Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 4106–4113, May 1995
Colloquium Paper

This paper was presented at a colloquium entitled "Self-Defense by Plants: Induction and Signalling Pathways," organized by Clarence A. Ryan, Christopher J. Lamb, André T. Jagendorf, and Pappachan E. Kolattukudy, held September 15–17, 1994, by the National Academy of Sciences, in Irvine, CA.

Signals involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants

(abscisic acid/electrical current/jasmonic acid/salicylic acid/systemin)

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ABSTRACT **Chemical and physical signals have been reported to mediate wound-induced proteinase inhibitor II (*Pin2*) gene expression in tomato and potato plants. Among the chemical signals, phytohormones such as abscisic acid (ABA) and jasmonic acid (JA) and the peptide systemin represent the best characterized systems. Furthermore, electrical and hydraulic mechanisms have also been postulated as putative *Pin2*-inducing systemic signals. Most of the chemical agents are able to induce *Pin2* gene expression without any mechanical wounding. Thus, ABA, JA, and systemin initiate *Pin2* mRNA accumulation in the directly treated leaves and in the nontreated leaves (systemic) that are located distal to the treated ones. ABA-deficient tomato and potato plants do not respond to wounding by accumulation of *Pin2* mRNA, therefore providing a suitable model system for analysis of the signal transduction pathway involved in wound-induced gene activation. It was demonstrated that the site of action of JA is located downstream to the site of action of ABA. Moreover, systemin represents one of the initial steps in the signal transduction pathway regulating the wound response. Recently, it was reported that heat treatment and mechanical injury generate electrical signals, which propagate throughout the plant. These signals are capable of inducing *Pin2* gene expression in the nontreated leaves of wounded plants. Furthermore, electrical current application to tomato leaves leads to an accumulation of *Pin2* mRNA in local and systemic tissues. Examination of photosynthetic parameters (assimilation and transpiration rate) on several types of stimuli suggests that heat-induced *Pin2* gene expression is regulated by an alternative pathway from that mediating the electrical current and mechanical wound response.**

Plants react to wounding and pathogen attack by activating a set of genes, most of which play a role in wound healing and prevention of a subsequent pathogen invasion. Some of these genes are expressed in the vicinity of the wound site while others are also systemically activated in the nondamaged parts of the plant. The potato and tomato proteinase inhibitor II (*Pin2*) gene families are the best studied examples of genes that are systemically activated upon mechanical damage (1, 2). This gene family is constitutively expressed in potato tubers and in the early stages of floral development. Young floral buds accumulate *Pin2* mRNA which is absent in the organs of the fully developed potato flower. In contrast to potato, tomato flowers accumulate *Pin2* mRNA, but adult flowers exhibit readily detectable levels in virtually every organ (3). In addition to its constitutive expression in tubers and flowers, *Pin2* mRNA accumulates in the foliage of both potato and tomato plants upon wounding by either mechanical damage or herbivore feeding. Transcriptional activation of the *Pin2* genes upon wounding is not confined to the site of injury. Indeed, the nondamaged leaves of a wounded plant readily accumulate *Pin2* mRNA, but after a short delay as compared to the directly wounded ones. Lower levels of *Pin2* mRNA are observed in the systemically induced leaves compared to the locally induced ones. This systemic induction is likely to be related to the synthesis or release of a wound signal at the site of the injury, which migrates throughout the plant activating *Pin2* genes in distal tissues. Nonwounded leaves both above and below the wound site accumulate *Pin2* mRNA, suggesting that the signal is most likely transported via the phloem (4).

Several different stimuli have been shown to induce *Pin2* mRNA accumulation in leaves and therefore have been suggested to play a role in the transduction of environmental or developmental cues to *Pin2* expression. Plant cell wall-derived oligosaccharides with different degrees of polymerization are able to activate *Pin2* gene expression, and they were therefore assumed to be the proteinase inhibitor inducing factor (5). Expression of the *Pin2* gene family is also initiated in detached leaves supplied with chitosan, a β-1,4-glucosamine homopolymer present in fungal cell walls (4). By using radiolabeled oligosaccharides, however, Baydon and Fry (6) demonstrated that molecules with a degree of polymerization greater than 6 do not travel long distances within the plant vascular system. This observation provides evidence that systemic activation of the *Pin2* gene is independent of these components. Rather, these compounds are thought to be released from the wounded tissues as early signals in the pathway that ultimately leads to both localized and systemic wound-induced expression of *Pin2* genes.

