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An Octadecanoid Pathway Mutant (JL5) of Tomato Is Compromised in Signaling for Defense against Insect Attack

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The activation of defense genes in tomato plants has been shown to be mediated by an octadecanoic acid–based signaling pathway in response to herbivore attack or other mechanical wounding. We report here that a tomato mutant (JL5) deficient in the activation of wound-inducible defense genes is also compromised in resistance toward the lepidopteran predator *Manduca sexta* (tobacco hornworm). Thus, we propose the name *defenseless1* (*def1*) for the mutation in the JL5 line that mediates this altered defense response. In experiments designed to define the normal function of DEF1, we found that *def1* plants are defective in defense gene signaling initiated by prosystemin overexpression in transgenic plants as well as by oligosaccharide (chitosan and polygalacturonide) and polypeptide (systemin) elicitors. Supplementation of plants through their cut stems with intermediates of the octadecanoid pathway indicates that *def1* plants are affected in octadecanoid metabolism between the synthesis of hydroperoxylinolenic acid and 12-oxo-phytodienoic acid. Consistent with this defect, *def1* plants are also compromised in their ability to accumulate jasmonic acid, the end product of the pathway, in response to wounding and the aforementioned elicitors. Taken together, these results show that octadecanoid metabolism plays an essential role in the transduction of upstream wound signals to the activation of antiherbivore plant defenses.

INTRODUCTION

The activation of defense genes in plants is triggered by signals generated at the site of insect or pathogen attacks. One well-characterized class of signaling molecules consists of oligosaccharide fragments, derived from enzymatic breakdown of either host plant or fungal cell walls during the initial stages of pest invasion (Darvill and Albersheim, 1984). Oligosaccharide fragments are released from leaf tissue by the action of lytic enzymes produced by invading pathogens (Hahn et al., 1981). The fragments are effective signals in eliciting defense gene induction in tissues adjacent to sites of pathogen ingression (Darvill and Albersheim, 1984). However, the inability of pectic oligosaccharides to be transported (basipetally) away from wound sites indicates that these substances are unlikely to be involved in long-distance signaling of defense responses (Baydoun and Fry, 1985; Aldington and Fry, 1996).

Defense genes are also induced by mechanical wounding inflicted by herbivores, both locally and in tissues far removed from the wound sites (Green and Ryan, 1972). Wounding of the lower leaves of several plant species results in the accumulation of defense proteins in wounded leaves and in unwounded leaves throughout the plants (Green and Ryan, 1972; Brown et al., 1985; Bradshaw et al., 1989; Eckelkamp et al., 1993; Cordero et al., 1994). Recent studies have provided evidence that a good candidate for the mobile wound signal for systemic gene induction in tomato plants is an 18–amino acid polypeptide called systemin (Pearce et al., 1991; McGurl et al., 1992; Narváez-Vásquez et al., 1995; Schaller and Ryan, 1996). However, other long-distance wound signals have been proposed, including abscisic acid (Peña-Cortes et al., 1989) and electrochemical potentials (Wildon et al., 1992).

Elicitors of the wound response have been proposed to activate defense gene expression by triggering the synthesis of lipid-derived second messengers (Farmer and Ryan, 1992). According to this model, elicitor binding to a putative receptor effects the intracellular release of linolenic acid from membrane lipids, which in turn is converted to 12-oxo-phytodienoic acid (PDA) and then to jasmonic acid (JA) via the octadecanoid pathway (Vick and Zimmerman, 1984). These two cyclopentanones are potent activators of defense gene transcription (Farmer and Ryan, 1992; Blechert et al., 1995). This scenario is analogous to signaling pathways in animals in which the release of arachidonic acid in response to polypeptide hormones results in the synthesis of prostaglandins and leukotrienes (Needleman et al., 1986). Recent studies suggest that linoleic acid, as well as linolenic acid, may be a precursor to biologically active oxylipins in plants (Blechert et al., 1995) and that PDA as well as JA may be terminal signals for the pathway (Weiler et al., 1993; Blechert et al., 1995).

Several lines of evidence support a role for the octadecanoid pathway in the signaling of plant defenses. First, octadecanoid precursors (e.g., linolenic acid) are liberated from intracellular lipid pools after elicitor treatment of plant suspension cultures

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(Mueller et al., 1993) or mechanical wounding of tomato leaves (Conconi et al., 1996). Second, application of JA or its octadecanoid precursors activates defense gene expression, as best exemplified by the accumulation of proteinase inhibitors in tomato leaves (Farmer and Ryan, 1992) and the induction of phytoalexin compounds in *Eschscholtzia californica* cell cultures (Mueller et al., 1993). Third, accumulation of JA is triggered by numerous elicitors of the defense response, including mechanical wounding (Creelman et al., 1992; Peña-Cortes et al., 1993; Blechert et al., 1995; Doares et al., 1995a), fungal elicitors (Gundlach et al., 1992), systemin (Doares et al., 1995a), oligosaccharides (Doares et al., 1995a), abscisic acid (Peña-Cortes et al., 1995), and octadecanoic acids (Peña-Cortes et al., 1995). Fourth, chemical inhibitors of the octadecanoid pathway, such as salicylic acid (Peña-Cortes et al., 1993; Doares et al., 1995b) and diethyldithiocarbamate (Farmer et al., 1994), block the expression of proteinase inhibitor genes in response to primary elicitors of the wound response in tomato leaves. Finally, transgene-mediated suppression of an Arabidopsis lipoxygenase gene (encoding the first enzyme in the conversion of linolenic acid to JA) results in inhibition of JA accumulation and activation of vegetative storage protein gene in response to wounding (Bell et al., 1995).

