation of AOS activity during the wound response may provide a mechanism to amplify the octadecanoid signaling pathway. On the other hand, others have shown that exogenous methyl JA stimulates oxylipin metabolism through the HPL pathway, and thus may shift oxylipin metabolism away from JA biosynthesis (Avdiushko et al., 1995; Kohlmann et al., 1999).

The aim of the present work was to gain an understanding of the molecular basis of Cyt P450-dependent metabolism of fatty acid hydroperoxides in tomato (*Lycopersicon esculentum*). Owing to the wealth of knowledge of plant-pest interactions in tomato, this system is likely to provide a good model for assessing the role of oxylipins in plant defense. The importance of HPL-derived volatiles in determining the flavor and aroma of fruits and vegetables provides additional incentive for investigating oxylipin metabolism in tomato (Kazeniac and Hall, 1970; Buttery and Ling, 1993).

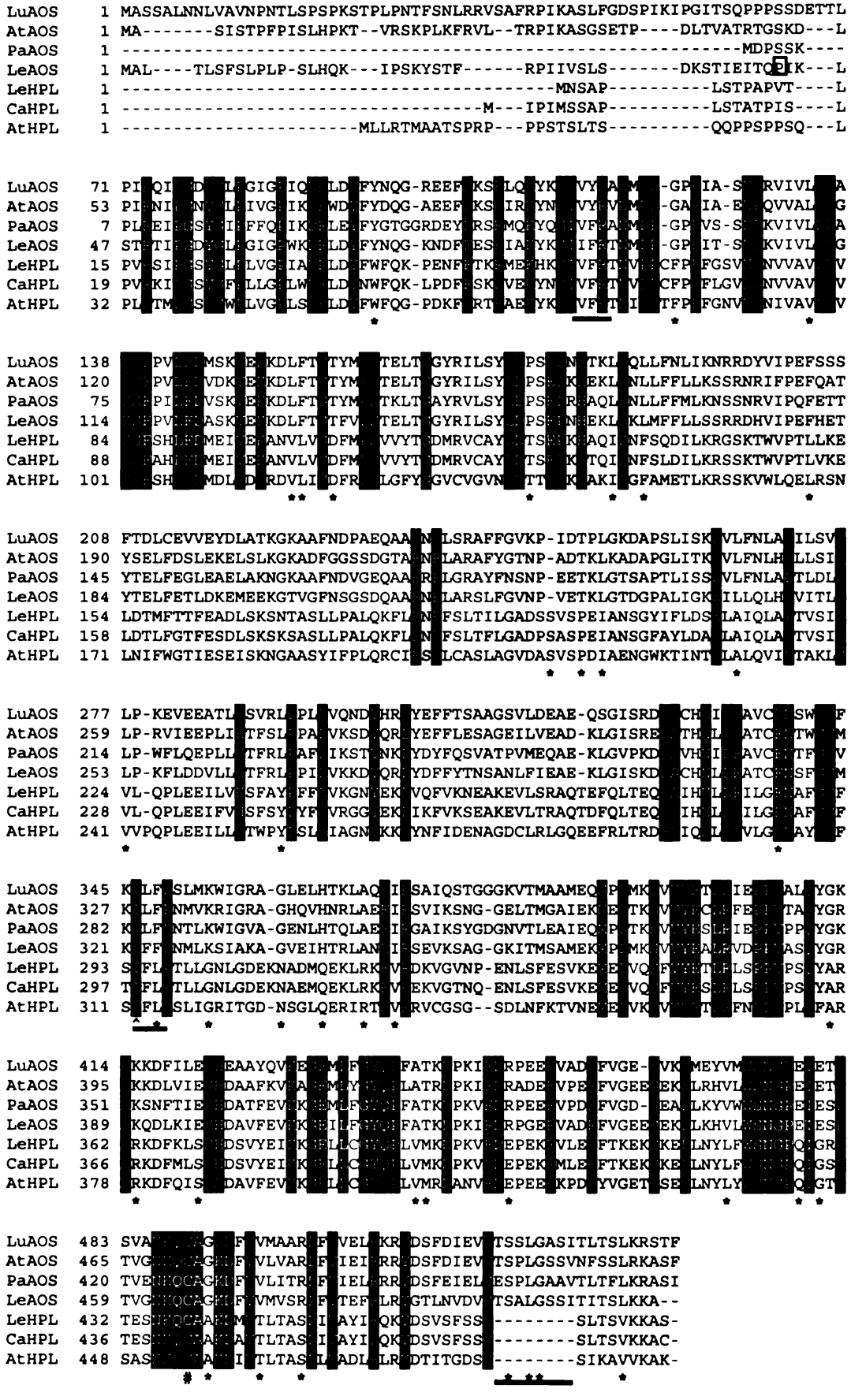
Toward this goal, we report here the isolation of cDNAs that encode functional members of the CYP74A (AOS) and CYP74B (HPL) subfamilies of P450 enzymes in tomato. The results of expression studies in *Escherichia coli* indicate a role for AOS and HPL in the commitment of 13-HPOT to the JA and C_6 aldehyde/traumatin pathways, respectively. We also report findings relevant to the developmental and defense-related expression of these two genes in planta. The significance of these results for understanding the regulation of fatty acid hydroperoxide metabolism is discussed.

Results

cDNA isolation and sequence analysis

An AOS-encoding cDNA from Arabidopsis (Laudert et al., 1996) was used to screen for related sequences in a tomato cDNA library. The longest clone obtained (designated as *LeAOS*) contained a 1,533-bp open reading frame, a 57-bp 5'-untranslated region (UTR), and a 111-bp 3'-UTR excluding the poly-(A) tail. The open reading frame was predicted to encode a 510-amino acid protein having a calculated molecular mass of 57,202 D. The presence of an in-frame stop codon (UAA) 30 nucleotides upstream of the putative AUG start codon indicated that *LeAOS* contained the full-length coding sequence. The deduced amino acid sequence of *LeAOS* was approximately 61% identical to *AOS* from flax (Song et al., 1993), guayule (Pan et al., 1995), and Arabidopsis (Laudert et al., 1996) (Table 6-1). Thus, *LeAOS* is classified as a new member of the CYP74A subfamily of Cyt P450s. The N-terminal region of *LeAOS*

Figure 6-2. Comparison of cDNA-deduced protein sequences of plant *AOS* and *HPL* genes. *LeAOS* and *LeHPL* sequences were aligned, using the ClustalW 1.7 program available at http://mbcr.bcm.tmc.edu/searchlauncher. AOS sequences were from flax (LuAOS; Song et al., 1993; accession no. U00428), guayule (PaAOS; Pan et al., 1995; accession no. X78166), and Arabidopsis (AtAOS; Laudert et al., 1996; accession no. Y12636). HPL sequences were from bell pepper (CaHPL; Matsui et al., 1996; accession no. U51674) and Arabidopsis (AtHPL; Bate et al., 1998; accession no. AF087932). Black boxes indicate amino acid residues that are conserved between all seven CYP74 members. Subfamily-specific substitutions are indicated with an asterisk. The three subfamily-specific motifs discussed in the text are underlined by the black bars. The ^ symbol denotes the T → (I/V) change within the I helix that is a hallmark of CYP74 enzymes. The conserved Cys within the heme-binding domain is marked by a # symbol. The boxed residue (Pro-43) at the N terminus of LeAOS denotes the site where the His-tag was added in the pQE-AOS expression construct.


LuAOS
AtAOS
PaAOS
LeAOS
LeHPL
CaHPL
AtHPL

displayed features of a typical chloroplast targeting peptide including an enrichment of hydroxylated amino acids. Conclusive evidence that *LeAOS* is localized to the chloroplast was recently obtained (Froehlich et al., 1999). These findings indicate that LeAOS is more similar to AOS from flax and Arabidopsis, which also reside in the chloroplast (Song et al., 1993; Harms et al., 1995; Laudert et al., 1996), than it is to the cytosolic AOS from guayule (Pan et al., 1995).

Values on the upper right diagonal of the matrix indicate the percentage of amino acid identity between different family members. Values on the lower left of the matrix indicate the percentage of nucleotide identity. Percentage identity within the open reading frame of each pair of sequences was calculated using DNA Star software (Clustal method). AOS sequences were from flax (*Linum usitatissimum*), guayule (*Parthenium argentatum*), Arabidopsis, and tomato. HPL sequences were from pepper, Arabidopsis, and tomato. GenBank accession nos. for the sequences are given in Figure 6-2.