On the other hand, the phytohormone auxin prevents wound-induced *Pin2* gene expression. Exogenous auxin is able to act as a repressor of the wound-inducible activation of a chimeric *Pin2*–CAT (chloramphenicol acetyltransferase) gene in transgenic tobacco callus and in whole plants (7). In addition, the endogenous levels of indole-3-acetic acid (IAA) decline 2- to 3-fold within 6 h after wounding. The kinetics of auxin decline correlate inversely with the activation kinetics of the *Pin2*–CAT construct in the foliage of transgenic tobacco. These results suggest that the endogenous levels of IAA in unwounded plant tissues are sufficient to maintain the inhibitor II gene system in a repressed state. However, upon wounding, the levels of IAA in bulk tissues decline, allowing

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Abbreviations: CAT, chloramphenicol acetyltransferase; ABA, abscisic acid; JA, jasmonic acid; LA, α-linolenic acid; LOX, lipoxygenase; 13HPLA, 13-hydroperoxylinolenic acid; MeJA, JA methyl ester; 12-oxoPDA, 12-oxophytodienoic acid; COX, cyclooxygenase.

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a derepression of the *Pin2* gene system with concomitant expression of the CAT protein under control of the *Pin2* promoter (8).

Additional signaling mechanisms involved in regulation of the wound response have been reported and diverse theories have been advanced regarding the nature of the systemic signal transmitted from wound sites. In particular, changes in membrane polarity are among the first effects detected in wounded plants. These alterations of the membrane polarity are systemically transmitted, but it is still unclear whether they are responsible for the systemic induction of genes or whether they are simply a result of the systemic changes that occurred upon wounding (9). Phytohormones such as abscisic acid (ABA) (10) and jasmonic acid (JA) derivatives (11–14) are able to induce *Pin2* gene activation. Other substances, including sucrose, also induced *Pin2* gene expression without wounding (14). In addition, a peptide mediating the systemic wound response of *Pin1* and *Pin2* genes in wounded tomato leaves has been reported (15). This molecule, systemin, is an 18-amino acid oligopeptide, rich in proline and basic amino acids, and it has been shown to move systemically in the phloem to distant plant tissues. On the other hand, many of the stimuli that initiate systemic responses in plants are also known to cause simultaneous electrical activity. Furthermore, an increasing number of plant species have been shown to be able to transmit action potentials and variation potentials (16). It has been reported recently that an electrical signal might be the messenger for systemic induction of *Pin2* gene expression (17). After wounding or heat treatment of tomato plants, a close relation is observed between the induced electrical signals and the activation of *Pin2* gene expression. The signal is propagated from the wound site to the systemic tissue via an unknown conductive pathway that does not involve the phloem. The signal travels apparently unhindered over significant distances along the stem, which consists of alternating live and dead (at least transitory) regions. Fromm and Eschrich (18) have recently reported that willow trees generate action potentials that are propagated throughout the plant at velocities of 2–5 $cm \cdot sec^{-1}$ after root stimulation. In the present study, we report on experiments to characterize the signal transduction pathway mediating wound-induced *Pin2* gene expression in tomato and potato plants.

Wound-Induced *Pin2* Gene Expression and Involvement of ABA

The plant growth regulator ABA appears to play a predominant role in the conversion of environmental signals into changes in plant gene expression (19). Recently, strong evidence has been provided for the involvement of ABA in the induction of *Pin2* gene expression upon wounding. In a series of experiments, potato plants were sprayed with 100 μM ABA. Subsequently, *Pin2* mRNA accumulates in the absence of any wounding (10). Tissue that was sprayed directly, as well as nontreated leaves, exhibited increased *Pin2* expression. This accumulation was tissue specific and detectable in leaves and stems but not in roots or the lower part of the stems. ABA that was sprayed on the leaves of a plant was able to trigger systemic induction of the *Pin2* gene with a pattern that was identical to the one described for wounded plants (4). In contrast, no activation of *Pin2* could be detected in tomato or transgenic tobacco plants that contained a wound-inducible potato *Pin2* gene when these were treated with ABA. An enhancement of *Pin2* mRNA accumulation was seen in these plants only upon incubation of detached leaves in an ABA solution, suggesting that differences in ABA absorption through the epidermis between potato and tomato or tobacco leaves might be responsible for the contrasting results. Conclusive evidence for the involvement of ABA in wound-induced *Pin2* activation was obtained by analyzing the wounding effect in mutant plants impaired in ABA synthesis. These plants provide an ideal control for the experiments that are based on external application of ABA. The potato mutant droopy and the sitiens (*sit*) mutant of tomato exhibit a mutation that blocks the last step in ABA biosynthesis, the conversion of ABA aldehyde to ABA (20, 21). The altered phenotype of these mutants is a result of a lower endogenous level of ABA (9–12%) and can be reversed by exogenous application of this hormone. Wound induction of *Pin2* was not observed in mutants of potato and tomato deficient in the synthesis of ABA (10). However, ABA treatment causes an accumulation of *Pin2* mRNA to levels normally found in wild-type plants upon wounding. Moreover, endogenous ABA levels increase, both locally and systemically, in wild-type plants upon wounding but not in the droopy mutant (3).

