Plant Physiol. (1997) 115: 817–826

Jasmonic Acid-Dependent and -Independent Signaling Pathways Control Wound-Induced Gene Activation in *Arabidopsis thaliana*[1]

Elena Titarenko, Enrique Rojo, José León, and Jose J. Sánchez-Serrano*

Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas, Campus de Cantoblanco Universidad Autónoma de Madrid, 28049 Madrid, Spain

Plant response to mechanical injury includes gene activation both at the wound site and systemically in nondamaged tissues. The model developed for the wound-induced activation of the proteinase inhibitor II (*Pin2*) gene in potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) establishes the involvement of the plant hormones abscisic acid and jasmonic acid (JA) as key components of the wound signal transduction pathway. To assess in *Arabidopsis thaliana* the role of these plant hormones in regulating wound-induced gene expression, we isolated wound- and JA-inducible genes by the differential mRNA display technique. Their patterns of expression upon mechanical wounding and hormonal treatments revealed differences in the spatial distribution of the transcripts and in the responsiveness of the analyzed genes to abscisic acid and JA. A correlation can be established between sensitivity to JA and the accumulation of the transcripts in systemic tissues upon wounding. A comparative study of the wound response in wild-type and JA-insensitive *coi1* mutant plants indicated that in *A. thaliana* wound signals are transmitted via at least two different pathways. One of them does not involve JA as a mediator and is preferentially responsible for gene activation in the vicinity of the wound site, whereas the other requires JA perception and activates gene expression throughout the aerial part of the plant.

Plants react to wounding and pest attack by activating a variety of genes. Some of them are expressed only in the vicinity of the wound site, whereas others are also systemically activated in the nondamaged parts of injured plants (Bowles, 1990). Well-characterized examples of systemically inducible genes are the proteinase inhibitor II (*Pin2*) gene family of potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*; Peña-Cortés et al., 1988; Farmer and Ryan, 1990) and two vegetative storage protein genes (*VspA* and *VspB*) of soybean (*Glycine max*; Mason and Mullet, 1990).

The mechanisms by which plants regulate wound-induced gene expression are not well understood. It has been shown that wounding triggers an increase in the endogenous levels of the plant growth regulator JA (Creelman et al., 1992; Albrecht et al., 1993; Laudert et al., 1996), and this increase is required for gene activation upon wounding (Peña-Cortés et al., 1993). Application of exogenous JA or its methyl ester at physiological concentrations can induce a variety of wound-responsive genes, including *Pin2* and *Vsp* (Mason and Mullet, 1990; Farmer et al., 1992).

In potato and tomato proteinase inhibitor genes can also be activated by oligosaccharide fragments generated from both plant and pathogen cell walls (Bishop et al., 1981) and by the 18-amino acid polypeptide systemin (Pearce et al., 1991). Systemin was found to act before JA in the wound signal transduction chain (Peña-Cortés et al., 1995). However, a role for systemin, or any related peptide hormone, in the transmission of the wound signal has been shown so far only in potato and tomato. The phytohormone ABA has also been postulated to participate in wound signaling in these plant species (Peña-Cortés et al., 1989, 1995).

Since JA, but not systemin, is able to activate *Pin2* expression in the *ABA*-deficient tomato (*sitiens*) and potato (*droopy*) mutants, in which *Pin2* genes are not induced by mechanical wounding, the site for ABA action has been located between systemin and JA (Peña-Cortés et al., 1996). These components would therefore link mechanical injury with an intracellular increase in JA concentration, which results in gene activation. Recently, the requirement of ethylene in mediating wound-induced gene activation has been demonstrated in tomato (O'Donnell et al., 1996), in which it acts together with JA to regulate *Pin2* gene expression.

Arabidopsis thaliana has served as a model plant to study different hormone signal transduction pathways. A number of ethylene-response mutants have been isolated, defining several loci in the ethylene signal transduction pathway (Kieber et al., 1993; Chang, 1996). Arabidopsis mutants affected in their responses to ABA (Leung et al., 1994; Meyer et al., 1994), auxins (Hobbie et al., 1994), and cytokinins (Kakimoto, 1996) have also been characterized. Three independent JA-insensitive mutants have already been identified. *jar1* was isolated by phenotypic screening for plants with root growth insensitive to jasmonate inhi-

[1] This research was supported in part by the European Communities' Biotech Program, as part of the Project of Technological Priority 1993–1996. Financial support was also provided by the Spanish Comisión Interministerial de Ciencia y Tecnología (grant nos. BIO93-0678-CO2-02, BIO94-1502-CE, and BIO96-0532-CO2-01). J.L. was the recipient of a postdoctoral contract from the Spanish Ministerio de Educación y Ciencia.

* Corresponding author; e-mail jjss@samba.cnb.uam.es; fax 34–1–585–4506.

Abbreviations: DD, differential display; JA, jasmonic acid.

bition (Staswick et al., 1992). The *coi1* mutant was isolated because of its resistance to the structurally related, chlorosis-inducing bacterial toxin coronatine (Feys et al., 1994). Upon JA treatment, both of these mutants showed reduced accumulation of 29- and 31-kD proteins, which were subsequently identified as VSPs that are immunologically related to soybean VspA and VspB (Staswick et al., 1992; Benedetti et al., 1995). The jasmonate-insensitive mutants *jin1* and *jin4* exhibited a reduction in the expression of the jasmonate-responsive gene *AtVsp*, which was induced in the leaves of seedlings upon treatment with methyl jasmonate (Berger et al., 1996). *jin4* and *jar1* mutated loci may be allelic.

To further understand the wound signal transduction pathway and the role of JA in this process, a search for novel wound- and JA-inducible genes was undertaken in *A. thaliana* to (a) obtain molecular markers to monitor wound-induced gene activation, (b) elucidate the role of JA in both local and systemic wound-induced gene activation, and (c) characterize wound- and JA-responsive genes and subsequently use their promoters in a transgenic approach for the isolation of new signaling mutants.

MATERIALS AND METHODS

Plant Material and Treatments

Arabidopsis thaliana ecotype Landsberg *erecta* plants were grown in soil in a greenhouse at 22°C with a 12-/12-h light/dark period. For wounding experiments one-half of the rosette leaves of 4- to 6-week-old plants were wounded with forceps, and the injured leaves (local), unwounded rosette leaves (systemic), and cauline leaves (upper) were harvested at different times after wounding (0.5, 1, 1.5, 2, 4, 8, 24, and 48 h). *A. thaliana* seeds from an F_2 population segregating for the *coi1* mutant were kindly provided by Dr. J.G. Turner (University of East Anglia, Norwich, UK) and were grown and selected as described previously (Benedetti et al., 1995). Wounding of *coi1* mutant plants was done as described above for wild-type plants.