A cDNA encoding HPL from bell pepper (*Capsicum annum*) was used to screen a tomato cDNA library for related sequences. Among the 15 positive clones identified, the longest cDNA (designated *LeHPL*) contained a 1,431-bp open reading frame that was predicted to specify a 476-amino acid protein with a molecular mass of 53,542 D. *LeHPL* contained a 169-bp 5'-UTR, and a 210-bp 3'-UTR excluding poly-(A) residues. The presence of an in-frame stop codon (UGA) 93 nucleotides upstream of the putative initiator AUG codon indicated that *LeHPL* encoded the entire protein. This was confirmed by DNA sequence analysis of RACE products derived from the 5' end of *LeHPL* transcripts ('Materials and Methods'). The deduced amino acid sequence of

	LuAOS	*AtAOS*	*PaAOS*	*LeAOS*	*LeHPL*	*CaHPL*	*AtHPL*
LuAOS		59.9	65.0	61.1	36.1	36.0	34.9
AtAOS	55.7		60.5	62.2	38.2	37.2	36.3
PaAOS	56.2	55.6		60.1	36.5	36.1	34.8
LeAOS	53.1	57.5	55.9		35.6	35.3	35.9
LeHPL	36.3	38.7	38.0	36.0		87.6	55.3
CaHPL	36.3	38.5	38.7	36.5	85.5		53.2
AtHPL	37.8	38.9	36.6	36.3	51.2	49.1	

Table 6-1. Percent amino acid and nucleotide identity between different *AOSs* and *HPLs*. Values on the upper right diagonal of the matrix indicate the percentage of amino acid identity between different family members. Values on the lower left of the matrix indicate the percentage of nucleotide identity. Percentage identity within the open reading frame of each pair of sequences was calculated using DNA Star software (Clustal method). AOS sequences were from flax (*Linum usitatissimum*), guayule (*Parthenium argentatum*), Arabidopsis, and tomato. HPL sequences were from pepper, Arabidopsis, and tomato. GenBank accession nos. for the sequences are given in Figure 6-2.

LeHPL was 88% and 55% identical to the published sequence of *HPL* from bell pepper (Matsui et al., 1996) and Arabidopsis (Bate et al., 1998), respectively (Table 6-1). This establishes LeHPL as a new member of the CYP74B subfamily of Cyt P450s. Unlike HPL from Arabidopsis (Bate et al., 1998), LeHPL does not appear to contain a typical chloroplast targeting sequence at the N terminus of the protein (Figure 6-2).

Comparisons between the primary structures of the seven known CYP74 members (four AOSs and three HPLs) showed 182 positions (38%) that were conserved in all members of both subfamilies (Figure 6-2). Many conserved residues were clustered at the N- and C-termini, and may be important for functions common to HPL and AOS (e.g. heme or substrate binding). We also noted subfamily-specific amino acid differences that might play a role in distinguishing AOS function from that of HPL. Specifically, there were 39 positions at which an amino acid was invariant among all AOSs, and was substituted to a different residue in all HPLs (Figure 6-2). One HPL-specific motif was PPxFP, which represents a variation of the N-terminal PPGP tetrapeptide that is important for stability and catalysis in many P450s (Szczesna-Skorpa et al., 1993). A hallmark of all CYP74 enzymes, including *LeAOS* and *LeHPL*, is a T→(V/I) substitution within the I helix that, in most P450s, participates in O_2 binding (Song et al., 1993). Sequences surrounding this site show a subfamily-specific character, with the AOS and HPL consensus sequences being KI(L/F)F and (S/T)IFL, respectively. Several other subfamily-specific signatures were located near the C-terminal heme-binding domain. The most striking of these was an eight-amino acid insertion in AOS sequences relative to HPL sequences, at the extreme C-terminal end of the protein (Figure 6-2).

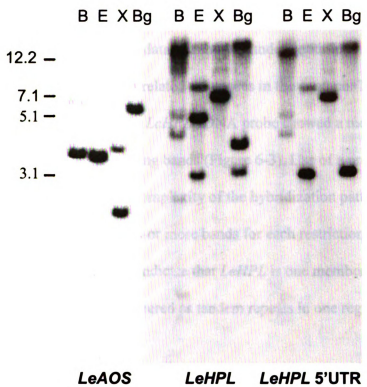


Figure 6-3. Southern-blot analysis of *LeAOS* and *LeHPL*. Genomic DNA from tomato was digested with restriction enzymes *Bam*HI (B), *Eco*RV (E), *Xba*I (X), or *Bgl*II (Bg). DNA blots were hybridized to labeled probes derived from the open reading frame of *LeAOS* (left), *LeHPL* (middle), or the *LeHPL* 5'-UTR (right). Blots were hybridized in 5× SSPE at 65°C and washed in 0.5× SSPE at the same temperature, as described in "Materials and Methods." Molecular mass standards (in kb) are indicated on the left.

Genomic DNA-blot analysis using the *LeAOS* cDNA as a probe showed a simple hybridization pattern for each of the restriction enzymes tested (Figure 6-3). This result indicates that *LeAOS* is derived from a single copy gene. However, detection of additional hybridizing bands (data not shown) under conditions of reduced stringency leaves open the possibility of related sequences in the genome. The results of hybridization analysis using a *LeHPL* cDNA probe showed a more complex pattern of weakly and strongly hybridizing bands (Figure 6-3). Use of a probe derived from the 5'-UTR of *LeHPL* reduced the complexity of the hybridization pattern as expected, but nevertheless still detected two or more bands for each restriction digest tested (Figure 6-3, right panel). These results indicate that *LeHPL* is one member of a family of highly related genes that may be clustered as tandem repeats in one region of the genome.

Functional expression of *LeAOS* and *LeHPL* in *E. coli*

To confirm that *LeAOS* and *LeHPL* encode the expected P450 enzymes, the cDNAs were subcloned into the pQE-30 expression vector to yield pQE-AOS and pQE-HPL, respectively, and transformed into an appropriate *E. coli* host. Bacterial cultures induced to express the constructs accumulated high levels of the recombinant proteins as determined by SDS-PAGE of bacterial lysates (data not shown). Crude lysates from cells expressing either pQE-AOS or pQE-HPL efficiently degraded 13-HPOT (Figure 6-4A) but did not metabolize 9-hydroperoxide derivatives of linolenic or linoleic acid (data not shown). Recombinant LeAOS and LeHPL metabolized the C_{20} hydroperoxide 15S-hydroperoxy-11(Z),13(E),17(Z)-eicosatrienoic acid at a rate comparable to that observed with 13-HPOT (Figure 6-4A), indicating that both enzymes can accommodate

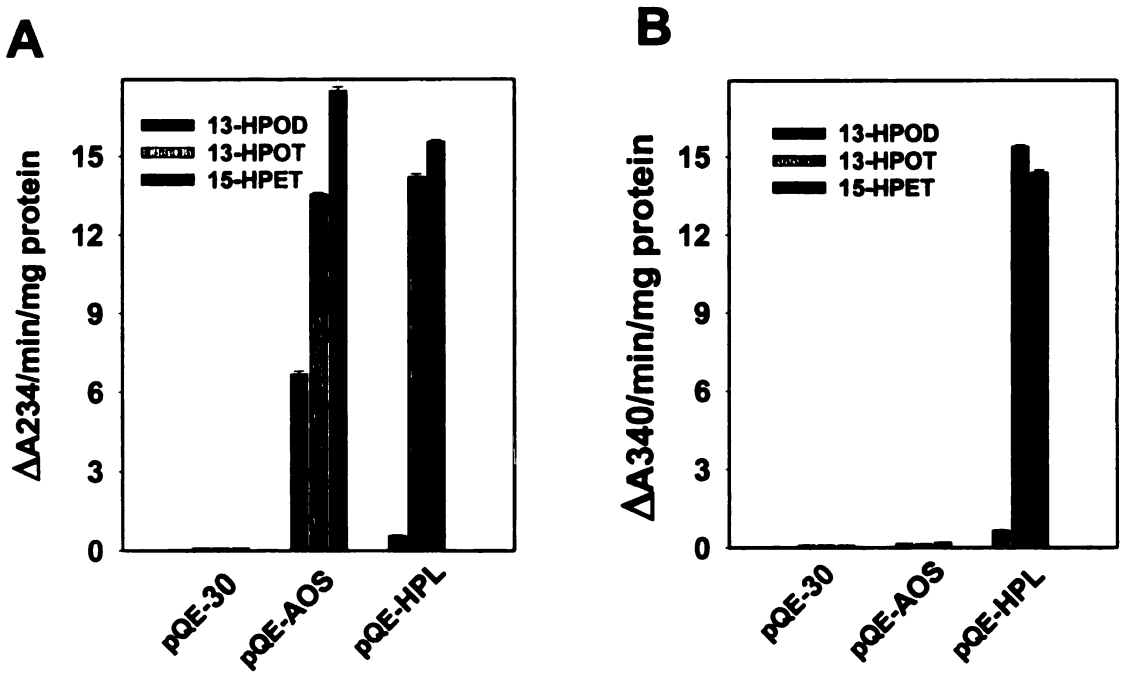


Figure 6-4. Activity of LeAOS and LeHPL expressed in *E. coli*. Total lysates of *E. coli* cells expressing pQE-AOS, pQE-HPL, or the empty vector (pQE-30) were tested for their ability to metabolize C_{18} (13-HPOD and 13-HPOT) and C_{20} (15-HPET) fatty acid hydroperoxides. Activity was measured either directly as a decrease in absorbance of the substrate at A_{234} (A) or indirectly as the production of aldehydes using a NADH-coupled assay (B). Error bars represent the mean and SD of activity determined from three enzyme preparations of each culture.

a range of fatty acid hydroperoxide substrates. However, the two enzymes differed in their ability to metabolize 13S-hydroperoxy-9(Z), 11(E)-octadecadienoic acid (13-HPOD), a common C_{18} hydroperoxide derived from linoleic acid. Whereas LeAOS utilized 13-HPOD at about one-half the rate observed with 13-HPOT, the rate of breakdown of 13-HPOD by LeHPL was less than 5% of that observed for 13-HPOT. Similar results were obtained using a coupled enzyme assay (Vick, 1991) to measure aldehyde production in the *in vitro* reactions. As expected from the known products of AOS and HPL (Figure 6-1), aldehyde production was associated with reactions catalyzed by LeHPL but not by LeAOS (Figure 6-4B). This assay also confirmed the strong preference of LeHPL for 13-HPOT over 13-HPOD.