Both the local accumulation of *Pin2* mRNA around the site of injury and the systemic activation of *Pin2* transcription in the distal nonwounded tissue are affected by ABA deficiency. In mutant plants, very low levels of *Pin2* mRNA were detected in the tissue closest to the wounding site, and *Pin2* mRNA concentration was below the limits of detection in the systemic foliage (3). This very low *Pin2* mRNA accumulation in the vicinity of the injury is consistent with the low ABA levels present in the mutant plants (21, 22) and suggests that ABA is involved in the release of a local wound signal or, alternatively, that it itself is the local signal. The lack of *Pin2* accumulation in the tissue distal to the wound site indicates that ABA is also involved in the systemic induction of *Pin2*, either by preventing the formation of a signal at the site of the injury, which subsequently migrates to the distal tissue, or by acting as the systemic signal itself. External ABA is also able to induce systemic expression of the *Pin2* gene family. Potato plants sprayed with ABA in the lower part of the foliage accumulate *Pin2* mRNA not only in the tissue treated directly but also in the upper part of the foliage, which was not sprayed (3). When performing this experiment in the ABA-deficient droopy plants, it was possible to demonstrate that the ABA levels increase in the distal nonsprayed tissue to levels normally found in the systemically induced wild-type plants (Fig. 1*A*). Accumulation of *Pin2* mRNA in the distal, nonsprayed

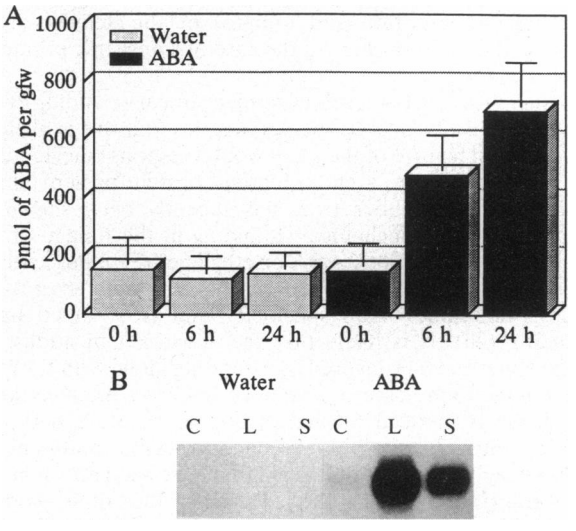

FIG. 1. (*A*) Systemic accumulation of ABA in droopy plants. Potato ABA-deficient plants were sprayed either with water or with 100 μM ABA. Nonsprayed leaves, located distal to the treated leaves, were harvested 6 and 24 h after treatment and the endogenous levels of ABA per g fresh weight (gfw) were measured. (*B*) ABA initiated *Pin2* mRNA accumulation in both local and systemic tissues. Total RNA was isolated from both sprayed leaves (lanes L) and from leaves located distal to the treated ones (lanes S). RNA gel blots were hybridized with *Pin2* cDNA (4). Lanes C, controls.

tissue correlates with the increase in the hormone concentration (Fig. 1*B*). Since droopy plants are not able to synthesize ABA *de novo*, the high ABA levels detected are most likely due to the migration of exogenous ABA to the distal, nonsprayed tissue. These results demonstrate the ability of ABA to migrate throughout the plant and thereby trigger the expression of the *Pin2* gene family in distal tissues in a manner similar to systemic induction upon wounding.