To examine hormone action, plants were grown in the greenhouse as described above and were sprayed with a 50 μM solution of ABA (mixed isomers, Sigma) or JA (mixed isomers, Apex Organics, Devon, UK). Rosette leaves were collected 2, 4, 8, 24, and 48 h after treatment. Tissue from different *A. thaliana* organs (roots, stems, different types of leaves, flowers, and green siliques) was harvested from 4- to 6-week-old plants grown in soil. Plants used for DNA purification were grown from sterilized seeds on agar plates with 0.5× Murashige-Skoog medium (Murashige and Skoog, 1962) supplemented with 1% Suc. Upper parts of the plants were harvested after 21 d of growth at 22°C under continuous light.

DD Technique

DD was performed as described previously (Liang and Pardee, 1992). Total RNA (1 μg) was reverse-transcribed using T_{11}MN 3′ primers (where M stands for a mixture of G, A, and C, and N stands for A, C, G, or T) using Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL). One-tenth of the reverse-transcription mixture was used as a template in a PCR reaction containing a given T_{11}MN primer in combination with one arbitrary 10-base primer (DD 10-mer kit, Operon Technologies, Alameda, CA).

PCR was performed using *Taq* polymerase (Perkin-Elmer), and reaction conditions were as follows: 94°C for 30 s; 40°C for 2 min; 72°C for 30 s for 40 cycles, followed by extension at 72°C for 5 min. Aliquots of PCR reactions were run through a 6% sequencing gel. On the same gel, reactions obtained from two different batches of mRNA were compared. Only bands consistently found to be differentially amplified were selected and analyzed further. The bands of interest were eluted in 100 μL of sterile water. Three microliters of each elution were reamplified using the appropriate pair of primers. The amplified fragments were purified from low-temperature melting point agarose (SeaPlaque FMC, Rockland, ME) using a purification kit (Qiaex, Qiagen, Düsseldorf, Germany), cloned in pUC18 vector, and sequenced on both strands using the Sequenase kit (version 2.0, Amersham). Partial cDNA clones (3′) were used as probes for northern and Southern hybridization analyses.

Nucleic Acid Isolation and Analysis

Total RNA was isolated as described previously (Logemann et al., 1987). RNA samples were separated on agarose-formaldehyde gels and transferred onto Hybond-N nylon membranes (Amersham) in 20× SSC using standard procedures (Sambrook et al., 1989). Equal RNA loading was visualized by staining the rRNAs with ethidium bromide (40 ng/μL). Replica blots were prepared and sequentially hybridized to at most three probes. Probes were labeled with [α-^{32}P]dCTP using the Rediprime kit (Amersham). The *Vsp* cDNA (clone 108B11) used as probe was obtained from the Arabidopsis Biological Resource Center (The Ohio State University, Columbus). Hybridization was at 42°C in 0.25 M phosphate buffer, pH 7.2, 0.25 M NaCl, 7% SDS, 1 mM EDTA, pH 8, 10% PEG 6000, 100 μg/mL denatured salmon sperm DNA, and 50% formamide (Amasino, 1986). After hybridization radioactive probes were stripped by washing the blots twice with 0.5% SDS in distilled water at 65°C.

Total DNA was isolated from 1 g of frozen tissue as described previously (Dellaporta et al., 1983). Ten micrograms of DNA was digested with restriction enzymes and subsequently separated on a 0.8% agarose gel. DNA transfer and hybridization were done according to standard procedures (Sambrook et al., 1989). Sequence comparison was performed using the GCG package (version 8, Genetics Computer Group, University of Wisconsin, Madison).

RESULTS

Cloning of *A. thaliana* Wound- and JA-Inducible genes

DD is a powerful tool for the detection of differentially expressed genes (Liang and Pardee, 1992). We have used this technique to identify genes with expression induced by mechanical damage. To this end, we have compared the

mRNA population present in the rosette leaves of fully developed *A. thaliana* plants with that expressed upon wounding, both in the locally damaged leaves and in the systemic, undamaged ones. A representative example of such an experiment is shown in Figure 1. In the lane corresponding to the wounded tissue, some differentially expressed bands can be seen. They were isolated, sequenced, and used as probes in northern hybridization experiments to determine their transcript levels in control and wounded plants (see below). Among them, four cDNA clones were found to hybridize to transcripts that accumulated in leaves upon wounding, whereas their expression levels were very low or absent in the leaves of control plants. Relevant characteristics of these clones are summarized in Table I.

Significant sequence similarity to genes from nonplant sources was found for the wound-induced *Tat* (tyrosine amino transferase), *Ck* (choline kinase), and *Aco* (acyl CoA oxidase) clones. The DD technique used for gene isolation in our study yielded the 3′ end of the corresponding cDNAs (Liang and Pardee, 1992). Because these parts routinely exhibit lower conservation than protein-coding regions, we did not succeed in finding homologies for some of the genes we isolated, *Wr3* (wound-responsive 3) among them. We have thus isolated nearly full-length cDNAs for some of the DD cDNA clones. However, sequencing of a full-length *Wr3* cDNA has not revealed any significant similarity in the databases (not shown).

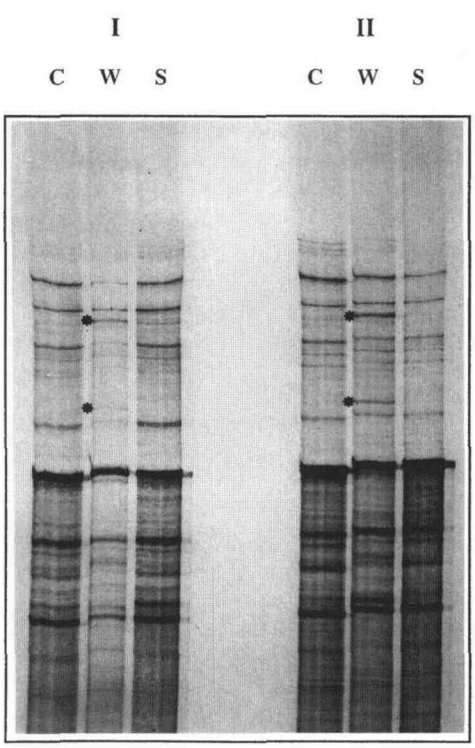

Figure 1. DD of *A. thaliana* leaf mRNAs. The autoradiogram shows duplicate samples (I and II) prepared from rosette leaves of control plants (C) and from the damaged rosette leaves (W) and unwounded, systemic leaves (S) of plants harvested 4 h after wounding. The positions of differentially expressed cDNAs are indicated by asterisks (*). The marked bands shown in this picture are derived from the *Wr3* transcript.