Gas chromatography-mass spectometry (GC-MS) was used to identify the trimethylsilyl (TMS) derivatives of metabolites produced upon incubation of 13-HPOT with lysates from bacteria that expressed either pQE-AOS, pQE-HPL, or the pQE-30 mock control. In the case of pQE-AOS, three prominent peaks (A, B, and C) that were not present among the products of the mock reaction were observed. The relative abundance of these compounds, as estimated by integration of the GC peak areas, was 22% (peak A), 100% (peak B), and 13% (peak C) (values normalized to peak B). The retention time (11 min 9 s), molecular ion ($[M]^{+\cdot}$ at m/z 364), and fragmentation pattern of peak A were identical to that of an authentic 12-oxo-phytodienoic (12-OPDA) standard (Cayman Chemical, Ann Arbor, MI). Peak B eluted at 12 min 37 s and gave the following mass spectrum as m/z (% relative intensity, ion structure): 526 (18%, $[M]^{+\cdot}$), 511 (13%, $[M - CH_3]^{+\cdot}$), 457 (100%, $[M - C_5H_9]^{+\cdot}$), 367 (8%), 221 (5%), 179 (4%), 147 (12%), and 73 (28%, TMS^+). This fragmentation pattern was consistent with

identification of the compound as the tri-TMS derivative of the α-ketol compound 12-oxo-13-hydroxy-9(Z), 15(Z)-octadecadienoic acid (enolization of the 12-oxo group provided an additional hydroxyl for derivatization). The major fragment at m/z = 457 ($[M - C_5H_9]^{+\cdot}$) indicated that this compound represented the α-ketol rather than the γ-ketol (12-oxo-9-hydroxy-10[E],15(Z)-octadecadienoic acid). Peak C eluted with a retention time of 13 min 10 s and produced a mass spectrum identical to that of peak B, indicating probable double bond isomerization during derivatization or GC analysis. α-Ketol and 12-OPDA, together with minor amounts of γ-ketol, are known to arise by spontaneous hydrolysis of the unstable epoxide product of AOS (Song and Brash, 1991) (Figure 6-1). The products of the pQE-HPL-catalyzed reaction were analyzed by GC-MS as the oxime, TMS derivatives. The major product eluted with a retention time of 8 min 59 s. A molecular ion, m/z 371 [$M^{+\cdot}$], was observed for the di-TMS derivative of this product, and the fragmentation pattern was identical to that of an authentic 12-oxo-trans-10-dodecenoic acid standard, the expected product of HPL (Figure 6-1). These results confirmed the identity of LeAOS and LeHPL as functional members of the CYP74A and CYP74B subfamilies of P450 enzymes, respectively.

Developmental expression of *LeAOS* and *LeHPL*

RNA-blot analysis was used to investigate the distribution of *LeHPL* and *LeAOS* mRNA in different organs of tomato (Figure 6-5). *LeHPL* transcripts accumulated to high levels in developing flowers, and decreased during flower maturation. *LeHPL* mRNA levels were also relatively high in leaf tissue, with greater expression detected in younger leaves compared to older leaves from the same plant (see Figure 6-7). Very

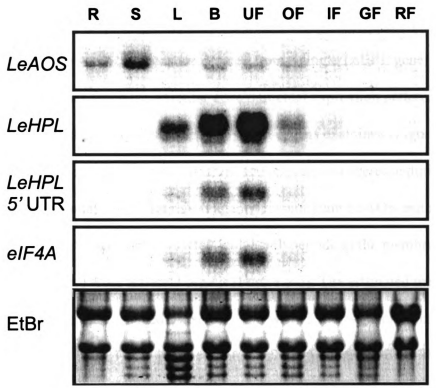


Figure 6-5. Expression of *LeAOS* and *LeHPL* genes in different organs of tomato. Total RNA was extracted from roots (R), stems (S), leaves (L), developing flower buds (B), mature unopened flowers (UF), mature opened flowers (OF), small (<0.5 cm) immature green fruit (IF), mature green fruit (GF), or mature red fruit (RF). Ten-microgram samples of RNA were subjected to RNA-blot analysis. Specific transcripts were detected by hybridization of blots to probes corresponding to full-length *LeAOS*, full-length *LeHPL*, the 5'-UTR of *LeHPL*, or an *eIF4A* probe used as a loading control. Also shown is a photograph of an ethidium bromide-stained gel of the RNA used for the experiment (EtBr).

low levels of LeHPL mRNA were detected in stems and immature green fruit, whereas roots and mature green and red fruit lacked detectable transcripts. Hybridization probes derived from either the full-length LeHPL cDNA or the LeHPL 5'-UTR showed a similar organ-specific expression pattern (Figure 6-5). This result showed that LeHPL transcripts detected by RNA-blot analysis are derived from a single LeHPL gene, or highly related LeHPL genes that have a similar developmental expression pattern.

LeAOS mRNA was broadly distributed among all organs examined (Figure 6-5). *LeAOS* transcript levels were relatively low in fruit, and appeared to decrease during fruit development. Polyclonal antibodies raised against recombinant LeAOS, but not preimmune serum from the same rabbit, reacted with a polypeptide in the membrane fraction of extracts prepared from different organs (Figure 6-6). The estimated mass of the cross-reacting polypeptide as judged by SDS-PAGE was 55 kD, which is consistent with that expected for the *LeAOS* gene product. Furthermore, the distribution of this polypeptide in different organs correlated with the distribution of *LeAOS* mRNA. Taken together, these results indicate that *LeAOS* is expressed in all tomato organs with the possible exception of ripe fruit. A second cross-reacting polypeptide of slightly lower *Mr* was often observed in immunoblot experiments, particularly in extracts derived from flowers (Figure 6-6). This band could represent a polypeptide that shares common epitopes with *LeAOS*, or a post-translationally modified form of *LeAOS*.

Wound-inducible expression of *LeAOS* is mediated by a *Def1*-independent signaling pathway

The importance of oxylipin metabolism for wound-inducible defense gene expression in tomato prompted us to examine the effect of wounding on *LeAOS* and *LeHPL* gene expression. Damage inflicted to tomato leaves by *Manduca sexta* larvae resulted in a modest (approximately 2-fold) increase in *LeHPL* mRNA accumulation (Figure 6-7). Wound-induced accumulation of *LeHPL* mRNA was more apparent in the lower damaged leaf than it was in the younger undamaged leaf. This is likely to reflect the higher constitutive expression of *LeHPL* in younger leaves. Wound-induced accumulation of *LeAOS* transcripts was much more apparent, and thus became the focus of additional experiments. The time course and amplitude of *LeAOS* expression differed in several ways from that of the well-characterized *proteinase inhibitor II* (*Inh-II*) gene (Figure 6-7). Whereas the maximum level of induction of *LeAOS* in local and systemic leaves was approximately 9- and 5-fold, respectively, *Inh-II* mRNA levels in these tissues increased by at least 60-fold. Wound-inducible accumulation of *LeAOS* mRNA was also more transient than that of *Inh-II*. These results indicated that the mechanism controlling wound-inducible expression of *LeAOS* might be different from that regulating *Inh-II* gene expression.