Tomato provides a useful experimental system with which to study many aspects of the wound response pathway. Given the amenability of tomato to genetic analysis, the use of mutants offers a powerful approach to complement the predominantly biochemical studies that have been used to characterize the wound response pathway thus far. For example, abscisic acid–deficient mutants have been used to provide evidence for the involvement of this phytohormone in the wound response pathway (Peña-Cortes et al., 1989, 1995). Recently, a more systematic screen for mutations that specifically disrupt the wound response in tomato led to the identification of two nonallelic recessive mutants (JL1 and JL5) deficient in the accumulation of proteinase inhibitors I and II in response to wounding (Lightner et al., 1993). Because both mutants accumulate inhibitors when exposed to methyl jasmonate, it was suggested that they are affected in a signaling step between the wound event and the downstream production of JA; however, no attempts were made to localize further the lesions in the pathway. In our study, we demonstrate that the JL5 mutant is deficient in a gene product, designated DEFENSELESS1 (DEF1), that acts after lipoxygenase conversion of linolenic acid to hydroperoxylinolenic acid but before the conversion of PDA to JA. This function is shown to play a critical role in the octadecanoid signaling pathway and in the defense of plants against insect predation.

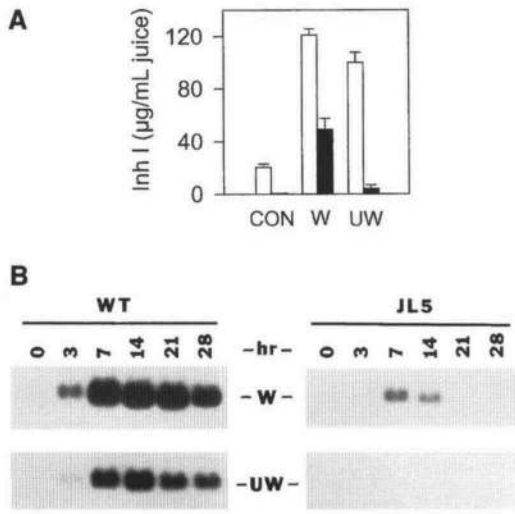

Figure 1. Proteinase Inhibitor I Protein and mRNA Levels in Response to Wounding.

Tomato seedlings (two-leaf stage) were wounded once across the midvein near the distal end of the lower leaf. Three hours later, a second wound was made parallel to the first wound, proximal to the petiole.
(A) Twenty-four hours after the initial wound, inhibitor I (Inh I) protein levels in the leaves of wild-type plants (open bars) and JL5 mutants (filled bars) were measured separately in the wounded leaf (W) and in the upper unwounded leaf (UW). As controls, inhibitor I levels were measured in the pooled upper and lower leaves of unwounded plants (CON). Data points are the means of 12 plants ± SE.
(B) Gel blot analysis of total RNA extracted separately from lower wounded (W) and upper unwounded (UW) leaves at various times (hr) after wounding is shown. Leaf tissue from eight plants was pooled for each RNA extraction to obtain an average response. RNA from wild-type (WT) plants and the mutants (JL5) was probed with the inhibitor I cDNA on the same blot and exposed to film for the same amount of time.

RESULTS

Temporal and Spatial Accumulation of Defense Gene Transcripts in Response to Wounding

We compared the wound response of JL5 to that of wild-type plants by using young (2-week-old) seedlings containing two fully expanded leaves. As shown in Figure 1A, wounding the lower leaf of wild-type plants caused the well-known accumulation of proteinase inhibitor I in both the wounded leaf and upper unwounded leaf. In contrast, JL5 plants accumulated reduced levels (40% of that of the wild type) of inhibitor I in the wounded leaf, and no accumulation of inhibitor I was observed in the upper unwounded leaf. Inhibitor I mRNA levels began to accumulate in wounded leaves of wild-type plants within 3 hr after wounding (Figure 1B), as previously found (Graham et al., 1986). Transcript levels reached a maximum 7 hr after wounding and then slowly declined during the remainder of the time course. A similar pattern of inhibitor I mRNA accumulation was observed in the unwounded leaf of wounded wild-type plants (Figure 1B). The amount of inhibitor I transcript in wounded leaves of the JL5 mutant was 5 to 10% of that in

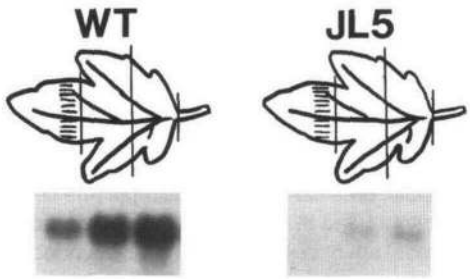

Figure 2. Accumulation of Proteinase Inhibitor I mRNA at Different Distances from the Wound Site.

A single wound (hatched line) was inflicted 0.5 cm from the tip of the lower leaf of wild-type (WT) plants and mutants (JL5). Eight hours later, the wounded leaf was dissected into three sections (as shown by the vertical lines) of ~1 cm each. Tissue segments from 12 plants were pooled before RNA extraction to obtain an average response. RNA prepared from pooled upper and lower leaves of unwounded plants did not contain detectable inhibitor I transcripts (data not shown). Gel blot analysis is as described in Figure 1.

the wild type (Figure 1B). The kinetics of this induction, however, paralleled that of wild-type plants with respect to both the onset of accumulation (3 hr) and the timing of maximal induction (7 hr). JL5 seedlings wounded on the lower leaves accumulated <5% of wild-type levels of inhibitor I mRNA in the upper unwounded leaf (Figure 1B).

The accumulation of low levels (5 to 10% of that of the wild type) of inhibitor I mRNA in wounded leaves of JL5 prompted us to examine the spatial pattern of inhibitor I transcript accumulation with respect to the wound site. As suggested previously (Lightner et al., 1993), it is possible that JL5 is defective in a systemic signaling pathway and that the residual signaling observed in the wounded leaf results from the action of "local" elicitors (e.g., oligosaccharide fragments) near the wound site. We tested this hypothesis by inflicting a single wound at the tip of a leaf and then by examining inhibitor I mRNA accumulation in the wounded section of the leaf or in sections further removed from the wound site. As shown in Figure 2, inhibitor I mRNA accumulation in JL5 is not restricted to cells close to the wound site. In fact, in both wild-type plants and mutants, the greatest accumulation of inhibitor I mRNA occurred in the leaf section farthest removed from the wound site. These data show that the residual wound signaling activity in JL5 is not specifically localized to tissue surrounding the wound site.