To investigate whether there is a link between wounding and JA in *A. thaliana*, the mRNA population from JA-treated leaves was compared with that from nontreated plants using the DD technique. Three JA-induced cDNA clones were isolated and their relevant characteristics are listed in Table II. No homologies were found in the databases for the *Jr1* and *Jr2* (jasmonate-responsive 1 and 2) cDNAs obtained from the DD gels. In contrast, the deduced partial *Jr3* amino acid sequence was 38% identical to the previously described *Ilr1* gene from *A. thaliana* (Bartel and Fink, 1995). The isolation and sequencing of a nearly full-length *Jr1* cDNA clone did not reveal any homology to sequences in the databases. In contrast, the sequence of a nearly full-length *Jr2* cDNA clone detected significant similarities to aminotransferase sequences (not shown). Nevertheless, *Jr2* is only 50% identical to *Tat* in their deduced amino acid sequences.

In addition, a cDNA clone with homology to the previously described *Vsp* from soybean (*Glycine max*; Mason and Mullet, 1990; Staswick, 1990) was obtained from the Arabidopsis Biological Resource Center and used in our study. This clone corresponds to the previously described wound- and JA-inducible *A. thaliana Vsp* cDNA (Berger et al., 1995).

Southern hybridization experiments were performed to investigate the copy number of the newly identified cDNAs in the *A. thaliana* genome. The results of such experiments are presented in Figure 2. According to the data obtained all analyzed wound- and JA-inducible genes are present in few copies (most likely a single copy) in the genome of *A. thaliana*.

Organ-Specific Expression in Noninduced Plants

We analyzed the accumulation of transcripts corresponding to the isolated cDNA clones in different organs of *A. thaliana*. The results from these experiments are presented in Figure 3. For most of the genes tested a basal, albeit low, level of expression could be detected in some parts of nontreated plants. *Ck* and *Vsp* cDNAs were found to hybridize on northern blots to different transcripts of approximately 1.6 and 2.1 kb and 1.1 and 1.2 kb, respectively (Tables I and II). The *Ck* transcript was observed principally in roots, as was that of *Jr1*. Both *Vsp* transcripts were strongly expressed in roots, flowers, and siliques. The expression of *Vsp* RNAs in different *A. thaliana* organs was previously described (Berger et al., 1995), but, in contrast to our results, no expression in roots or green siliques was detected. There are two *Vsp* genes in *A. thaliana*, and the probe used by Berger et al. (1995) was derived from a different *Vsp* cDNA; this may explain the slightly different genomic hybridization and gene expression results. Differences in the age of the plants used or in growing conditions may also account for discrepancies in expression patterns.

Table I. *Relevant characteristics of the cDNA clones corresponding to wound-inducible genes isolated by DD*

Clone	Size of 3′ Fragment	mRNA Length	Induction	Highest-Scored Homology (Amino Acid Sequences)
	bp	*kb*		
Tat	489	1.7	Wounding JA	30% Identity with *Tat* of *Trypanosoma cruzi* (Bontempi et al., 1993); 25% identity with human *Tat* (Rettenmeier et al., 1990); 23% identity with rat *Tat* (Hargrove et al., 1989).
Wr3	483	1.0	Wounding	No significant similarity found.
Ck	253	1.6 2.1	Wounding	50% Identity with rat *Ck* (Uchida and Yamashita, 1992); 46% identity with yeast *Ck* (Hosaka et al., 1989).
Aco	543	2.6	Wounding	55% identity with rat peroxisomal component of *Aco* (Miyazawa et al., 1987); 52% identity with human *Aco* (Aoyama et al., 1994); 45% identity with yeast *Aco* (Dmochowska et al., 1990).

Specific mRNA Accumulation in *A. thaliana* Plants upon Wounding

Results from DD experiments indicated that mRNA accumulation of the identified genes was higher in damaged leaves 4 h after wounding than in leaves of control plants or systemic (nonwounded) leaves of injured plants. To ascertain the time course of induction upon wounding and to elucidate whether transcript accumulation was restricted to the locally damaged tissue, northern analysis was performed with total RNA from different leaves (local, systemic, and upper) of both control and wounded plants (Fig. 4). For all genes analyzed, large, transient increases in the steady-state mRNA levels were observed upon wounding. According to their expression patterns the analyzed clones could be separated into three major groups.

One group with clones *Tat*, *Vsp*, *Jr1*, and *Jr2* exhibited similar or stronger induction in the leaves that were not directly wounded compared with that observed in locally damaged leaves, although at the latest times after wounding, *Jr2* transcript levels were higher in the locally damaged tissue. *Wr3* and *Ck* genes formed a second group with a higher level of transcript accumulation in the locally injured leaves and very little in the systemic leaves, where higher transcript levels could be observed only shortly after wounding, especially in *Wr3*. The *Aco* and *Jr3* genes formed a third group. Soon after wounding, their expres-

Table II. *Relevant characteristics of the cDNA clones corresponding to JA-inducible genes isolated by DD*

Sequence similarities found in the databases for a nearly full-length *Jr2* cDNA clone (discussed in "Results") are not indicated. The *Vsp* clone encompasses most of the coding region and was obtained from the Arabidopsis Biological Resource Center.

Clone	Size of 3′ Fragment	mRNA Length	Induction	Highest-Scored Homology (Amino Acid Sequences)
	bp	*kb*		
Jr1	250	1.7	Wounding JA	No significant similarity found.
Jr2	217	1.5	Wounding JA ABA	No significant similarity found.
Jr3	490	1.7	Wounding JA	38% Identity with *Ilr1* aminohydrolase of *A. thaliana* (Bartel and Fink, 1995).
Vsp	600	1.1 1.2	Wounding JA	47% Identity with soybean *VspA* and *VspB* (Mason and Mullet, 1990).