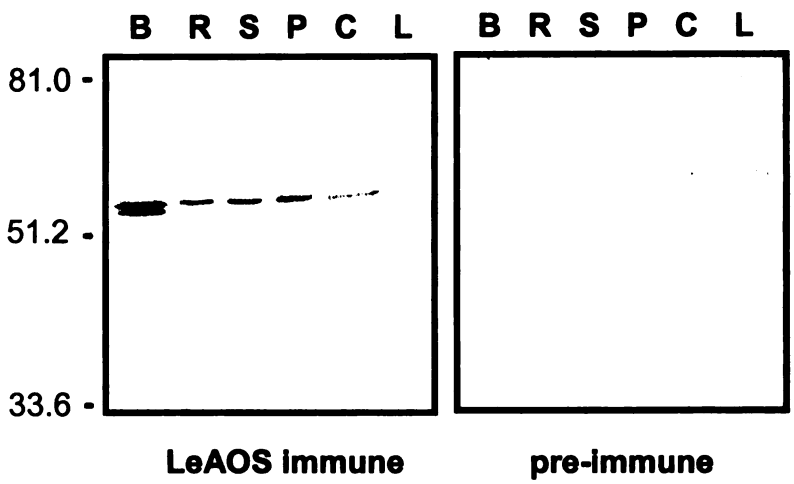


Figure 6-6. Accumulation of LeAOS protein in different organs of tomato. Fifteen-microgram samples of membrane protein prepared from young flower buds (B), roots (R), stems (S), petioles (P), cotyledons (C), and leaves (L) were separated by SDS-PAGE. Protein was transferred to Immobilon-P membranes and probed with either antiserum raised against LeAOS (left) or an equivalent amount of preimmune serum (right). The numbers on the left of the figure indicate the position of *M*r standards.

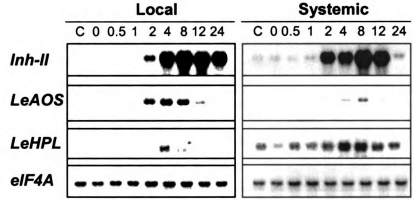


Figure 6-7. Accumulation of *LeAOS*, *LeHPL*, and *Inh-II* mRNAs in tomato plants in response to herbivory. Tobacco hornworm larvae (third instar) were placed onto the lower leaf of 3-week-old cv Micro-Tom plants and allowed to feed for 5 to 10 min. During this period, approximately 5% to 10% of the area of the attacked leaf was consumed by the larvae. Leaf tissue was harvested for extraction of total RNA immediately after removal of the larvae (0 point) or at the times indicated (in hr). RNA was prepared separately from the lower damaged leaf (Local response) and from the third leaf (counted from the base of the plant) (Systemic response). RNA was also prepared from a set of control plants that received no damage (C). Duplicate RNA blots containing 5 μg of RNA per sample were hybridized to cDNA probes for proteinase inhibitor II (*Inh-II*), *LeAOS*, *LeHPL*, and *eIF4A* as a loading control.

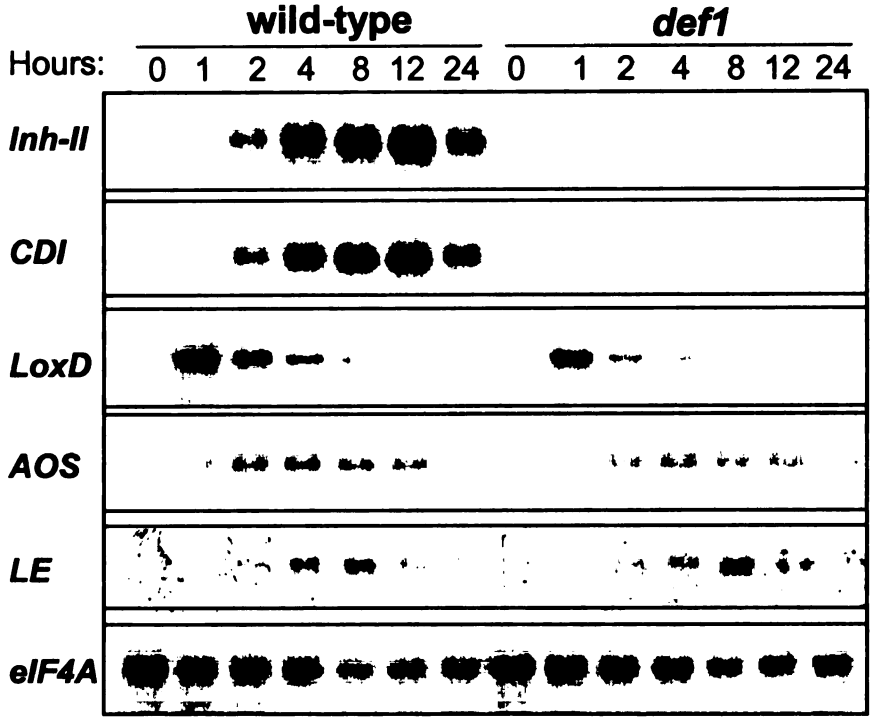


Figure 6-8. Analysis of wound-induced gene expression in wild-type and *def1* mutant plants. Fifteen-day-old wild-type (cv Castlemart) and *def1* mutant seedlings were mechanically wounded at the distal end of the terminal leaflet of the lower leaf. Undamaged tissue on the same leaflet was harvested for RNA extraction at the indicated times after wounding. RNA blots were hybridized to cDNA probes for *proteinase inhibitor II* (*Inh-II*), *cathepsin D inhibitor* (*CDI*), *TomLoxD* (*LoxD*), *LeAOS* (*AOS*), *LE* RNase (*LE*), and *eIF4A* as a loading control.

To further test this idea, we examined *LeAOS* expression in the tomato *defenseless1* (*def1*) mutant that is deficient in the octadecanoid-based signaling pathway that mediates the expression of *Inh-II* and other defense-related genes (Howe et al., 1996). Previous characterization of *def1* showed that it is deficient in JA accumulation in response to wounding and other elicitors (Howe et al., 1996). Genetic mapping studies have shown that the *def1* phenotype does not result from a defect in the *LeAOS* gene (A. Itoh and G.A. Howe, unpublished data). Moreover, direct measurements of 12-OPDA levels in *def1* and wild-type plants indicated that the mutant has both AOS and allene oxide cyclase activity (Stelmach, E. Weiler, G.A. Howe, unpublished data). Taken together, the available evidence indicates that the *Def1* gene product plays a role in the regulation of a late step in the biosynthesis of JA, or in the further metabolism of JA (e.g. transport or stability). A dramatic aspect of the *def1* phenotype is the lack of wound-induced accumulation of defensive proteinase inhibitor genes such as *Inh-II* and *cathepsin D inhibitor* (*CDI*) (Figure 6-8). In contrast to this, the pattern of wound-inducible *LeAOS* mRNA accumulation in *def1* plants was comparable to that in wild-type plants. This effect was not specific for *LeAOS*, as other transcripts, including those for lipoxygenase (*LoxD*) (Heitz et al., 1997) and a senescence-induced RNase (*LE*) (Lers et al., 1998), were also induced by wounding in both mutant and wild-type plants. These results demonstrate that wound-induced expression of *LeAOS*, *LoxD*, and *LE* mRNA is *Def1* independent.

Discussion

Fatty acid hydroperoxides derived from lipoxygenase are precursors for an array of oxylipins that function in diverse aspects of plant growth and development. In this paper we report the isolation and characterization of tomato cDNAs encoding AOS and HPL, two similar P450 enzymes that commit 13-HPOT to different branches of oxylipin metabolism. The *LeAOS* and *LeHPL* proteins are 36% identical at the amino acid level, and are classified as members of the CYP74A and CYP74B subfamilies of Cyt P450s, respectively. Identification of *AOS* and *HPL* genes in tomato brings the total number of reported AOS and HPL sequences to seven. In comparing the primary structures of these, we noted 39 positions at which all AOS members contained one common amino acid and all HPLs contained a different residue. The significance of these subfamily-specific substitutions will become more or less apparent as additional CYP74 genes are identified. Subfamily-specific substitutions might reflect differences in the catalytic properties or substrate specificity of the two classes of enzymes. The facile expression of recombinant forms of AOS and HPL in *E. coli* should facilitate studies aimed at understanding the structure-function relationship that defines the catalytic identity of these unusual P450s.

A major difference between the predicted amino acid sequence of *LeAOS* and *LeHPL* was the presence of a typical chloroplast targeting sequence at the N-terminus of LeAOS (Figure 6-2). Previous studies indicate that chloroplast targeting peptides are present on AOS from flax and Arabidopsis (Song et al., 1993; Harms et al., 1995; Laudert et al., 1996), as well as HPL from Arabidopsis (Bate et al., 1998). A plastid location for AOS and HPL is consistent with biochemical studies demonstrating that

AOS and HPL activity is associated with chloroplasts (Vick and Zimmerman, 1987; Gardner et al., 1991; Blée and Joyard, 1996; Zhuang et al., 1996). Recently, we have shown that LeAOS is imported into chloroplasts where it specifically targets to the inner membrane of the chloroplast envelope (Froehlich et al., 1999). This finding indicates that LeAOS obtains its hydroperoxide substrates from one or both of the plastid-localized lipoxygenases (TomLoxC and TomLoxD) that have been described in tomato (Heitz et al., 1997). In contrast to LeAOS, the deduced N-terminus of LeHPL lacked a typical transit peptide. That the N-terminal sequence of LeHPL is very similar to that of bell pepper HPL (Figure 6-2) indicates that these proteins share a similar subcellular location. Given the preponderance of evidence indicating that HPL activity is associated with the chloroplast, additional experiments aimed at determining the subcellular location of LeHPL are clearly warranted.