Proteinase inhibitor I belongs to a group of >15 defense-related proteins known as systemic wound response proteins (SWRPs; Schaller and Ryan, 1996). Because the accumulation of SWRPs is coordinately regulated by wounding (Hildmann et al., 1992; Constabel et al., 1995; Schaller and Ryan, 1996), it was of interest to examine the expression in JL5 of SWRP-encoding transcripts other than proteinase inhibitor I. As shown in Figure 3, transcripts encoding serine proteinase inhibitor II (Graham et al., 1985b), an aspartic acid proteinase inhibitor (cathepsin D inhibitor) (Hildmann et al., 1992), and leucine aminopeptidase (Hildmann et al., 1992; Pautot et al., 1993) accumulate to <10% of wild-type levels in both the wounded leaf and the unwounded leaf of wounded plants. In JL5 plants treated with JA, however, these mRNAs accumulated to wild-type levels, in agreement with the observed accumulation of proteinase inhibitor I and II protein in methyl jasmonate–treated JL5 plants (Lightner et al., 1993; data not shown). These results demonstrate that the JL5 mutant is blocked in a signaling step that precedes the transcriptional activation of numerous defense-related genes.

Susceptibility of Mutants to Insect Attack

The finding that JL5 plants are severely deficient in wound-inducible accumulation of numerous defense-related transcripts (Figure 3) suggested that these plants might also be compromised in their resistance to herbivorous insects. To test this hypothesis, newly hatched *Manduca sexta* (hornworm) larvae were placed on leaves of either 3-week-old (experiment 1) or 6-week-old (experiment 2) tomato plants to initiate the

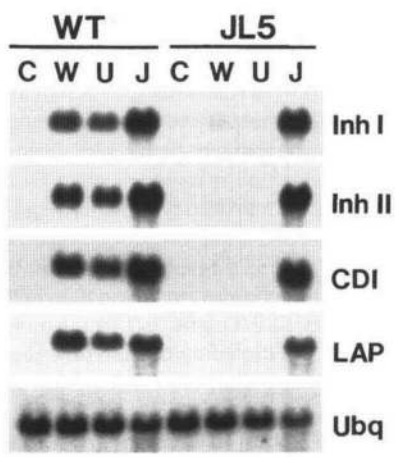

Figure 3. Accumulation of Defense-Related Transcripts in Response to Wounding and JA.

Wild-type (WT) plants and mutants (JL5) were wounded as described in Figure 1. RNA was extracted 7 hr after wounding from either the lower wounded (W) or upper unwounded (U) leaves. RNA was also prepared from unwounded control plants (C) and from plants treated for 10 hr with jasmonic acid (J) (10 nmol of JA applied to the upper leaf surface of each plant). Eight plants were pooled for each RNA extraction to obtain an average response. RNA gel blots were hybridized with cDNA probes for proteinase inhibitors I and II (Inh I and Inh II), cathepsin D inhibitor (CDI), leucine aminopeptidase (LAP), and ubiquitin (Ubq) as a loading control.

Table 1. Hornworm Feeding Trials with Wild-Type Plants and Mutants

Experiment[a]	Plant	Inh I (μg/mL)	Inh II (μg/mL)	Larval Weight (g)
1	Wild type	155 ± 24	190 ± 13	0.61 ± 0.10 (n = 15)
1	JL5	8 ± 1	8 ± 1	1.29 ± 0.09 (n = 15)
2	Wild type	257 ± 31	275 ± 37	1.75 ± 0.15 (n = 22)
2	JL5	26 ± 14	11 ± 2	5.01 ± 0.35 (n = 23)

[a] In experiment 1, 15 newly hatched larvae (~5 mg each) were placed randomly on the leaves of 29 tomato plants (3 weeks old) of each genotype grown together in the same flat. Larvae were allowed to move freely between plants of the same genotype. In experiment 2, six newly hatched larvae were placed on leaves of four separately potted plants (6 weeks old) of each genotype (larvae remained on the same plant for the duration of the trial). Experiments 1 and 2 were terminated 9 and 11 days, respectively, after the start of the trial, at which time inhibitor I (Inh I) and inhibitor II (Inh II) levels in the remaining leaf tissue and larval weights were measured. Data represent means ±SE. The detection limit of inhibitors I and II was 13 μg/mL.

feeding trial. Larval weights were determined when the foliage of JL5 plants was >90% consumed, as were the proteinase inhibitor I and II contents in the remaining leaf tissue. As shown in Table 1, the level of proteinase inhibitors accumulated in wounded leaves of JL5 plants at the end of the feeding trial was 10- to >25-fold less than that produced in wild-type plants. The average weights of larvae feeding on JL5 plants were approximately two (experiment 1) and three (experiment 2) times that of larvae feeding on wild-type plants. Moreover, it was readily apparent that the larvae consumed much more foliage from JL5 plants than they did from wild-type plants (Figure 4). These data indicate that the JL5 mutant is compromised in a natural defense response, and as a result, foliage from this plant is a better food source for hornworm larvae than is that of wild-type plants. We propose the name *def1* for the mutation in the JL5 line that mediates the compromised defense response.