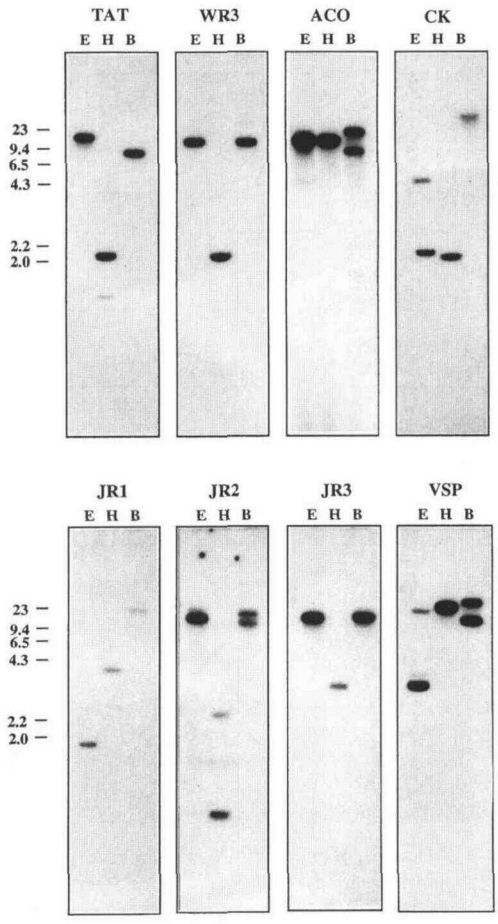

Figure 2. Southern analysis of the wound- and JA-inducible genes. *A. thaliana* genomic DNA (10 μg per lane) was digested with *Eco*RI (E), *Hin*dIII (H), and *Bam*HI (B) and subjected to Southern analysis. Blots were hybridized with probes from the JA- and wound-inducible cDNAs. Size markers (in kilobase pairs) are indicated at the left.

sion increased both in the damaged and in the systemically induced leaves to a similar extent. At later times, however, their expression was restricted to the locally damaged tissues.

Large differences in persistence of the wound-induced levels of expression of these genes were observed. Whereas *Wr3-*, *Aco-*, and *Jr2*-induced levels were maintained up to 48 h, the expression in injured plants of *Tat*, *Vsp*, *Jr1*, *Jr3*, and *Ck* had returned to almost basal levels by 24 h after wounding (Fig. 4B). The persistence of the *Vsp* transcript in the systemically induced cauline leaves has not been reproducibly observed.

Wounding caused a rapid activation of the genes analyzed. As shown in Figure 4A, significant increases in transcript levels for *Tat*, *Ck*, *Wr3*, and *Jr3* were already detectable 30 min after wounding, whereas *Aco*, *Jr1*, *Jr2*, and *Vsp* transcript accumulation started later. It was interesting that two hybridizing bands were detected with the *Vsp* and *Ck* probes, which exhibited different temporal and spatial expression patterns in wounded *A. thaliana* plants. The larger *Vsp* transcript was detectable 1 h and peaked 2 h after wounding in systemic leaves of injured plants, whereas 8 h after injury only the mRNA coding for the smaller transcript was detectable. The northern experiment with mixed RNAs shown in Figure 5 confirmed the presence of two differentially expressed *Vsp* transcripts. Wounding strongly induced two *Ck* transcripts in locally damaged leaves, whereas only the smaller band was present in the control (nonwounded) plants (Fig. 4).

Hormonal Regulation of Wound-Inducible Genes

The expression of some wound-inducible genes previously described (such as proteinase inhibitors, chalcone synthase, or *Vsp*) has been shown to be strongly induced by application of exogenous JA (Creelman and Mullet, 1995). The three genes *Jr1*, *Jr2*, and *Jr3* obtained from JA-treated plants were shown to be induced by mechanical injury,

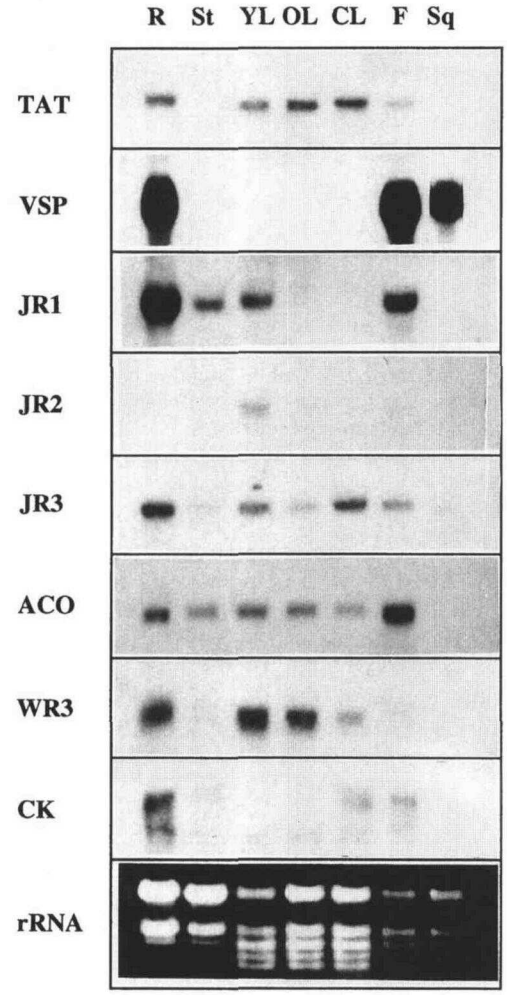

Figure 3. Constitutive expression of wound-responsive genes in the different organs of *A. thaliana* plants. Northern analysis was performed with total RNA (5 μg per lane) from root (R), stem (St), and rosette leaves from 4-week-old plants (YL), rosette leaves from 6-week-old plants (OL), and cauline leaves (CL), flowers (F), and siliques (Sq). Blots were hybridized to radioactive probes from the wound-inducible cDNAs (see text for information concerning the cDNAs used as probes). The ethidium bromide-stained rRNAs are shown as a control for loading in all RNA gels.