While ABA is involved at some stage during wound-induced gene activation, constitutive *Pin2* expression in potato flowers and tubers is apparently not affected by the block in the ABA biosynthetic pathway. The ABA-deficient droopy mutant has wild-type *Pin2* mRNA levels in tubers and flower buds, suggesting that the presence of different or additional factors is involved in *Pin2* gene activation in tubers and flowers (3). Alternatively, the different modes of expression of *Pin2* reflect a differential expression of members of the gene family. Another possibility is that one or several members of the gene family might be active in some or all of these states. Transgenic potato plants containing a gene fusion consisting of a *Pin2* promoter fused to a β-glucuronidase (GUS) reporter showed constitutive GUS activity in tubers and floral buds and would-induced activity in leaves (23). In addition, this construction endows transgenic ABA-deficient droopy plants with constitutive GUS activity in tuber and floral buds and ABA-induced activity in leaves (3). Therefore, the expression of a single promoter element mirrors the expression pattern of the whole gene family.

ABA Involvement in Wound Response

The *in vivo* involvement of ABA in the gene activation processes that follows mechanical damage of the plant tissue is supported by the fact that the endogenous ABA concentration increases 3- to 5-fold upon wounding. This elevation is not restricted to the tissue that has been damaged directly but can also be detected in the nonwounded systemically induced tissue (3). This phenomenon is common to several plant species. ABA increases upon wounding have been detected in potato, tomato, and tobacco leaves (24). Furthermore, in all three plant species, a correlation is established between the ABA increase and either the expression of the *Pin2* gene family (in the case of potato and tomato) or the activity of an introduced *Pin2* promoter (in the case of transgenic potato or tobacco plants) (10).

An increase of ABA levels after mechanical wounding of the foliage of nonsolanaceous species suggests that this is likely to be a common feature of the plant wound response mechanism. For instance, hevein, a chitin-binding protein present in the laticifers of the rubber tree, has recently been shown to accumulate upon mechanical wounding in the foliage of the tree but not in the roots. ABA or ethylene treatment leads to an increase in the hevein mRNA level, whose expression displays the same organ specificity that is obtained upon wounding (25). It is likely that the activation of additional potato genes can be triggered by wounding along with the *Pin2* gene family. Expression of a gene coding for a highly anionic peroxidase is induced during healing of potato tubers and tomato fruits. This gene also responds to ABA treatment; its mRNA accumulates in potato calli that have been grown in 100 μM ABA (26). Similarly, the isolation of four other wound- and ABA-responsive genes was recently reported (27). The distribution of the corresponding mRNAs in the different organs of nonwounded, wounded, and ABA-treated wild-type and mutant potato plants shows that wounding or ABA treatment leads to a pattern of expression of these genes that is very similar to that of *Pin2*, thus supporting the direct involvement of this hormone in signal transduction of mechanical damage. ABA's involvement in signal transduction has been found in monocots. A *Pin2* promoter activity is induced in transgenic rice plants locally and systemically after wounding or after ABA or JA application. This finding suggests that there is also a systemic signaling mechanism operating in rice that responds to mechanical wounding and that the wound response signal and certain steps of the transduction pathways are conserved among dicots and monocots (28). Furthermore, wound-inducible proteinase inhibitor from maize, displaying 39% sequence identity to the tomato *Pin1*, accumulated in leaves upon ABA treatment (29). Together, these results indicate that ABA might mediate the wound response in both dicotyledonous and monocotyledonous species.

Wound-Induced *Pin2* Gene Expression and Involvement of JA

JA treatment results in plant responses similar to those caused by ABA treatment. Several ABA-induced proteins can be detected upon incubation of barley leaves in a JA solution, and these JA-induced proteins are immunologically related to the proteins accumulating upon ABA treatment (30). Interestingly, both ABA and JA have been associated with wound-induced gene expression in diverse plant species (31). However, the expression of some of these genes is also affected by other factors, showing the complexity of their regulation. JA is synthesized from α-linolenic acid (LA) by a lipoxygenase (LOX)-mediated oxygenation leading to 13-hydroperoxylinolenic acid (13HPLA), which is subsequently transformed to JA by the action of hydroperoxide dehydratase and additional modification steps (32). Both JA and its methyl ester (MeJA) are thought to be significant components of the signaling pathway regulating the wound response in higher plants. Airborne MeJA and JA (11), as well as intermediates of the JA biosynthetic pathway such as LA, 13HPLA, and 12-oxophytodienoic acid (12-oxoPDA) also lead to an accumulation of *Pin2* mRNA in tomato leaves (33). Likewise, we have demonstrated that JA strongly induces *Pin2* (13, 14) as well as other ABA-responsive/wound-induced genes in potato leaves (27). More interestingly, treatment of potato leaves with JA results in similar levels of mRNA accumulation in both wild-type and ABA-deficient mutant plants (Fig. 2). These data suggest that JA is involved in a step downstream of ABA in the pathway that links wounding to *Pin2* gene activation. In other words, JA could bypass the initial recognition events requiring ABA and thus trigger the induction of the genes even in the absence of ABA. Nevertheless, we have observed that JA-induced *Pin2* gene activation, like ABA-induced *Pin2* gene expression, can be blocked by cycloheximide. This result suggests that some later step, necessary for *Pin2* gene activation, depends on *de novo* protein biosynthesis.