Response of *def1* Plants to Elicitors and Octadecanoid Pathway Intermediates

To better define the position of the wound response pathway affected by the *def1* mutation, we examined the responsiveness of mutants to various elicitors of defense gene expression. Previously, it was shown that ectopic expression of prosystemin, the precursor of systemin (McGurl et al., 1992), in transgenic tomato plants results in the constitutive accumulation of high levels of inhibitor I and inhibitor II proteins in the absence of wounding (McGurl et al., 1994). Grafting experiments suggest that this phenotype is mediated by the continual generation of a systemic wound signal (McGurl et al., 1994). To investigate whether *def1* can block the constitutive activation of defense genes in these transgenic plants, we constructed (see Methods) a mutant line of tomato that is homozygous for both the cauliflower mosaic virus 35S promoter–expressed prosystemin (35S:PS) transgene that mediates prosystemin overproduction and the *def1* mutation. As shown in Figure 5A, several wound-inducible, defense-related transcripts (the serine proteinase inhibitors I and II, cathepsin D inhibitor, and leucine aminopeptidase) are highly expressed in unwounded 35S:PS plants but are not expressed in the 35S:PS *def1* mutant. This

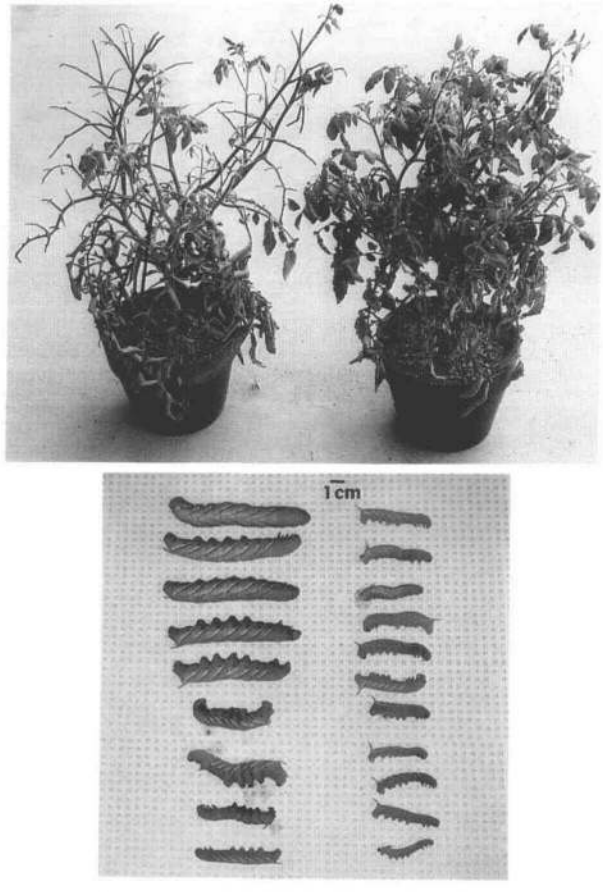

Figure 4. Effect of Feeding of Hornworm Larvae on Wild-Type Plants and Mutants.

Eight newly hatched larvae (~5 mg each) were placed on the leaves of each of four plants (8 weeks old) of each genotype. Larvae were allowed to feed for 13 days and remained on the same plant for the duration of the trial.

(Top) A representative wild-type plant (right) and JL5 mutant (left) at the end of the feeding trial.

(Bottom) Representative hornworm larvae recovered at the end of the trial from wild-type plants (right) and mutants (left). The average weight (in grams) of the larvae recovered from wild-type plants and mutants was 1.2 ± 0.1 and 5.3 ± 0.4, respectively.

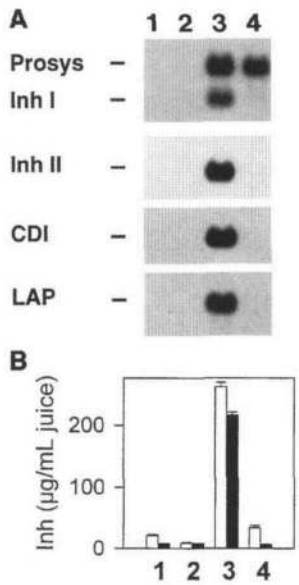

Figure 5. Suppression of Defense Gene Expression by *def1* in Plants Overproducing Prosystemin.

(A) Gel blot analysis of RNA prepared from unwounded leaf tissue of 15-day-old plants of the following genotypes: lane 2, *def1/def1*; lane 3, 35S:PS/35S:PS; and lane 4, 35S:PS/35S:PS *def1/def1*. Lane 1 contains RNA from the wild type. Blots were hybridized with cDNA probes for prosystemin (Prosys), proteinase inhibitors I and II (Inh I and Inh II), cathepsin D inhibitor (CDI), and leucine aminopeptidase (LAP), as described in Figure 1B.

(B) Proteinase inhibitor I (open bars) and II (filled bars) levels in leaves of unwounded, 15-day-old plants. Genotypes 1 to 4 are the same as those given in **(A)**. Data points are the means ±SE of 12 plants.

effect is not due to suppressed expression of the 35S:PS transgene, as shown by the high constitutive level of accumulation of prosystemin mRNA (Figure 5A) and protein (data not shown) in 35S:PS *def1* plants. We also observed that the high levels of proteinase inhibitor I and II protein accumulated in 35S:PS plants is reduced in 35S:PS *def1* plants to a level comparable to that seen in unwounded wild-type plants (Figure 5B). Thus, the constitutive activation of the defense gene program in prosystemin-overexpressing plants requires the wild-type *def1* gene product.

The induction of proteinase inhibitors in mutants in response to elicitors was examined by using (1) the 18–amino acid polypeptide systemin from tomato leaf cells, (2) polygalacturonic acid (PGA) derived from the tomato cell wall, and (3) chitosan oligomers derived from fungal cell walls. All three elicitors have been shown to induce the accumulation of inhibitor I and II proteins in wild-type plants in a dose-dependent manner. These compounds were largely ineffective in inducing the accumulation of inhibitor I (Figure 6) or inhibitor II protein (data not shown) in the mutants, although slight induction was observed when the elicitors were supplied at high concentrations.