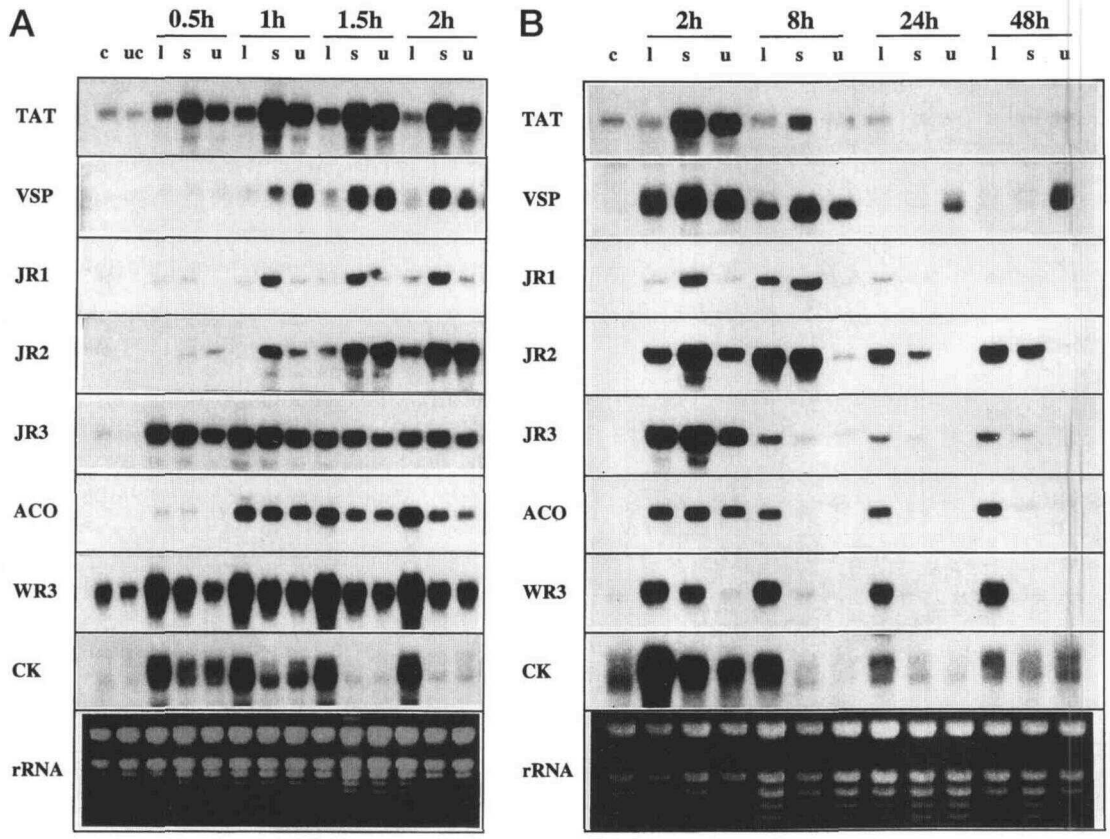

Figure 4. Time course of wound-induced mRNA accumulation in leaves of *A. thaliana*. Collection times correspond to plant tissues harvested 0.5, 1, 1.5, and 2 h after wounding (A) and 2, 8, 24, and 48 h after wounding (B). Results are representative of those obtained in separate, independent experiments. c, Control, rosette leaves from unwounded plants; uc, cauline leaves from unwounded plants; l, locally wounded rosette leaves; s, unwounded (systemic) rosette leaves; and u, cauline (upper) leaves of wounded plants. Five micrograms of total RNA was loaded per lane and blots were hybridized to radioactive probes of the wound-induced cDNAs, as indicated on the left.

suggesting that JA may regulate wound-inducible gene expression in *A. thaliana*. In potato and tomato ABA has been shown to be involved in *Pin2* activation in response to wounding (Peña-Cortés et al., 1995). To assess whether the model for wound signaling established for potato and tomato holds true in *A. thaliana*, the effects of ABA or JA treatments on the accumulation of specific transcripts from the identified wound-responsive genes were studied, and the results are presented in Figure 6.

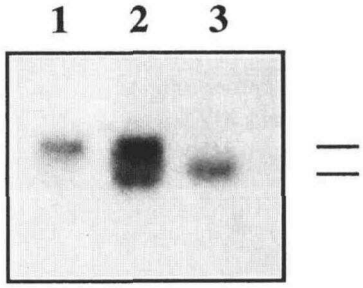

Figure 5. Two *Vsp* transcripts accumulate differentially upon wounding. The RNAs from cauline leaves of plants 2 and 8 h after wounding were run either separately (lane 1, cauline leaves 2 h after wounding; lane 3, 8 h after wounding; 5 μg per lane) or mixed (lane 2, 5 μg of RNA from cauline leaves after 2 h and 5 μg 8 h after wounding). Blot hybridization was done with a *Vsp* probe.

A group of genes including *Tat*, *Vsp*, *Jr1*, *Jr2*, and *Jr3* was strongly induced by JA, suggesting that this hormone may mediate activation upon wounding. The application of exogenous 50 μM JA caused a more pronounced accumulation of *Tat*, *Vsp*, and *Jr1* transcripts than mechanical damage, since their elevated levels were still detectable after 24 and even 48 h (Fig. 6), whereas upon wounding their expression declined to control values by 24 h (Fig. 4B). These differences may reflect a persistence of elevated JA levels in the exogenously treated plants compared with more transient increases in endogenous JA concentration in the wounded plants.

In contrast, treatment with JA produced no effects on the level of the *Ck* transcript and only weak and transient increases over the basal levels of *Wr3* and *Aco* transcripts. These data suggest that in these genes factors other than or in addition to JA are involved in the regulation of wound-induced expression.

The expression patterns observed after ABA treatment were also different among the genes analyzed. Only the *Jr2* gene was induced to similar levels by both ABA and JA; other genes showed increases upon ABA treatment, which

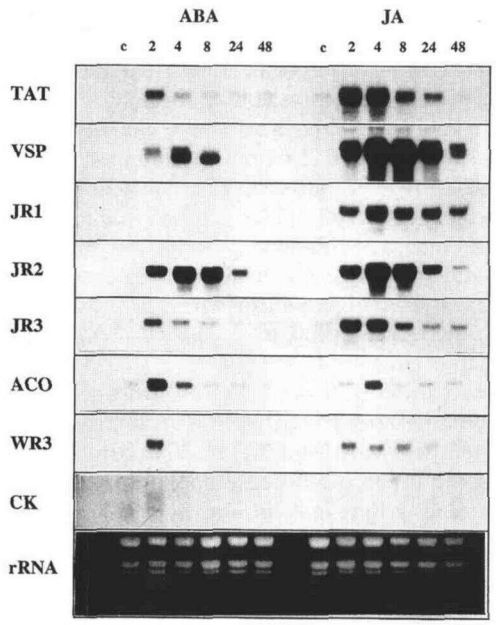

Figure 6. Hormonal induction of wound-responsive genes. *A. thaliana* plants were treated with ABA (50 μM) or JA (50 μM), as indicated in "Materials and Methods." Rosette leaves were collected 2, 4, 8, 24, and 48 h after treatment. c, Control leaves from untreated plants. Five micrograms of total RNA was loaded per lane and hybridized as described in Figure 4.

for *Tat*, *Vsp*, and *Jr3* were more transient and below the levels attained upon treatment with JA (Fig. 6). In *Jr1* and *Ck* no transcript accumulation was observed in ABA-treated plants.