Characterization of *LeAOS*

Expression of *LeAOS* in *E. coli* showed that the open reading frame encodes an authentic CYP74A enzyme (LeAOS) that metabolizes 13- but not 9-hydroperoxides of linoleic and linolenic acids. *LeAOS* expression was detected in all organs of the plant except mature red fruit. Similar expression patterns were observed for *AOS* in Arabidopsis and flax (Harms et al., 1998; Laudert and Weiler, 1998). Accumulation of *LeAOS* mRNA and protein in flowers is consistent with previous studies in Arabidopsis showing that *AOS* promoter activity is high in flowers, particularly in pollen sacs and pollen grains (Kubigsteltig et al., 1999). It is presently not known whether AOS-derived products are required for pollen development in tomato, as they are in Arabidopsis

(McConn and Browse, 1996). Detection of *LeAOS* mRNA and protein in leaves supports previous reports of AOS activity (Caldelari and Farmer, 1997) and inducible JA synthesis in tomato leaves (Peña-Cortés et al., 1993; Doares et al., 1995; Conconi et al., 1996). The relatively high accumulation of *LeAOS* mRNA in stems (Figure 6-5) compared to that in leaves indicates that *LeAOS* is expressed in vascular bundles, as was reported to be the case in wounded leaves of Arabidopsis (Kubigsteltig et al., 1999). *LeAOS* mRNA and protein were also detected in tomato roots. Given that root development appears normal in JA-deficient mutants of Arabidopsis (McConn and Browse, 1996), this result indicates that AOS-derived oxylipins serve a non-developmental role in roots. *LeAOS* mRNA expression in green fruit, while being relatively low, is consistent with previous studies showing increased levels of *cis*-JA during the early stages of tomato fruit ripening (Fan et al., 1998). However, our results do not exclude the possibility that JA synthesis in tomato fruit involves a different AOS-encoding gene that is undetectable by high stringency nucleic acid hybridization.

Characterization of *LeHPL*

Expression of *LeHPL* in *E. coli* confirmed that this cDNA encodes a functional member of the CYP74B subfamily of enzymes. LeHPL was similar to LeAOS in its ability to use 13- but not 9-hydroperoxides of C_{18} fatty acids. However, LeHPL was clearly distinguishable from LeAOS in its strong preference for 13-HPOT over 13-HPOD. This feature is shared by HPL isolated from other sources, including bell pepper (Shibata et al., 1995b), tea leaves (Matsui et al., 1991), and Arabidopsis (Bate et al., 1998). Fauconnier et al. (1997) reported the purification from tomato leaves of an HPL that,

like recombinant LeHPL, did not utilize 9-hydroperoxides and showed a strong preference for 13-HPOT over 13-HPOD. However, the purified enzyme displayed a molecular mass (73 kD) much greater than that predicted for LeHPL (53.5 kD). Additional experiments are needed to clarify the relationship between LeHPL and this purified form of tomato leaf HPL.

LeHPL mRNA was most abundant in developing flowers. This finding indicates that HPL-derived products might have a role in the production of floral scent. Relatively high levels of *LeHPL* mRNA were also detected in leaves. The overlapping expression pattern of *LeAOS* and *LeHPL* in leaves is consistent with the idea that these two enzymes compete for the same pool of substrate (Avdiushko et al., 1995; Blée and Joyard, 1996; Blée, 1998). However, additional studies aimed at determining the subcellular and tissue-specific location of both enzymes are needed to substantiate this hypothesis. The paucity of *LeHPL* mRNA accumulation in mature green and red fruit was surprising since *cis*-3-hexenal, derived from the action of HPL on 13-HPOT, is a prominent volatile component of the aroma and flavor of tomato fruit (Buttery and Ling, 1993). A possible explanation for these results is that *LeHPL* plays only a minor, if any, role in the production of C_6 volatiles during tomato fruit ripening. Additional insight into the contribution of *LeHPL* to fruit aroma and flavor might be gained by altering *LeHPL* expression in transgenic plants.

Wound-inducible expression of *LeAOS* is mediated by a *Def1*-independent signaling pathway

Damage inflicted to tomato leaves by hornworm larvae triggered the accumulation of *LeAOS* mRNA both in the damaged leaf and in the upper undamaged leaves of the plant (Figure 6-7). Similar changes in *LeAOS* expression occurred in plants subjected to mechanical wounding (Figure 6-8; data not shown). The transient accumulation of *LeAOS* transcripts in these experiments was a consequence of the limited damage inflicted to the plant (e.g. 5-10 min of feeding by the insect). It is likely that sustained feeding by herbivores, such as that occurring in natural and agricultural ecosystems, would result in much greater increases in *LeAOS* expression. Increased expression of AOS, and possibly other octadecanoid pathway enzymes, could serve to amplify the JA signaling cascade as a means of enhancing the induced resistance response. Wound-inducible increases in *AOS* mRNA, protein, and activity have been documented in Arabidopsis (Laudert and Weiler, 1998; Kubigsteltig et al., 1999) and flax (Harms et al., 1998). *LeHPL* transcript levels also appeared to increase in response to insect attack (Figure 6-7) and mechanical wounding (data not shown), but only by about 2- fold relative to unwounded controls. Additional studies are needed to determine whether the expression of *LeHPL* and *LeAOS* in tomato leaves is affected by other defense signals, or by interactions with pathogens.

The wound-inducible expression pattern of *LeAOS* differed in several respects from that of proteinase inhibitor genes. First, the time course of *LeAOS* mRNA accumulation was more transient than that of the inhibitor genes. Second, the amplitude of *LeAOS* mRNA accumulation in both damaged and systemic leaves was 10- to 20-

fold lower than that of *Inh-II* mRNAs. Finally, wound-inducible expression of *LeAOS* was observed in the *def1* mutant, while that of the *Inh-II* and *CDI* genes was not. Two additional genes, *LoxD* and *LE*, were also wound inducible in the *def1* background. These results demonstrate the existence of two classes of genes whose requirements for wound induction in tomato can be defined as being either *Def1*-dependent or *Def1*-independent. Given the involvement of *Def1* in wound-inducible JA accumulation (Howe et al., 1996), we indicate that endogenous JA is a signal for *Def1*-dependent, but not *Def1*-independent, wound responses. It is noteworthy that some genes exhibiting *Def1*-independent expression, such as *LeAOS* and *LoxD*, are inducible by exogenous JA (Heitz et al., 1997; G.I. Lee and G.A. Howe, unpublished data). This raises the possibility that genes whose expression is altered by exogenous JA might not be under the control of the JA signaling pathway as it operates *in planta*. Alternatively, *LeAOS* and *LoxD* may be controlled by both JA-dependent and -independent wound-response pathways. This interpretation is consistent with the observation that wound-induced accumulation of *LeAOS* and *LoxD* mRNA in *def1* plants was slightly less than that in wild-type plants (Figure 6-8). Regulation of AOS and LOX activities by both JA-dependent and -independent signaling pathways might allow amplification or increased sensitization of wound responsiveness under different conditions. This notion is consistent with other studies showing that wound and defense responses in tomato involve multiple signaling pathways (Chao et al., 1999; Ryan, 2000). In Arabidopsis, JA-dependent and -independent wound responses have been shown to be differentially regulated by Ca^{2+}/calmodulin, as well as by reversible protein phosphorylation events (Titarenko et al., 1997; León et al., 1998; Rojo et al., 1998). Thorough analysis of

mutants such as *def1* or those that are suppressed in the action of systemin (Howe and Ryan, 1999) may provide further insight into the role of oxylipins in wound and defense signaling pathways in tomato.