The inability of the mutant to activate defense gene expression in response to prosystemin, systemin, PGA, and chitosan indicated that *def1* blocks the wound response pathway at a step downstream of the initial reception event mediated by these elicitors. Recent biochemical evidence (Doares et al., 1995a) showing that these elicitors, like mechanical wounding, activate defense genes via the octadecanoid pathway further suggested that the *def1* mutation affects a signaling step within this pathway. To investigate this possibility, we tested

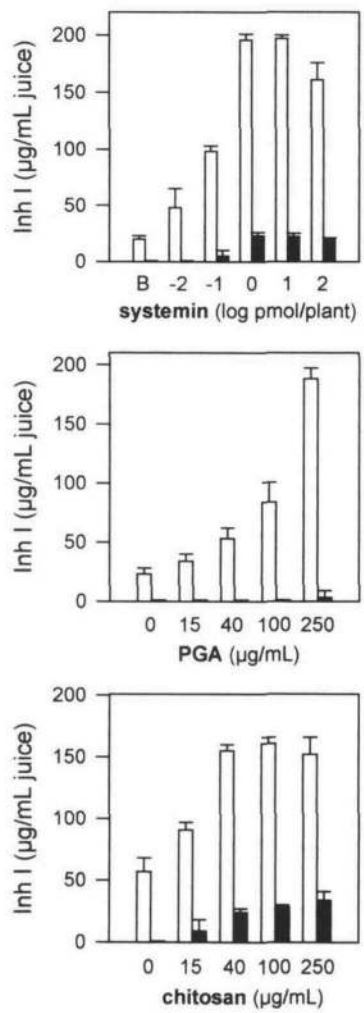

Figure 6. Accumulation of Proteinase Inhibitor I Protein in Response to Elicitors.

(Top) Systemin.
(Middle) PGA.
(Bottom) Chitosan.
Elicitors were supplied at the indicated concentrations through the cut stems of wild-type plants (open bars) or mutants (filled bars). B indicates plants supplied with phosphate buffer alone. Data points represent the means ±SE of six plants. Inh I, inhibitor I.

def1 plants for the accumulation of proteinase inhibitors in response to octadecanoic acids. Application of linolenic acid (18:3) to wild-type plants strongly induced the accumulation of inhibitor I and inhibitor II mRNA (Figure 7). This effect appears to be specific to linolenic acid, because the application of linoleic acid (18:2) was largely ineffective. In contrast to wild-type plants, linolenic acid induced only low levels (<20% of that of the wild type) of inhibitor I and II mRNA accumulation in the mutants. This effect was also measurable at the level of inhibitor protein accumulation; the amount of linolenic acid–induced inhibitor II protein in mutant plants was 21% of that in the wild type (Table 2). In a segregating F_2 population derived from wild-type and *def1*/*def1* parents, the deficiency in inhibitor II in response to linolenic acid cosegregated with the mutant wound response phenotype (Table 2). These data are consistent with the hypothesis that the wound and linolenic acid response phenotypes result from the same mutation.

The ability of *def1* plants to accumulate inhibitor I and II proteins in response to two other octadecanoic acid intermediates, 13(*S*)-hydroperoxylinolenic acid (HPLA) and PDA, was also tested. HPLA elicited only a weak response in the mutants (25 and 13% of wild-type inhibitor I and II levels, respectively). In contrast, PDA, like JA, was a potent inducer of the inhibitors in both wild-type and mutant plants (Figure 8). This result suggests that the octadecanoid pathway in the *def1* mutant is blocked downstream of HPLA action but upstream of PDA action.

Mutant Plants Are Deficient in JA Accumulation in Response to Wounding and Elicitation by Systemin

Defense gene induction in plants by several elicitors (wounding, systemin, PGA, chitosan, and abscisic acid) has been correlated with increases in JA accumulation (Gundlach et al., 1992; Peña-Cortes et al., 1993, 1995; Doares et al., 1995a). To further test the hypothesis that *def1* inhibits the octadecanoid pathway leading to JA biosynthesis, we examined JA levels in the mutants in response to wounding and elicitation by systemin. Although the levels of JA in uninduced wild-type plants and mutants were similar, mechanical wounding increased JA accumulation eightfold in wild-type plants and only two- to threefold in the mutants (Table 3). In plants elicited with systemin, JA levels increased 20-fold in wild-type plants and twofold in the mutants. Thus, the deficiency in defense protein accumulation observed in the mutants in response to wounding and systemin correlates with a deficiency in JA accumulation.

DISCUSSION

The use of mutants offers a powerful approach to dissecting the signaling cascade that couples wounding to changes in defense gene expression. As a first step toward this goal, tomato mutants deficient in the accumulation of wound-inducible proteinase inhibitors were isolated (Lightner et al., 1993). In this report, we show that the inability of one of these mutants (JL5) to produce proteinase inhibitors in response to wounding results from a deficiency in accumulation of inhibitor mRNA. The induction kinetics of inhibitor I mRNA accumulation in wounded tissue indicate that JL5 is affected in the amplitude of the wound response rather than the timing of the response (Figure 1B).

The impaired wound response of JL5 is associated with a dramatic reduction in resistance toward hornworm larvae (Table 1 and Figure 4). The mutation (*def1*) in JL5 that mediates the altered defense phenotype reduces the natural resistance of tomato to hornworm larvae to a level similar to that in tomato plants transformed with an antisense prosystemin gene (Orozco-Cardenas et al., 1993). As is the case for prosystemin antisense plants, the increased susceptibility of *def1* plants to insect attack is presumably the result of a deficiency in the accumulation of SWRPs (Schaller and Ryan, 1996) that function to deter insect feeding. A wealth of evidence indicates that plant proteinase inhibitors play an important role in protecting plants against attack by herbivorous insects (Green and Ryan, 1972; Broadway and Duffey, 1986; Hilder et al., 1987; Johnson et al., 1989). Although the failure of *def1* plants to accumulate serine, aspartic acid, and cysteine proteinase inhibitors (Figures 1 to 3; G.A. Howe and C.A. Ryan, unpublished results) is likely to account for a large component of the reduced resistance, there may be other SWRPs (e.g., polyphenol oxidase) that also serve an important function in protecting plants against insects (Constabel et al., 1995).