Comparative Study of the Wound Response in Wild-Type *A. thaliana* and JA-Insensitive *coi1* Mutant Plants

A few nonallelic, JA-insensitive *A. thaliana* mutants have been described so far (Staswick et al., 1992; Feys et al., 1994; Berger et al., 1996). Among them, the *coi1* showed normal growth in the presence of JA and did not express the 29- and 31-kD Vsp homologs upon treatment with jasmonates (Benedetti et al., 1995).

To elucidate the role of JA as a mediator in wound signaling in *A. thaliana*, we performed a comparative study of specific mRNA accumulation upon wounding in wild-type and *coi1* mutant plants using the isolated clones as molecular markers. The results are presented in Figure 7. In *coi1* plants part of the wound-responsive genes was not induced by wounding, either in locally injured leaves or in unwounded, systemic tissues, throughout the time course analyzed. This set of genes coincides with the group of transcripts inducible by JA, further supporting the idea that this phytohormone mediates their expression upon wounding.

It was interesting that the wound induction of *Ck*, *Wr3*, and *Aco* was similar in wild-type and *coi1* plants, suggesting the presence of a signaling pathway independent of JA perception for wound-induced gene activation. The wound-induced accumulation of *Aco* transcript in the *coi1* mutant was lower than in wild-type plants and was observed only in the locally wounded leaves (Fig. 7). This suggests that JA perception may be required for attaining maximal levels of *Aco* expression, especially in the systemically induced parts of the plant.

DISCUSSION

Mechanical injury triggers the activation of a large array of genes. Whereas some of them are strongly expressed at the wound site, others accumulate preferentially in distal, nondamaged tissues. Whether the same signals (including JA) coordinate the wound-induced expression of these genes has not been elucidated. Although the involvement of JA in regulating gene activation upon wounding has been firmly established in several plant species (Creelman et al., 1992; Hildmann et al., 1992; Laudert et al., 1996), the processes occurring immediately after wounding are poorly characterized; therefore, other component(s) that may also participate in wound signaling are still unknown.

We have isolated and characterized new *A. thaliana* wound- and JA-inducible genes, which provide a more complete picture of the plant response to mechanical damage. The wound response in this plant species involves the activation of gene expression that is remarkably fast compared with other plants studied, such as *Vsp* in soybean or *Pin2* in potato and tomato. As in these other cases, activation of certain wound-inducible genes in *A. thaliana* is not restricted to the damaged tissue, and a systemic accumulation in distal tissues also occurs (Peña-Cortés et al., 1988; Mason and Mullet, 1990). According to the spatial distribution of their expression in injured plants, the genes analyzed can be separated into three groups: the first group

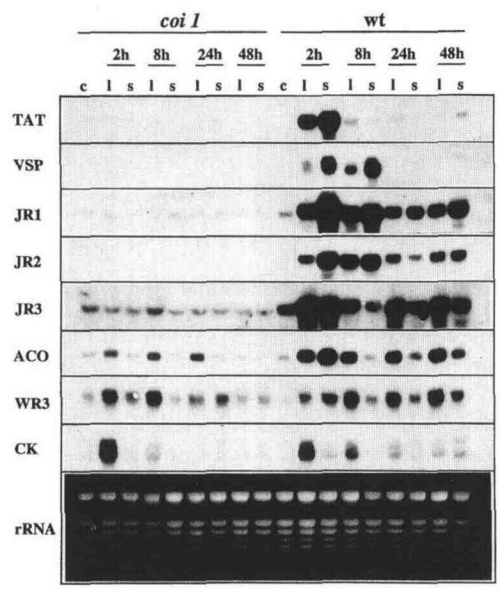

Figure 7. Wound-induced gene activation in wild-type (wt) and JA-insensitive *coi1 A. thaliana* plants. Collection times correspond to 2, 8, 24, and 48 h after wounding. c, Control, unwounded plants; l, locally wounded; and s, systemic (unwounded) leaves of injured plants. Five micrograms of total RNA was loaded per lane.

includes *Ck* and *Wr3* and shows a stronger induction in locally injured leaves than in systemic leaves; the second includes *Vsp*, *Tat*, *Jr1*, and *Jr2* and is generally characterized by higher levels of systemic gene activation; a third group is formed by *Aco* and *Jr3* genes and displays mixed characteristics, being expressed to the same extent in both local and systemic tissues shortly after wounding but later persisting only in the locally damaged tissue.

The isolated clones have been used as molecular markers to analyze the role of JA in mediating wound responses in plants. Exogenously applied JA was able to induce only a subset of the characterized genes, which included those clones showing a stronger systemic accumulation in wounded *A. thaliana* plants. At the same time, for *Ck*, *Wr3*, and *Aco* transcripts, low levels (if any, in the case of *Ck*) of accumulation were detected upon JA treatment, suggesting that their induction upon wounding may occur via a JA-independent pathway.

Further support for the presence of two distinct wound signal transduction pathways was obtained from the study of the wound response in the JA-insensitive *coi1* mutant. In *coi1* plants JA perception is impaired; therefore, plants are able to grow normally on JA-containing medium, but Vsp proteins do not accumulate (Feys at al., 1994). In contrast to wild-type plants, the JA-responsive group of genes, including *Tat*, *Vsp*, *Jr1*, *Jr2*, and *Jr3*, are not induced in *coi1* plants upon wounding, thus confirming the role of JA in mediating their activation in injured plants. It is interesting that transcripts for the group of non-JA-responsive genes accumulate in *coi1* in response to wounding, revealing the presence of a wound-signaling pathway that does not depend on JA perception for its activity. The existence of a JA-independent pathway is also supported by the wound-induced expression of the glutathione *S*-transferase gene in a JA-deficient *A. thaliana* mutant (McConn et al., 1997).

The levels of *Aco* transcript attained in wounded *coi1* plants are lower than in the wild-type plants and are restricted to the damaged tissue, indicating that, although its wound activation is not dependent on JA as a mediator, the expression of *Aco* in wild-type plants in response to wounding may involve both JA-dependent and -independent pathways. Wound-induced *Ck* and *Wr3* levels are similar in *coi1* and wild-type plants, both in local and systemic leaves, indicating that, in contrast to *Aco*, induction of *Ck* and *Wr3* expression is fully independent of JA. Taken together, our results suggest that at least two signaling pathways are activated upon wounding: one of them does not require JA and is principally responsible for gene activation in the vicinity of the wound site, and the other involves JA perception and most likely an increase in the endogenous levels of this hormone (Laudert et al., 1996), which would activate gene expression throughout the aerial part of the plant, both locally and systemically to the wound site. Some components that are differentially involved in the JA-dependent and -independent wound-signaling pathways have been identified (Rojo et al., 1997). Their mode of regulation suggests cross-talk between the pathways in the control of the wound response.