Both the chemical structure as well as the biosynthetic pathway of JA resemble those of the mammalian eicosanoids (prostaglandins and leukotrienes), which are derived from LOX- and cyclooxygenase (COX)-mediated reactions (32, 34).

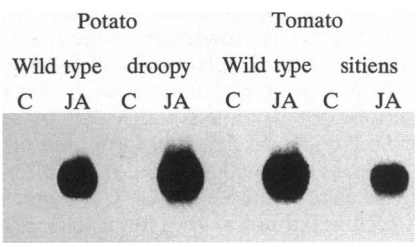

FIG. 2. JA-activated *Pin2* gene expression in both wild-type and ABA-deficient plants. Potato and tomato plants (wild type and ABA mutants) were sprayed with an aqueous solution containing 50 μM JA. Total RNA was isolated from treated leaves (lanes JA) and from plants treated with water (lanes C). Northern blot analysis was done as described (4).

Tomato and potato plants treated with different mammalian LOX and COX inhibitors show different levels of *Pin2* mRNA as well as other wound-inducible genes (i.e., cathepsin d inhibitor and threonine deaminase) (35). The mammalian COX inhibitor aspirin (acetylsalicylic acid) is one of the most effective inhibitors, blocking both local and systemic accumulation of wound-induced *Pin2* gene activation. This result suggests a common feature in both local and systemic activation of this wound-induced gene (35).

Mechanical wounding increases the endogenous level of JA in soybean (36). In tomato, wounding also leads to an increase of JA levels after 6 h. Thereafter, levels of JA decline, being slightly higher at 24 h than in the control leaves. Tomato plants pretreated with aspirin and subsequently wounded, however, show the same low levels of JA as nonwounded plants (35). Therefore, aspirin blocks a step in the JA biosynthetic pathway, thus preventing the synthesis of JA upon wounding and wound-induced *Pin2* gene expression. These results strongly support the involvement of endogenous JA levels in wound response.

Detached leaves of tomato plants supplied with different intermediates of the JA biosynthetic route in the presence or absence of COX and LOX inhibitors demonstrate that neither wounding, ABA, LA, nor 13HPLA is able to complement the inhibition mediated by all inhibitors tested (Table 1). Conversely, 12-oxoPDA and JA are also able to overcome the inhibitory effect of the substances examined. The effect of ABA on aspirin-mediated inhibition was difficult to assess because of the low levels of *Pin2* gene expression. The fact that LA and 13HPLA do not restore the accumulation of *Pin2* mRNA in the presence of aspirin or propyl gallate, whereas 12-oxoPDA does overcome this inhibition, suggests that hydroperoxide dehydrase, which mediates the formation of 12-oxoPDA from 13HPLA, is the target of these inhibitors. Furthermore, the fact that ABA appears not to be able to overcome the inhibitory effect of aspirin in tomato and that JA suppresses the inhibition by all inhibitors supports the assumption that the step in the signal transduction chain in which JA is involved is located downstream of ABA (35).

Systemin-Induced *Pin2* Gene Activation

Pearce *et al.* (15) recently described a peptide termed systemin, which is able to move throughout the vascular tissue of the plant, inducing *Pin2* gene expression. Farmer and Ryan (33) proposed that this peptide may be released upon wounding and may be recognized by a membrane receptor in the distal tissue, which activates JA biosynthesis by triggering lipase activity.

In all the above-mentioned cases, putative wound signals have been demonstrated to induce *Pin2* gene activation without wounding. The hierarchy within the signaling pathway that regulates wound response is still unknown. Whether a similar or different set of signal transduction steps is active in both the wounded and the unwounded leaf needs to be investigated further (38, 39).

Table 1. Effect of animal LOX and COX inhibitors on *Pin2* mRNA accumulation

	Aspirin	Sham	p-Gallate	zk139
Wounding	−	−	−	−
ABA	−	−	−	−
LA	−	−	−	−
13HPLA	−	+	−	+
12oxoPDA	+	+	+	+
JA	+	+	+	+

Detached tomato leaves were pretreated with 1 mM aspirin, 1 mM salicylhydroxamic acid (sham), 1 mM propyl gallate (p-Gallate), or 100 μM zk139 and subsequently wounded or supplied with 100 μM ABA, 100 μM LA, 100 μM 13HPLA, 50 μM 12oxoPDA, or 50 μM JA. Total RNA was isolated and analyzed as described (37). +, Detectable *Pin2* mRNA levels; −, absence of *Pin2* mRNA.