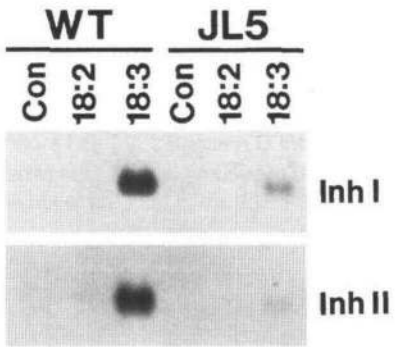

Figure 7. Accumulation of Proteinase Inhibitor I and II mRNA in Response to Octadecanoic Acids.

Wild-type (WT) plants and mutants (JL5) were sprayed with either a vehicle solution (Con) (0.1% Triton X-100 and 15 mM phosphate, pH 6.5) or the same solution containing 10 mM linoleic acid (18:2) or 10 mM linolenic acid (18:3). Total RNA was isolated from leaf tissue (eight pooled plants per isolate) 24 hr after spraying. RNA gel blots were hybridized with inhibitors I and II (Inh I and Inh II) cDNA probes as described in Figure 1B.

Table 2. Proteinase Inhibitor II Protein Accumulation in Wild-Type, JL5, and Segregating F_2 Plants in Response to Wounding and Linolenic Acid

		Inhibitor II Concentration in Response to[c]	
Plant[a]	No.[b]	Wounding	Linolenic Acid
WT	12	48 ± 10	125 ± 18
JL5	12	<13	26 ± 8
F_2	29	39 ± 8 (25 to 54)	122[d] ± 20 (98 to 172)
F_2	15	<13	45 ± 9 (25 to 58)

[a] Twelve-day-old seedlings were assayed for wound-inducible inhibitor II protein accumulation in the cotyledons. Four days later, the same plants were tested for inhibitor II accumulation in leaf tissue in response to linolenic acid. WT, wild type.

[b] Of 44 F_2 plants tested, 29 showed the wild-type response to both wounding and linolenic acid, and 15 plants showed the mutant response to both treatments. $\chi^2 = 1.9$ ($P > 0.1$) for the monogenic recessive hypothesis.

[c] Inhibitor II concentrations are in micrograms per milliliter of leaf juice. Data show the means ± SD for the number of plants indicated. Numbers within parentheses indicate the range of values obtained for individual plants. The detection limit of inhibitor II was 13 μg/mL.

[d] Wounding and subsequent excision of cotyledons from wild-type plants induced the accumulation of inhibitor II to <65 μg/mL before the application of linolenic acid.

An interesting aspect of the *def1* wound response is that significant amounts of inhibitor I protein and mRNA accumulate in mechanically wounded leaves but little, if any, accumulation was observed in unwounded leaves of the same plant (Figure 1). This phenotype originally suggested that the JL5 mutant is capable of responding to local elicitors near the wound site, but is defective in systemic wound signaling (Lightner et al., 1993). However, we found that the accumulation of defense gene transcripts in wounded leaves of the mutants is not restricted to the area encompassing the wound site (Figure 2). This finding indicates that the *def1* gene product is required for defense signaling in tissue both adjacent to and distal from the wound site.

One explanation for the "leaky" wound response phenotype of the mutant is that the *def1* mutation does not completely abolish the function of the affected signaling step. This interpretation is consistent with the finding that several elicitors (e.g., systemin, chitosan, and linolenic acid), when used at high concentrations, evoke a measurable response in the mutant (Figures 6 and 7, and Table 2). Given that the *def1* gene product appears to function in octadecanoid metabolism (see below), it is possible that *def1* plants are affected not in the production of a mobile wound signal but rather in the downstream, intracellular transduction of the signal. Thus, the absence of signaling in remote unwounded leaves of the mutant (Figure 1B) could be accounted for if the concentration of mobile wound signal in that tissue is below what is required to activate the octadecanoid pathway in the mutant. A second explanation for the residual signaling in *def1* plants is the existence of a redundant pathway for octadecanoid metabolism that relies on membrane degradation initiated by severe mechanical wounding. It is possible that fatty acids released during this process are converted, via a DEF1-independent pathway, into oxylipins (e.g., PDA or jasmonate) that can act as signals for defense gene induction.

def1 mutants are deficient in the accumulation of defense proteins in response to known elicitors of the wound response, including transgene-mediated prosystemin overproduction, PGA, chitosan, and systemin (Figures 5 and 6). The simplest interpretation of this result is that a single mutation (*def1*) blocks the wound response pathway at a point downstream from the initial reception events mediated by these elicitors. This interpretation is supported by recent biochemical evidence showing that defense gene induction by wounding, PGA, chitosan, and systemin can be blocked by inhibitors of the octadecanoid pathway, that is, diethyldithiocarbamate (Farmer et al., 1994) and salicylic acid (Peña-Cortes et al., 1993; Doares et al., 1995b). At the mechanistic level, these findings provide indirect evidence that a common step in the activation of defense gene expression by wounding, systemin, chitosan, and PGA is the release of C:18 fatty acid from the membrane. It will be interesting to determine whether other proposed wound signals require DEF1 for the activation of defense gene expression. Finally, it should be noted that our data do not exclude

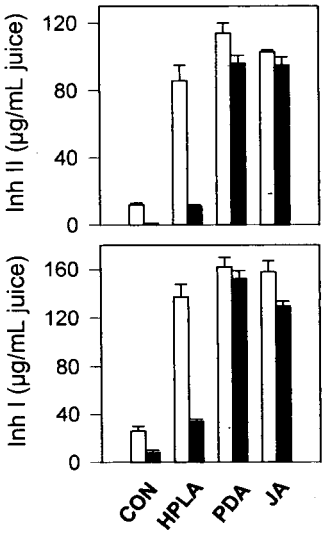

Figure 8. Accumulation of Proteinase Inhibitors in Response to Octadecanoid Pathway Intermediates.