The requirement of ABA for wound-induced *Pin2* expression in tomato and potato is well established (Peña-Cortés et al., 1989; Hildmann et al., 1992; Peña-Cortés et al., 1995), although the role ABA plays in these plant species may differ (Peña-Cortés et al., 1989). The fact that ABA is able to activate the expression of the majority of wound-inducible genes analyzed here above basal levels suggests a role for this hormone in wound signaling in *A. thaliana*. In most cases, however, only weak activation of gene expression has been observed, which may be due to poor uptake or may be related to side effects from other processes (such as stomatal closure), which are also triggered by ABA treatment. The involvement of ABA in mediating wound-inducible gene activation in *A. thaliana* will certainly require further analysis.

It is known that genes involved in stress responses often show a complex pattern of organ-specific expression. The potato *Pin2* gene, for example, is constitutively expressed in tubers and immature floral buds (Hildmann et al., 1992). The *Vsp* genes from soybean and *A. thaliana* are active in young, developing organs and flowers (Mason and Mullet, 1990; Berger et al., 1995). The wound-inducible genes described here have low basal levels of expression in nontreated plants. However, *Vsp* transcripts accumulate to high levels in flowers, roots, and green siliques. The *Jr1* transcript also has a high level of accumulation in roots and, to a lesser extent, in flowers. The role of these gene products in the physiology of these organs is not known. Vsp may serve as a temporary reservoir for N_2, as has already been postulated for its wound-induced presence in leaves (Staswick, 1990). In the case of *Jr1*, further speculation is hampered by the lack of significant similarities in the databases, which could give some indication of function.

Some of the wound-induced genes identified show striking homologies to housekeeping genes previously described in nonplant species; examples are choline kinase, acyl-CoA oxidase, and aminotransferases. In potato and tomato the expression of several metabolic genes is upregulated by wounding in a similar way as the *Pin2* gene, but their role in the wound response is still poorly understood (Hildmann et al., 1992; Bergey et al., 1996). We can speculate on the implication of the metabolic genes described here in maintaining basal cell functions with additional requirements in the wound response, for instance, in cell membrane synthesis or maintenance. The *Aco* enzyme catalyzes the first step in the fatty acid β-oxidation pathway and, therefore, may be involved in the last steps of JA synthesis. However, in animals *Aco* activation forms part of the response to leukotriene-mediated inflammation (Devchang et al., 1996) by catabolizing these stress-related second messengers. Because the chemical structure and biosynthetic pathway of JA are very similar to those of prostaglandins and leukotrienes (Vick and Zimmerman, 1987), we speculate that the *Aco* action in the plant response to wounding may include a role in JA catabolism, thus temporally limiting the extension of the JA-mediated wound response.

One isolated clone (*Jr3*) has significant homology to *IIr1*, an amidohydrolase of *A. thaliana* that releases active IAA (auxin) from conjugates (Bartel and Fink, 1995). Because auxins inhibit the activation of stress-related genes in dif-

ferent plant species (Kernan and Thornburg, 1989; DeWald et al., 1994; Rojo et al., 1997), a possible role of that enzyme in the damaged plant may be the release of free auxins to check the strength of the wound response. Therefore, *Aco* and *Jr3* gene products may play a role in feedback regulation of wound-induced gene expression.

It is interesting that the induction of *Aco* and *Jr3* upon wounding appears to be coordinately regulated with regard to spatial distribution, sensitivity to JA, and duration of expression. The generation of transgenic plants bearing the sense or antisense gene constructions for these genes will enable us to establish their role in stress responses. Characterization of the promoter regions of genes belonging to JA-dependent and -independent wound-signaling pathways will help us to clarify the role of this phytohormone and other regulators in mediating the plant response to mechanical wounding.

ACKNOWLEDGMENTS

We are indebted to J.G. Turner for kindly providing the *coi1* seeds and to C. Castresana, S.D. Jackson, and G. Vancanneyt for critical reading of the manuscript. The *A. thaliana* cDNA libraries used for the isolation of homologs to DD fragments were kindly provided by G. Vancanneyt. The excellent technical assistance of Tomás Cascón is gratefully acknowledged. We also would like to thank Inés Poveda and Angel Sanz for the photographic work.

Received April 21, 1997; accepted July 11, 1997.

LITERATURE CITED

Albrecht T, Kehlen A, Stahl K, Knofel H-D, Sembdner G, Weiler EW (1993) Quantification of rapid, transient increases in jasmonic acid in wounded plants using a monoclonal antibody. Planta **191:** 86–94

Amasino RM (1986) Acceleration of nucleic acid hybridisation rate by polyethylene glycol. Anal Biochem **152:** 304–307

Aoyama T, Tsushima K, Souri M, Kamijo T, Suzuki Y, Shimozawa N, Orii T, Hashimoto T (1994) Molecular cloning and functional expression of a human peroxisomal acyl-coenzyme A oxidase. Biochem Biophys Res Commun **198:** 1113–1118

Bartel B, Fink GR (1995) *ILR1*, an amidohydrolase that releases active indole-3-acetic acid from conjugates. Science **268:** 1745–1748

Benedetti CE, Xie D, Turner JG (1995) *COI1*-dependent expression of an *Arabidopsis* vegetative storage protein in flowers and siliques and in response to coronatine or methyl jasmonate. Plant Physiol **109:** 567–572

Berger S, Bell E, Mullet JE (1996) Two methyl jasmonate-insensitive mutants show altered expression of *AtVsp* in response to methyl jasmonate and wounding. Plant Physiol **111:** 525–531

Berger S, Bell E, Sadka A, Mullet JE (1995) *Arabidopsis thaliana AtVsp* is homologous to soybean *VspA* and *VspB*, genes encoding vegetative storage protein acid phosphatases, and is regulated similarly by methyl jasmonate, wounding, sugars, light and phosphate. Plant Mol Biol **27:** 933–942

Bergey DR, Howe GA, Ryan CA (1996) Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proc Natl Acad Sci USA **93:** 12053–12058

Bishop PD, Makus DJ, Pearce G, Ryan CA (1981) Proteinase inhibitor-inducing factor activity in tomato leaves resides in oligosaccharides enzymically released from cell walls. Proc Natl Acad Sci USA **78:** 3536–3540