It has been demonstrated that the phytohormones ABA and JA and the peptide systemin are involved in modulating the expression of *Pin2* genes. In addition, both ABA and JA initiate *Pin2* mRNA accumulation in ABA-deficient sitiens and droopy plants. These plants do not accumulate *Pin2* mRNA upon wounding, thus allowing examination of the relative position of systemin, ABA, and JA in triggering signaling gene expression. Systemin initiates *Pin2* mRNA accumulation in both tomato and potato wild-type plants. Conversely, the peptide does not activate *Pin2* gene expression in both sitiens and droopy plants (Fig. 3). In contrast, ABA-deficient plants treated with ABA do accumulate *Pin2* mRNA. These results suggest that systemin alone is not sufficient to trigger the induction of *Pin2* gene expression in ABA-deficient plants. Several reasons could account for this observation: (*i*) systemin does not trigger JA biosynthesis directly but requires the presence of another factor(s); (*ii*) ABA-deficient plants are unable to synthesize JA *de novo*; and (*iii*) the putative systemin receptor does not recognize the peptide.

LA, the precursor of JA, activates *Pin2* gene expression in both wild-type and ABA-deficient plants (Fig. 4*A*). In contrast, water alone or γ-linolenic acid does not initiate *Pin2* mRNA accumulation. Quantitation of endogenous levels of JA from the same leaf material showed an increase of endogenous JA levels in both wild-type and ABA-deficient plants after LA treatment (Fig. 4*B*). These data suggest that ABA-deficient plants are able to synthesize JA *de novo*, most likely by processing the applied LA.

Wounding initiates the accumulation of *Pin2* mRNA in damaged tissues as well as in unwounded distal leaves. In addition, mechanical damage leads to an increase of endogenous levels of either ABA or JA in both potato and tomato plants (10, 35). It has been proposed that changes in endogenous levels of ABA and JA mediate the wound response in these plants. Potato and tomato wild-type leaves also show increased endogenous levels of ABA and JA after systemin treatment (Table 2). Similar to wounding, the peptide does not affect the endogenous concentration of ABA or JA in ABA-deficient plants. Most importantly, exogenous ABA promotes an increase of endogenous JA levels in both wild-type and ABA-deficient plants. Conversely, potato or tomato leaves treated with JA do not show any change of internal ABA levels (Table 2) (27).

Electrical Current-Activated *Pin2* Gene Expression in Tomato Leaves

Many of the stimuli that initiate systemic responses in plants are also known to be able to transmit action potentials and variation potentials (16). Evidence for a link between electrical signaling and biochemical response was presented recently by Wildon *et al.* (17). The authors showed that wounding the cotyledon mechanically or treating with heat resulted in the

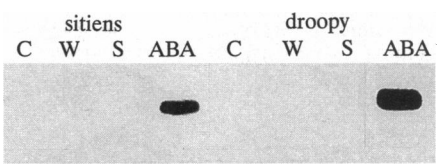

FIG. 3. Systemin did not activate *Pin2* gene expression in ABA-deficient plants. Tomato ABA-deficient (sitiens) and potato ABA-deficient (droopy) detached leaves were supplied with water (lanes C), with water and mechanically wounded (lanes W), with 1 μM systemin (lanes S), or with 100 μM ABA (lanes ABA). After 20 h of incubation, the detached leaves were harvested and total RNA was isolated. RNA gel blots were hybridized as described (4).