Wild-type plants (open bars) and JL5 mutants (filled bars) were supplied through their cut stems with a buffer control (CON), HPLA (25 nmol per plant), PDA (10 nmol per plant), or JA (10 nmol per plant). Data points represent the means ± SE of six plants. Inh I and Inh II, inhibitors I and II.

Table 3. Accumulation of JA in Wild-Type and JL5 Plants in Response to Wounding and Elicitation by Systemin

			Plants Supplied with[c]	
Plant[a]	Unwounded[b]	Wounded[b]	Buffer	Systemin
WT	36 ± 5	302 ± 14	51 ± 8	1021 ± 67
JL5	46 ± 5	114 ± 15	49 ± 5	96 ± 8

[a] WT, wild type.

[b] Intact plants were wounded multiple times on each of the two primary leaves with a hemostat. Leaf tissue from the wounded plants or from unwounded control plants was harvested for JA determination 90 min later.

[c] Excised plants were supplied with systemin (2.5 pmol per plant) or buffer alone through the cut stem, and leaf tissue was collected for JA determination 2 hr and 15 min later. Concentrations of JA are in picomoles per gram fresh weight of tissue. Values are the means ±SE of three independent experiments.

the possibility that some elicitors may activate signaling pathways that operate at the same time as and/or independently of octadecanoid signaling. The phosphorylation of plasma membrane proteins in response to PGA (Farmer et al., 1989), for example, may be an early event in the octadecanoid pathway or may be part of an alternate signaling pathway (Reymond et al., 1995). In this regard, *def1* plants may be useful for elucidating the octadecanoid-independent events mediated by various elicitors.

The low responsiveness of *def1* plants to several primary elicitors of the wound signaling pathway together with the fact that these plants respond normally to JA (Figure 3) suggest that the octadecanoid pathway serves as the primary defense signaling mechanism for both wound and pathogen-derived signals. Direct evidence for this was obtained by showing that treatment of *def1* plants with downstream metabolites of the octadecanoid pathway (PDA and JA) promotes the accumulation of proteinase inhibitors, whereas elicitors and the upstream precursors linolenic acid and HPLA are poorly active (Table 2, and Figures 7 and 8). Thus, *def1* appears to block the octadecanoid pathway between the action of HPLA and that of PDA.

Several biochemical activities could be affected in this part of the pathway. For example, *def1* could disrupt the activity of allene oxide synthase (AOS) or the next enzyme in the pathway, allene oxide cyclase. AOS catalyzes the dehydration of HPLA to an unstable allene oxide intermediate (Song and Brash, 1991), which is then cyclyzed to PDA by the action of allene oxide cyclase (Hamberg and Fahlstadius, 1990). Experiments are in progress to determine whether these activities are affected in *def1* plants.

It is also possible that *def1* affects the transport or subcellular compartmentalization of an octadecanoid intermediate. This possibility seems particularly relevant in the case of PDA, which, when supplied to mutants through their cut stems, may bypass a requirement for an intracellular transport step. The localization of several JA biosynthetic enzymes to the chloroplast (Vick, 1993; Bell et al., 1995; Blée and Joyard, 1996) suggests that PDA may also be synthesized in this compartment. The conversion of PDA to JA requires a reduction step and subsequent β-oxidation steps. Given that β-oxidation in plants has been proposed to occur exclusively in peroxisomes (Gerhardt, 1983), it is possible that a block in the transport of PDA from the plastid to the peroxisome could account for the wound response deficiency observed in *def1* plants. Another possible route of PDA transport is suggested by the recent finding that PDA can activate defense gene expression in *E. californica* without its prior conversion to JA (Blechert et al., 1995). If this is the case in tomato leaves, then PDA may be transported directly from its site of synthesis to a location at which it interacts with a putative receptor to initiate gene expression.

The proposal that *def1* disrupts the flux of linolenic acid through the octadecanoid pathway is consistent with our finding that mutant plants accumulate reduced levels of JA in response to wounding and to elicitation by systemin. However, the mutant appears to be less affected (relative to wild-type plants) in wound-inducible JA accumulation than it is in systemin-elicited JA accumulation (Table 3). This suggests that JA biosynthesis is regulated differently in response to wounding than it is in response to systemin. As recently discussed by Creelman and Mullet (1995), it is possible that wounding stimulates localized JA accumulation as a consequence of simple mixing of JA biosynthetic enzymes with substrates not normally available in unwounded tissue. Such a process could account for the residual wound signaling activity observed in wounded leaves of *def1* plants. Alternatively, elicitors such as PGA and systemin may activate JA synthesis in one subcellular compartment, whereas mechanical wounding, drought stress, and developmental signals activate JA synthesis in other compartments (Creelman and Mullet, 1995). This notion is supported by a recent study showing that JA accumulates to high levels in AOS-overexpressing transgenic potato plants without activating the expression of proteinase inhibitor genes (Harms et al., 1995). Clearly, the *def1* mutant should be useful in elucidating the pathways by which octadecanoid metabolism influences diverse developmental and environmental responses.

METHODS

Plant Material

Wild-type and mutant (JL5) tomato plants (*Lycopersicon esculentum* cv Castlemart) were grown under 17-hr days at 28°C with light at >300 μE m^{-2} sec^{-1} and 7-hr nights at 17°C in total darkness. The original JL5 mutant (Lightner et al., 1993) was repeatedly backcrossed to the wild-type parent (cv Castlemart) with selection for the wound response deficiency of proteinase inhibitors I and II. All experiments were performed with homozygous (*def1/def1*) lines that were backcrossed at least twice.