Bomtempi EJ, Búa J, Aslund L, Porcel B, Segura EL, Henriksson J, Orn A, Pettersson U, Ruiz AM (1993) Isolation and characterization of a gene from *Trypanosoma cruzi* encoding a 46-kilodalton protein with homology to human and rat tyrosine aminotransferase. Mol Biochem Parasitol **59:** 253–262

Bowles D (1991) The wound response of plants. Curr Biol **1:** 165–167

Chang C (1996) The ethylene signal transduction pathway in *Arabidopsis*: an emerging paradigm? Trends Biochem Sci **21:** 129–133

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA **92:** 4114–4119

Creelman RA, Tierney ML, Mullet JE (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc Natl Acad Sci USA **89:** 4938–4941

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep **1:** 19–21

Devchang PR, Keller H, Peters JM, Vazquez M, Gonzalez FJ, Wahli W (1996) The PPARa-leukotriene B_4 pathway to inflammation control. Nature **384:** 39–43

DeWald DB, Sadka A, Mullet JE (1994) Sucrose modulation of soybean *Vsp* gene expression is inhibited by auxin. Plant Physiol **104:** 439–444

Dmochowska A, Dignard D, Maleszka R, Thomas DY (1990) Structure and transcriptional control of the *Saccharomyces cerevisiae POX1* gene encoding acyl-coenzyme A oxidase. Gene **88:** 247–252

Farmer EE, Johnson RR, Ryan CA (1992) Regulation of expression of proteinase inhibitor genes by methyl jasmonate and jasmonic acid. Plant Physiol **98:** 995–1002

Farmer EE, Ryan CA (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA **87:** 7713–7716

Feys BJF, Benedetti CE, Penfold CN, Turner JG (1994) Arabidopsis mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6:** 751–759

Hargrove JL, Scoble HA, Mathews WR, Baumstark BR, Biemann K (1989) The structure of tyrosine aminotransferase. J Biol Chem **264:** 45–53

Hildmann T, Ebneth M, Peña-Cortés H, Sánchez-Serrano JJ, Willmitzer L, Prat S (1992) General roles of abscisic and jasmonic acids in gene activation as a result of mechanical wounding. Plant Cell **4:** 1157–1170

Hobbie L, Timpte C, Estelle M (1994) Molecular genetics of auxin and cytokinin. Plant Mol Biol **26:** 1499–1519

Hosaka K, Kodaki T, Yamashita S (1989) Cloning and characterization of the yeast *CK1* gene encoding choline kinase and its expression in *Escherichia coli*. J Biol Chem **264:** 2053–2059

Kakimoto T (1996) CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science **274:** 982–985

Kernan A, Thornburg RW (1989) Auxin levels regulate the expression of a wound-inducible proteinase inhibitor II-chloramphenicol acetyl transferase gene fusion *in vitro* and *in vivo*. Plant Physiol **91:** 73–78

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (1993) CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell **72:** 427–441

Laudert D, Pfannschmidt U, Lottspeich F, Hollander-Czytko H, Weiler EW (1996) Cloning, molecular and functional characterization of *Arabidopsis thaliana* allene oxide synthase (CYP 74), the first enzyme of the octadecanoid pathway to jasmonates. Plant Mol Biol **31:** 323–335

Leung J, Bouvier-Durand M, Morris P-C, Guerrier D, Chefdor F, Giraudat J (1994) *Arabidopsis* ABA response gene *ABI1*: features of a calcium-modulated protein phosphatase. Science **264:** 1448–1452

Liang P, Pardee A (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science **257:** 967–971

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

Mason HS, Mullet JE (1990) Expression of two soybean vegetative storage protein genes during development and in response to water deficit, wounding, and jasmonic acid. Plant Cell **2:** 569–579

McConn M, Creelman RA, Bell E, Mullet JE, Browse J (1997) Jasmonate is essential for insect defense in *Arabidopsis*. Proc Natl Acad Sci USA **94:** 5473–5477

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in *Arabidopsis thaliana*. Science **264:** 1452–1455

Miyazawa S, Hayashi H, Hijikata M, Ishii N, Furuta S, Kagamiyama H, Osumi T, Hashimoto T (1987) Complete nucleotide sequence of cDNA and predicted amino acid sequence of rat acyl-CoA oxidase. J Biol Chem **262:** 8131–8137

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 493–497

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ (1996). Ethylene as a signal mediating the wound response of tomato plants. Science **274:** 1914–1917

Pearce G, Strydom D, Johnson S, Ryan CA (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science **253:** 895–898

Peña-Cortés H., Albrecht T, Prat S, Weiler EW, Willmitzer L (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta **191:** 123–128

Peña-Cortés H, Fisahn J, Willmitzer L (1995) Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. Proc Natl Acad Sci USA **92:** 4106–4113

Peña-Cortés H, Prat S, Atzorn R, Wasternack C, Willmitzer L (1996) Abscisic acid-deficient plants do not accumulate proteinase inhibitor II following systemin treatment. Planta **198:** 447–451

Peña-Cortés H, Sanchez-Serrano JJ, Mertens R, Willmitzer L, Prat S (1989) Abscisic acid is involved in the wound-induced expression of the proteinase inhibitor II gene in potato and tomato. Proc Natl Acad Sci USA **86:** 9851–9855

Peña-Cortés H, Sanchez-Serrano JJ, Rocha-Sosa M, Willmitzer L (1988) Systemic induction of proteinase inhibitor II gene expression in potato plants by wounding. Planta **174:** 84–89

Rettenmeier R, Natt E, Zentgraf H, Scherer G (1990) Isolation and characterization of the human tyrosine aminotransferase gene. Nucleic Acid Res **8:** 3853–3861

Rojo E, Titarenko E, Leon J, Berger S, Jancanneyt G, Sánchez-Serrano JJ (1997) Reversible protein phosphorylation regulates jasmonic acid dependent and independent wound signal transduction pathways in *Arabidopsis thaliana*. Plant J (in press)

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Staswick PE (1990) Novel regulation of vegetative storage protein genes. Plant Cell **2:** 1–6

Staswick PE, Su W, Howell SH (1992) Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc Natl Acad Sci USA **89:** 6837–6840

Uchida T, Yamashita S (1992) Molecular cloning, characterization, and expression in *Escherichia coli* of a cDNA encoding mammalian choline kinase. J Biol Chem **267:** 10156–10162

Vick BA, Zimmerman DC (1987) Oxidative systems for modification of fatty acids: the lipoxygenase pathway. *In* PK Stumpf, EK Conn, eds, The Biochemistry of Plants, Vol 9: Lipids. Academic Press, New York, pp 53–90