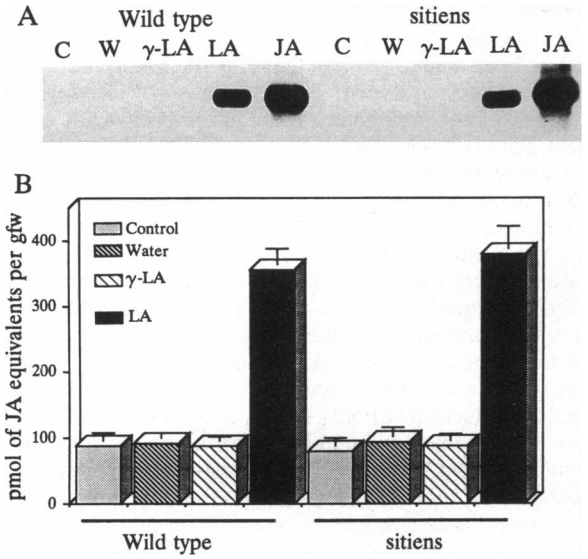

FIG. 4. (*A*) LA-activated *Pin2* gene expression in both wild-type and ABA-deficient tomato plants. Tomato wild-type and sitiens plants were sprayed with water containing 0.1% methanol (lane W), or with solution containing 300 μM γ-linolenic acid (lane γ-LA), 300 μM LA (lane LA), or 50 μM JA (lane JA). As a control (lane C), RNA was isolated from nontreated tomato leaves. Northern blot analysis was performed as described (4). (*B*) LA led to an accumulation of JA in both tomato wild-type and sitiens plants. The concentration of JA per g fresh weight (gfw) attained upon treatment was also determined.

slow transmission of an action potential out of the cotyledon and into the first leaf, where this correlated in all cases with the induction of *Pin2*. Thus, a propagated electrical signal might be the messenger for systemic induction of *Pin2*. However, definitive evidence demonstrating that electrical signals were propagated throughout the plant was not provided.

A convincing experiment to demonstrate the transmission of electrical signals in plant tissues would be the application of

Table 2. Systemin led to an increase of both ABA and JA levels

	ABA, pmol/gfw	JA equivalents, pmol/gfw
	Tomato	
Wild type		
Control	30	50
Wounding	1672	455
Systemin	1312	369
ABA		390
sitiens		
Control	5.0	65
Wounding	4.5	90
Systemin	3.3	78
ABA		480
	Potato	
Wild type		
Control	260	148
Wounding	1318	841
Systemin	966	1243
ABA		1166
droopy		
Control	94	135
Wounding	101	145
Systemin	98	120
ABA		740

Detached tomato and potato (wild type and ABA deficient) leaves were supplied with water (control), with water and wounded (wounding), with 1 μM of systemin per plant (systemin), or with 100 μM ABA. Probes were collected 8 h after treatment and endogenous levels of ABA and JA equivalents per g fresh weight (gfw) were analyzed.

electric current. We applied electrical currents to tomato leaves and investigated the effect of this stimulus on *Pin2* gene expression. Interestingly, electrical current stimulation leads to an accumulation of *Pin2* mRNA in both local and systemic tissue in a manner similar to that observed by wounding or heat treatment (Fig. 5*A*). Therefore, this suggests that plant tissues carry the electrical current stimulation, allowing both local and systemic activation of the pathway mediating wound-induced *Pin2* gene expression. Furthermore, systemic leaves from electrical current-treated plants showed increased levels of ABA similar to those observed after either mechanical wound or heat treatment (Fig. 5*B*), suggesting that all three stimuli might be translated by similar mechanisms.

Early observations have shown that action potentials can be generated and propagated in higher plants and regulate a wide variety of physiological responses. Fluctuations in CO_2 uptake were measured after the generation of action potentials in the root by potassium chloride (40). Van Sambeek and Pickard (41) measured shifts in CO_2 and H_2O exchange in undamaged leaves after the arrival of an electrical current released from adjacent leaves by damage. Modification of stomatal aperture detected by modifications in the transpiration and assimilation rate is always coupled to the flow of ions across the guard cell plasma membrane (42).

Gas exchange measurements before and after stimulus application (mechanical wound, heat, or current) show that mechanical injury and electrical current lead to a complex relaxation kinetics that is characterized by two time constants in the assimilation rate (*A*) and transpiration rate (*E*) (Fig. 6, local mechanical stimulation). The first time constant appears 2–3 min after stimulus application in both local and systemic tissue. In the 2- to 3-min period, a transient decline in the assimilation rate (*A*) is observed. This deflection is reversed within 5–7 min. At ≈8 min, the assimilation rate decreases again until a new steady state is reached. The transpiration rate (*E*) reflects the relaxation kinetics of the assimilation rate (Fig. 6). Both time constants observed in the assimilation rate are also present in the response of the transpiration rate.