Materials and Methods

Plant Material and Growth Conditions

cv Micro-Tom seed (*Lycopersicon esculentum* cv Micro-Tom) was obtained from Dr. Avraham Levy (Weizmann Institute, Rehovot, Israel). Seed for the tomato *def1* mutant was collected from a *def1/def1* homozygous line that had been back-crossed four times to *L. esculentum* cv Castlemart, the wild-type parent of *def1*. Seedlings were grown in Jiffy peat pots (Hummert International, St. Louis) in a growth chamber maintained under 17 hr of light (300 $\mu E\ m^2\ s^{-1}$) at 28°C and 7 hr of dark at 18°C. Flowers and fruit were collected from plants maintained in a greenhouse.

cDNA Cloning and Sequencing

A 1.1-kb *Xho*I fragment derived from the coding region of an Arabidopsis AOS cDNA (Laudert et al., 1996) was labeled with [α-^{32}P]dCTP and used to screen a tomato cDNA library constructed from tomato plants that overexpress the prosystemin gene, as described by Heitz et al. (1997). Duplicate filters were hybridized at 42°C in a solution containing 5× SSPE, 50% (v/v) formamide, 5× Denhardt's reagent, 0.5% (w/v) SDS, and 50 μg/mL denatured salmon sperm DNA. Filters were washed at 42°C in a solution containing 5× SSPE and 0.5% (w/v) SDS, followed by an additional wash at 65°C. Four

positive clones were obtained among approximately 4×10^5 plaque-forming units screened. Following excision of the cDNA from the phagmid, DNA sequence analysis showed that all clones were identical with the exception of minor differences in the length of the 5' end. The longest cDNA insert, designated *LeAOS*, was sequenced completely on both strands using a primer walking approach. The sequence of the *LeAOS* cDNA was deposited to GenBank (accession no. AF230371). A bell pepper (*Capsicum annum*) cDNA encoding HPL was isolated using a reverse-transcription PCR (RT-PCR) kit (Life Technologies/Gibco-BRL, Cleveland) and total RNA isolated from bell pepper as a template. Two gene-specific primers were designed from the cDNA sequence reported by Matsui et al. (1996) (GenBank accession no. U51674). The sequence of the forward and reverse primers used for RT-PCR was 5'-(GTG-GAT-CCA-TTC-ATA-AAA-CAA-CAA-CTA-C)-3' and 5'-(GTG-AAT-TCA-GCA-ACC-TTT-AGT-ACC-TAC-C)-3', respectively. An amplified 1,477-bp product was subcloned into pBluescript SK(-) (Stratagene, La Jolla, CA) and sequenced to confirm its identity to the published sequence (Matsui et al., 1996). This clone was used to screen a tomato leaf cDNA library as described above but with the following modifications. Filters were washed at 65°C in a solution containing 5× SSPE and 0.5% (w/v) SDS. Fifteen positive plaques were identified among 3×10^5 plaque-forming units screened. DNA sequence analysis of eight cDNA inserts showed that all cDNAs were identical except for minor differences in the length of the 5' end and the number of poly-(A) residues at the 3' end. The longest clone, designated *LeHPL*, was subcloned into smaller fragments and sequenced in its entirety on both strands. The sequence of the *LeHPL* cDNA was deposited to GenBank (accession no. AF230372). A RACE

procedure (Life Technologies/Gibco-BRL, Cleveland) was used to obtain additional sequence information at the 5' end of *LeHPL*. First strand cDNA was synthesized from total RNA prepared from either tomato leaves or flowers as a template. The sequence of the gene-specific primer used for this reaction was 5'-(ACT-TCC-TTG-GCT-TCA-TTT-T)-3'. PCR amplification of the dC-tailed cDNA was performed using the manufacturer's abridged anchor primer and a gene-specific primer having the sequence 5'-(AGC-GCC-GAG-GAT-AGT-GAG-GGA-GAA)-3'. PCR products were re-amplified using the manufacturer's abridged universal amplification primer and a nested gene-specific primer having the sequence: 5'-(TGG-AGT-GCA-GGA-AGA-AGA-GAA-G)-3'. Amplified PCR products were subcloned into pBluescript SK(-). DNA sequencing of 5' RACE products derived from both leaf and flower mRNA confirmed the structure of the 5'-UTR of *LeHPL*, including the presence of the in-frame stop codon upstream of the initiator Met.

Expression of *LeAOS* and *LeHPL* in *Escherichia coli*

A PCR strategy was employed to subclone the *LeAOS* cDNA into the *E. coli* expression vector pQE-30 (Qiagen USA, Valencia, CA). Forward and reverse primers were designed to contain *Bgl*II and *Pst*I restriction sites, respectively. The sequence of the forward primer was 5'-(GCT-AGA-TCT-CCT-ATA-AAA-TTA-TCT-ACC-AGG)-3' and that of the reverse primer 5'-(GTT-CTG-CAG-CCG-ATA-GTG-ACA-GTG-TAG-ACC)-3'. Using the *LeAOS* cDNA as a template, the PCR-amplified product was cut with *Bgl*II and *Pst*I and cloned into *Bam*HI and *Pst*I sites of pQE-30. The resulting expression vector was called pQE-AOS. This strategy removed the first 42 amino acids

from the N terminus of *LeAOS*, and added the sequence MRGSHHHHHHGS to Pro 43 of *LeAOS* (Figure 6-2). A similar strategy was used to construct a vector for expression of *LeHPL*. Forward and reverse primers were designed to contain *Bam*HI and *Sst*I sites, respectively. The sequence of the forward primer was 5'-(CGG-GAT-CCC-CGA-TAA-TGA-ATT-CTG-CTC)-3' and that of the reverse primer 5'-(GCG-AGC-TCT-CAT-AAG-TCA-GAA-CAG)-3'. PCR products obtained using the *LeHPL* cDNA as a template were digested with *Bam*HI and *Sst*I, and cloned into *Bam*HI and *Sac*I sites of pQE-30 to give pQE-HPL. This strategy added the sequence MRGSHHHHHHGSPI to the deduced initiator Met of *LeHPL*.

Expression constructs pQE-AOS and pQE-HPL were transformed into *E. coli* strain M15. Bacteria grown under standard conditions (37°C in Luria-Bertani medium) and induced with isopropylthio-β-galactoside produced recombinant protein that was associated with inclusion bodies (data not shown). Induction of cultures using the following procedure significantly enhanced the recovery of active enzyme in the soluble fraction of lysed cells. Bacterial cultures (50 mL) were grown in Terrific Broth medium at 37°C to logarithmic phase (A_{600} of 0.6), at which time the culture was induced by the addition of isopropylthio-β-galactoside to a final concentration of 0.5 mM. Cultures were incubated for an additional 8 hr at 26°C with gentle shaking (150 rpm). Bacteria were harvested by centrifugation and resuspended in 5 mL of a solution containing 50 mM potassium phosphate (pH 7.5) and 5% (v/v) glycerol. Following one freeze-thaw cycle, cells were broken by sonication and centrifuged at 10,000g for 20 min. SDS-PAGE analysis of supernatant protein from induced culture extracts showed the

presence of the recombinant protein, migrating with the expected molecular mass (data not shown).

Enzyme Assays and Preparation of Fatty Acid Hydroperoxides

The hydroperoxide-degrading activity of recombinant LeAOS and LeHPL was measured spectrophotometrically using two methods described by Vick (1991). One assay, which does not distinguish between AOS and HPL activity, involved monitoring the decrease in A_{234} that results from disruption of the conjugated diene bond in the substrate. The second method was specific for HPL and involved an NADH-coupled assay for detection of aldehyde reaction products. The protein content of cell extracts was determined by the Bradford assay. Fatty acid hydroperoxide substrates (9- and 13-substituted) were prepared using soybean lipoxygenase (Sigma-Aldrich, St. Louis) or corn seed lipoxygenase as described (Vick, 1991). Fatty acids for these reactions were obtained from Nu-Chek-Prep, Inc (Elysian, MN). Substrate specificity results were confirmed using purified hydroperoxides (9-HPOD, 9-HPOT, 13-HPOD, 13-HPOT) purchased from Cayman Chemical.

Identification of Metabolites

Two micromoles of 13-HPOT, dissolved in 30 mL of 50 mM potassium phosphate (pH 7.0), was mixed with 1 mg of soluble protein (enzyme source) obtained from *E. coli* cells expressing either pQE-AOS, pQE-HPL, or the pQE-30 vector control. The reaction was allowed to proceed for 10 min at room temperature and then stopped by acidification to pH 4.0 with 1 M citrate. Products were extracted twice with diethyl

ether and dried under N_2 gas. TMS derivatives of pQE-AOS reaction products were prepared by treatment of the extract with 30 μL of BSTFA (bis[TMS] trifluoroacetamide/trimethylchlorosilane) (99:1, v/v) (Supelco, Bellefonte, PA) and 10 μL of pyridine for 1 hr at 80°C. Oxime TMS derivatives of pQE-HPL products were prepared by first reacting enzyme products with hydroxylamine hydrochloride at 80°C for 1 hr, followed by treatment with BSTFA and pyridine as described above. One to 2 μL of the derivatized compounds was used for GC-MS analyses, which were carried out on an AX 505H double focusing mass spectrometer (JEOL, Peabody, MA) equipped with a 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA). GC separations employed a DB-1 methyl silicone capillary column (30 m × 0.25 mm i.d.) (J&W Scientific, Folsom, CA) interfaced directly to the ion source via a heated transfer block. The temperature program was initiated at 50°C and ramped to 225°C at 20°C min^{-1}. The temperature was then increased to 270°C at 2°C min^{-1}. The ion source was operated at 70 eV with the scan rate of the instrument set to approximately 1 spectrum s^{-1}.