Insect Feeding Trials

Tobacco hornworm (*Manduca sexta*) eggs and hornworm artificial diet were obtained from Carolina Biological Supply Company (Burlington, NC). The eggs were placed on one side of a hatching apparatus (a piece of circular Whatman paper inside a 150 × 15 mm Petri dish), and the hornworm artificial diet was placed at the opposite end. A temperature of 26°C was maintained with a 100-W light bulb. Typically, the larvae hatched and established feeding on the diet within 3 days of shipment. Newly hatched larvae were reared on the artificial diet for 3 days (attaining a weight of 5 to 6 mg and a length of 7 to 8 mm) before being placed on tomato plants to initiate the feeding trials.

Elicitors and Bioassay

Wounding and chemical elicitor experiments were performed with 15-day-old seedlings, except where otherwise indicated. A hemostat was used to inflict wounds near the distal end of the leaflet, perpendicular to the midvein. All elicitors except linolenic acid were supplied to plants through their cut stems as follows. Plants were excised at the base of the stem with a razor and placed immediately into a 0.5-mL plastic tube containing the elicitor diluted into 300 μL of phosphate buffer (15 mM sodium phosphate, pH 6.5). When most of the elicitor solution had been imbibed (~45 min), the plants were transferred to vials containing 20 mL of fresh water. Proteinase inhibitor levels in the leaf tissue were measured by radial immunodiffusion assay (Ryan, 1967) 24 hr after elicitation. All incubations were performed under constant light in a closed plexiglass box containing an open jar of 10 N NaOH as a CO_2 trap. 13(*S*)-Hydroperoxylinolenic acid (HPLA) and 12-oxophytodienoic acid (PDA) were obtained from Cayman Chemical Co. (Ann Arbor, MI) as a solution in ethanol. Before use, the ethanol was evaporated (but not dried completely) in a Speedvac (Savant Instruments, Inc., Hicksville, NY), and the compound was redissolved in 15 mM sodium phosphate buffer. Jasmonic acid (JA) (Farmer et al., 1992), systemin (Pearce et al., 1991), polygalacturonic acid (PGA) (degree of polymerization ~20) (Bishop et al., 1981), and nitrous acid–treated chitosan (Hadwiger and Beckman, 1980; Walker-Simmons and Ryan, 1984) were diluted from aqueous stock solutions into phosphate buffer before use.

Intact plants were sprayed with a freshly prepared solution of linolenic acid (10 mM linolenic acid [Sigma], 0.1% Triton X-100, 15 mM sodium phosphate, pH 6.5) or buffer control (0.1% Triton X-100, 15 mM sodium phosphate, pH 6.5) and incubated for 24 hr under constant light before determination of proteinase inhibitor levels.

Isolation of the 35S:PS *def1* Mutant

The construction of transgenic plants expressing the prosystemin cDNA under the control of the cauliflower mosaic virus 35S promoter (referred to as the 35S:PS transgene) has been described by McGurl et al. (1994). A transgenic line homozygous for a single insertion of the 35S:PS transgene (G.A. Howe and C.A. Ryan, unpublished data) was used for the experiment shown in Figure 5. A cross between JL5 (*def1/def1*) and a homozygous transgenic line (35S:PS/35S:PS) produced F_1 progeny (*DEF1/def1* 35S:PS/+) that constitutively accumulate proteinase inhibitors I and II (data not shown). Thus, the 35S:PS transgene is dominant and *def1* is recessive with respect to the constitutive accumulation of inhibitors I and II. To simplify the identification of prosystemin overproducing *def1/def1* plants, a testcross was made between the F_1 (*DEF1/def1* 35S:PS/+) and *def1* homozygous parents. Of 40 testcross progeny analyzed by polymerase chain reaction, 19 harbored the transgene. Nine of these plants (genotype *def1/DEF1* 35S:PS/+) showed constitutive accumulation of inhibitors I and II, whereas the remaining 10 plants (genotype *def1/def1* 35S:PS/+) failed to express the inhibitors to levels greater than that observed in unwounded wild-type plants. These results are consistent with the hypothesis that the *def1* and 35S:PS loci are unlinked ($\chi^2 = 0.15$; $P > 0.9$). Following self-fertilization of a *def1/def1* 35S:PS/+ F_1 plant, several independent F_2 plants were tested for 35S:PS transgene homozygosity by scoring the ratio of kanamycin-resistant to kanamycin-sensitive seedlings in the F_3 generation. An F_2 line that produced only kanamycin-resistant progeny (53 of 53 F_3 plants tested) was chosen as the true-breeding *def1/def1* 35S:PS/35S:PS line.

RNA Gel Blot Analysis

Total RNA was isolated from tomato leaf tissue and analyzed by gel blot hybridization, as previously described (McGurl et al., 1995). Five micrograms of total RNA was also electrophoresed and stained with ethidium bromide before gel blotting to ensure equal loading of samples. Nitrocellulose filters were hybridized to the following tomato cDNAs labeled with a T7 Quickprime Kit (Pharmacia Biotech, Uppsala, Sweden): proteinase inhibitor I (Graham et al., 1985a), proteinase inhibitor II (Graham et al., 1985b), prosystemin (McGurl et al., 1992), cathepsin D inhibitor (Schaller et al., 1995), and leucine aminopeptidase (gift from L. Walling, University of California at Riverside; Pautot et al., 1993). Washed filters were visualized by autoradiography by using Kodak XAR-5 film. Alternatively, the amount of radioactive probe hybridizing with specific mRNAs was quantified by electronic autoradiography on an InstantImager (Packard, Meriden, CT), using rRNA or ubiquitin mRNA to normalize the values.

JA Measurements

For each experiment, extracts were prepared in duplicate from two 5-g (fresh weight) portions of tissue (leaves plus petioles). A competitive enzyme-linked immunoassay based on a monoclonal antibody directed against methyl jasmonate was used to determine the levels of JA in the extracts (Albrecht et al., 1993).

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