Antibody Production and Western-Blot Analysis

A 0.5-L culture of *E. coli* was induced for the expression of pQE-AOS as described above, except that induced cells were grown at 37°C for 4 hr in Luria-Bertani medium. Cells were harvested by centrifugation and resuspended in 1/10 volume of lysis buffer (50 mM sodium-phosphate, pH 8.0, 10 mM imidazole, 300 mM NaCl, and 0.25% [v/v] Emulgen 911 [Kayo Corporation, Tokyo]). Following one freeze-thaw cycle, cells were broken by two passes through a French press calibrated at 17,000 pounds per square inch. Insoluble inclusion bodies containing LeAOS were recovered by centrifugation

for 20 min at 8,000g. The pellet was washed twice with 40 mL of lysis buffer and recovered by centrifugation. Washed pellets were solubilized at 4°C in 35 mL of a solution containing 6 M guanidine HCl, 100 mM sodium-phosphate, pH 8.0, and 10 mM Tris (tris [hydroxymethyl]aminomethane) HCl, pH 8.0. The mixture was sonicated for 10 min at 4°C to facilitate solubilization, and then centrifuged at 12,000g for 20 min. Recombinant LeAOS in the supernatant was purified by nickel affinity chromatography as described by the manufacturer (Qiagen). His-tagged LeAOS, which eluted from the nickel column at pH 4.5, was dialyzed twice for 4 hr against 100 volumes of 50 mM Tris, and 0.2% (v/v) Emulgen 911. Approximately 0.5 mg of protein was solubilized in Laemmli sample buffer and further purified by preparative SDS-PAGE (12% [w/v] gel). Acrylamide gel slices containing His-tagged LeAOS were stained with Coomassie Brilliant Blue and macerated through a syringe as described (Harlowe and Lane, 1988). For the initial immunization, 100 μg of antigen in the mashed gel slice was mixed with Freund's complete adjuvant and injected at multiple subcutaneous and intramuscular sites of a New Zealand white rabbit. Four boosts, each consisting of 50 μg of antigen mixed with Freund's incomplete adjuvant, were administered over the course of a 90-d immunization schedule.

Protein extracts for western-blot analysis were prepared from fresh plant tissue that was extracted with a mortar and pestle at 4°C in a buffer containing 50 mM sodium-phosphate, pH 7.0. The buffer to tissue ratio (w/w) was about 2:1. Crude cellular debris was removed by centrifugation at 2,000g for 10 min. The membrane fraction of the resulting supernatant was recovered by centrifugation at 100,000g for 15 min at 2°C. Pelleted membranes were washed with 1 M NaCl, and recovered by

centrifugation as described above. Membrane material equivalent to 15 μg of total protein was solubilized in Laemmli sample buffer, boiled for 5 min, and separated by SDS-PAGE (10% [w/v] gels). Separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA) in a solution consisting of 25 mM Tris, 192 mM Gly, and 20% (v/v) methanol, using standard procedures (Harlowe and Lane, 1988). Membranes were probed with anti-LeAOS antibodies used at a 1:2,000 dilution in a Tris-buffered saline solution containing 1% (w/v) bovine serum albumin as a blocking agent and 0.05% (w/v) Tween 20 to reduce non-specific binding. Antigen-antibody complexes were detected with the use of an alkaline phosphatase-conjugated second antibody as described by the manufacturer (Kirkegaard and Perry, Gaithersburg, MD).

Southern-Blot Analysis

Genomic DNA from young leaves of cv Micro-Tom plants was purified as described by Rogers and Bendich (1985). Ten-microgram aliquots of DNA were digested with restriction enzymes, electrophoresed on a 0.8% (w/v) agarose gel, and blotted to Duralon-UV membranes (Stratagene) as indicated by the manufacturer. Blots were pre-hybridized at 65°C in a solution containing 5× SSPE, 5× Denhardt's solution, 100 μg/mL denatured salmon sperm DNA, and 0.5% (w/v) SDS. Blots were hybridized at 65°C and washed at the same temperature in a solution containing 0.5× SSPE and 0.5% (w/v) SDS. DNA probes were prepared using a T7 Quickprime Kit (Pharmacia Biotech, Piscataway, NJ). The following cDNA fragments were labeled for use as probes: a 1.7-kb *Eco*RI-*Xho*I fragment containing full-length *LeAOS*; a 1.4-kb *Eco*RI-*Hind*III

fragment containing the coding region of *LeHPL*; and a 0.2-kb *Eco*RI-*Eco*RI fragment containing the *LeHPL* 5'-UTR.

Wounding Experiments

Manducta sexta larvae were reared on artificial diet as described by the vendor (Carolina Biological Supply, Burlington, NC) from which the eggs were purchased. One larva (third instar) was placed on the terminal leaflet of the oldest leaf of a 3-week-old cv Micro-Tom plant that contained three fully expanded leaves. Larvae were allowed to feed on the leaf for 5 to 10 min, during which time 5% to 10% of the area of the leaf was consumed. Plants were sampled for RNA analysis at different times after the challenge. Leaf tissue from six to eight plants per time point was pooled prior to RNA extraction. Mechanical wounding of plants was performed using a hemostat as described previously (Howe et al., 1996).

RNA Gel Blot Analysis

Total RNA was isolated from tomato tissue and analyzed by RNA-blot hybridization as previously described (Howe et al., 1996), except that Duralon-UV membranes were used in place of nitrocellulose. All gels were run in duplicate, with one set stained with ethidium bromide to ensure equal loading of the samples and intactness of the RNA. Hybridization signals were visualized by autoradiography using Kodak XAR-5 film, or were measured using a Phosphorimager (Molecular Dynamics). These signals were normalized to signals obtained using a probe for translation initiation factor *eIF4A* mRNA (Taylor et al., 1993). Hybridization and subsequent washing of *eIF4A*-probed

blots was performed at 60°C in 2× SSPE. DNA probes were prepared as described above.

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Chapter 7

Conclusions and future perspectives

The systemic wound response of tomato is an important defense mechanism against insect herbivores. The discovery of systemin provided a breakthrough in our understanding of how local injury induces systemic expression of defense genes such as *Proteinase Inhibitors* (*PIs*; McGurl et al., 1992: Pearce et al., 1991). Manipulation of prosystemin expression in transgenic plants demonstrated that this polypeptide signal is an essential regulator of systemic *PI* expression (McGurl et al., 1992; McGurl et al., 1994). Subsequent studies showed that systemin induces *PI* expression by activating the octadecanoid pathway for jasmonic acid (JA) biosynthesis (Doares et al., 1995; Howe et al., 1996).

At the outset of this thesis research, a wealth of circumstantial evidence indicated that systemin might function as a long-distance signal for *PI* expression (Ryan and Pearce, 1998). To investigate this hypothesis, I characterized tomato mutants that are defective in the systemic wound response. Systemin-insensitive *spr1* plants were previously isolated as *suppressor of prosystemin-mediated responses* (*spr*), and were shown to be deficient in wound-induced systemic *PI* expression (Howe and Ryan, 1999). As described in Chapter 2, systemin failed to induce JA biosynthesis and subsequent expression of *PIs* in *spr1* plants (Figure 2-5; Figure 2-6). In contrast, application of oligogalacturonic acid (OGA), which is regarded as a local wound signal, resulted in normal *PI* expression in *spr1* plants (Figure 2-3; Figure 2-4). These results indicate that the *spr1* mutation defines the systemin-dependent pathway of systemic wound signaling, and further indicate that the *Spr1* gene product plays a role in coupling systemin perception at the plasma membrane to the initiation of JA biosynthesis in the chloroplast (Figure 7-1).

Reciprocal grafting experiments between wild-type and *spr1* plants were used to investigate how systemin regulates systemic *PI* expression (Figure 2-7). Whereas *spr1* plants were impaired in the generation of the systemic wound signal in wounded leaves, the mutant was able to perceive that signal in undamaged leaves. Because *Spr1* is required for the recognition of systemin, these results indicate that systemin is likely not the long-distance signal for *PI* expression. Systemin appears to act at or near the site of wounding (i.e. in rootstock tissues) to increase JA synthesis to a level that is required for the systemic response. Consistent with this interpretation, grafting experiments using JA-insensitive and JA-deficient mutants indicated that JA or a derivative of JA is the systemic wound signal (Li et al., 2002b). These experiments demonstrated that JA biosynthesis is required for the generation of the systemic wound signal, and that JA perception is essential for recognition of this signal in unwounded leaves. This finding supports the idea that systemin amplifies the systemic wound signal in damaged tissue by stimulating JA biosynthesis.

The role of JA in systemic wound signaling was investigated further by characterization of JA-deficient JL1 plants. Gas chromatography-mass spectrometry (GC-MS) analysis showed that JL1 plants are compromised in the conversion of 12-oxo-phytodienoic acid (OPDA) to JA (Figure 3-2). OPDA application experiments showed that this octadecanoid pathway intermediate is not an active signal for *PI* expression in tomato (Figure 3-3). Reciprocal grafting experiments with wild-type and JL1 plants confirmed that JA biosynthesis is required for the generation of the long-distance signal for *PI* expression (Figure 3-4).

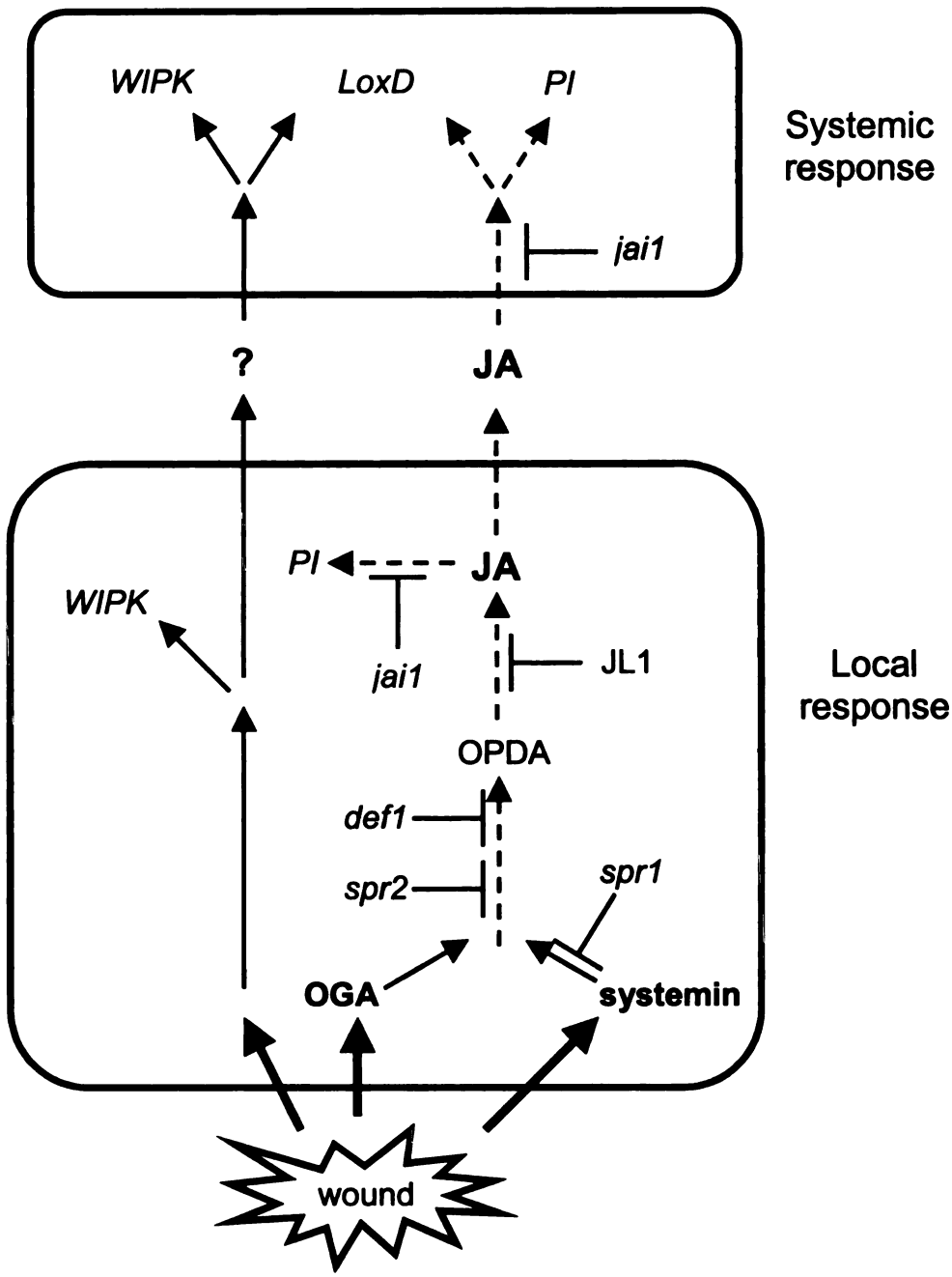

Figure 7-1. Revised model of systemic wound signaling in tomato. Wounding activates systemin/JA-dependent (broken arrows) and JA-independent signaling (solid arrows) pathways that regulate distinct sets of wound response genes in undamaged leaves (systemic response). See text for details.

It was indicated that two related cytochrome P450s, allene oxide synthase (AOS) and hydroperoxide lyase (HPL), regulate oxylipin levels in plants by competing for a common substrate, 13-hydroperoxy linolenic acid (13-HPOT; Bate et al., 1998; Blee and Joyard, 1996; Lauder et al., 1996). AOS and HPL metabolize 13-HPOT to JA and volatile C_6 aldehyde, respectively. To investigate the regulation of JA biosynthesis, genes encoding these two enzymes were cloned from tomato. Expression of these genes in *E. coli* confirmed that AOS and HPL efficiently metabolize the same substrate, 13-HPOT, but do not metabolize other hydroperoxy fatty acid substrates (Figure 6-4). Both genes are expressed in leaves, which supports the idea that AOS and HPL compete for the same substrate in wounded tissue (Figure 6-5).

Previous studies indicated that a JA-independent signaling pathway mediates some systemic wound responses in tomato (O'Donnell et al., 1998; Stratmann and Ryan, 1997). This idea is consistent with the observation that expression of *AOS* was up-regulated by wounding in both wild-type and JA-deficient *defenseless1* (*def1*) plants (Figure 6-7). In addition, early wound response genes such as *lipoxygenaseD* (*LoxD*) were systemically expressed upon wounding of *spr1* plants, which indicates that a systemin-independent signaling pathway is involved in this systemic response (Figure 2-2; Figure 2-5). Wounding activated the systemic expression of a mitogen-activated protein kinase (MAPK) gene homologue (*WIPK)* in JA-deficient and JA-insensitive mutants. This observation indicates the existence of a JA-independent signaling. Expression of *LoxD* was determined to be under regulation by both JA-independent and -dependent signaling pathways.

Based on the findings from this thesis study, a model of systemic wound signaling in tomato is proposed (Figure 7-1). In this model, wounding activates two distinct signaling pathways for systemic gene expression. One is the JA-dependent signaling pathway, which requires the action of *Spr1, Spr2, Def1,* and *Jai1*. β-oxidation is involved in this pathway, which is compromised in JL1 plants. This pathway regulates systemic expression of late genes such as *PIs*. JA or a derivative of JA that is transported by the phloem may be the long-distance signal in this pathway. Systemin promotes the synthesis of JA at or near the site of wounding. The other systemic wound signaling pathway operates independently from JA and systemin, and is poorly understood. A subset of early wound response genes is regulated by this signaling pathway. The JA-independent pathway also regulates the systemic wound induction of a MAPK. In this case, the long-distance signal appears to be xylem-borne (Stratmann and Ryan, 1997).

Further biochemical studies are needed to determine whether JA or one of its derivatives is the long-distance signal of systemic *PI* expression. Because the systemic wound signal for *PI* expression moves though the phloem (Nelson et al., 1983), the relative level of the endogenous signal in phloem is expected to rapidly increase in response to wounding. Measurement of JA and related oxylipins in phloem sap will be useful to address this question. In addition, application of radiolabeled JA to the wound site may be helpful to monitor the movement of JA *in vivo*.

As an initial step to identifying the *Spr1* gene by map-based cloning, a mapping population was generated by crossing the *spr1* mutant to the wild tomato species *Lycopersicon pennellii* (Appendix of Chapter 2). Bulk segregant analysis and amplified

fragment length polymorphism (AFLP) analysis would be useful to find molecular markers closely linked to the *Spr1* locus. A similar approach was used to map the chromosomal location of *Def1* and *Spr2* (Li et al., 2003; Li et al., 2002a). Molecular cloning of the *Spr1* gene will provide insight into how the binding of systemin to the SR160 receptor on the plasma membrane activates JA biosynthesis in chloroplasts (Scheer and Ryan, 2002).

Map-based cloning of the gene defined by the JL1 mutant line is in progress in the Howe lab (C. Li and G. Howe, unpublished results). This gene product is presumably involved in β-oxidation, which is required for both degradation of fatty acids and conversion of OPDA to JA. The cloning of this gene will extend our understanding of how the conversion of OPDA to JA regulates endogenous JA levels. OPDA itself is not an active signal for wound-induction of *PI-II*, which is contrary to the finding that OPDA regulates expression of defense genes in Arabidopsis (Stintzi et al., 2001). Microarray experiments with JL1 plants will be helpful to investigate the putative role of OPDA as a signal for gene expression in tomato plants.

In vitro import assays have established that AOS is targeted to the inner envelope membrane of chloroplasts, whereas HPL is targeted to the outer envelope membrane (Froehlich et al., 2001). This finding further supports the possibility that the two enzymes compete for the same substrate located in the envelope membrane. To further address this hypothesis, it will be useful to determine whether the two enzymes co-localize in the same cell and the same chloroplast.

Microarray experiments using JA-deficient and JA-insensitive mutants would be helpful to identify wound-inducible genes that are regulated by the JA-independent

signaling pathway. It may also be informative to examine the expression of *WIPK* using steam-girdled tomato plants in which the xylem but not the phloem is intact. This experiment would determine whether the JA-independent signal for *WIPK* expression is phloem-borne or xylem-borne.

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