Leaves located distal to the site of wounding or current application (Fig. 6, systemic mechanical, or electrical stimu-

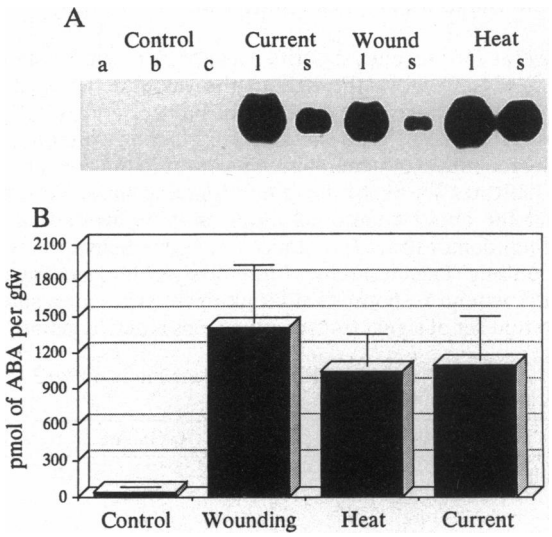

FIG. 5. (*A*) Electrical current-induced *Pin2* gene expression. Tomato plants were treated with 10 V (current), mechanically damaged (wound), or treated with fire (heat). After 8 h, the treated leaves (lanes l) as well as the distal nontreated leaves (lanes s) were collected, and total RNA was isolated. Control (lanes a–c) represents untreated plants. (*B*) Current stimulation, mechanical injury, and heat treatment led to an increase of endogenous levels of ABA. In nontreated, distally located leaves (see *A*, lanes s), ABA concentration per g fresh weight (gfw) was analyzed as described (10).

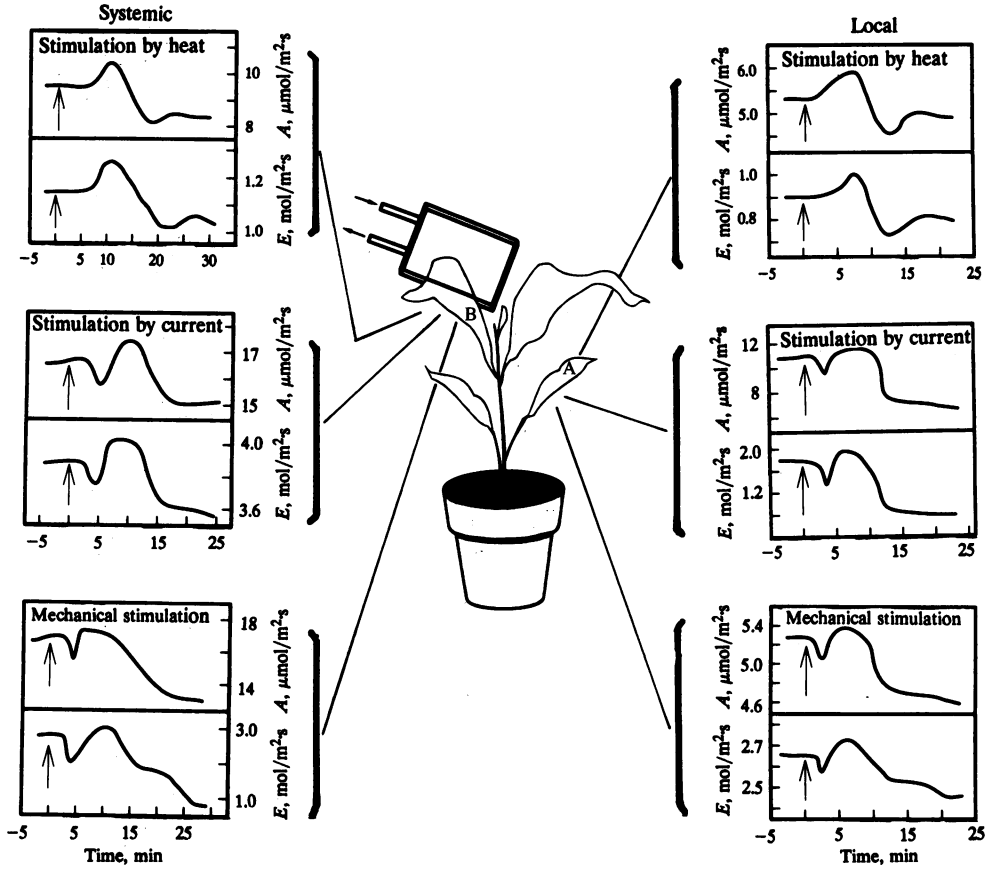

FIG. 6. Gas exchange experiments. Wound response was stimulated by electrical current, by mechanical damage, or by heat in tomato plants. Assimilation rate (*A*) and transpiration rate (*E*) were measured in both the treated leaf (leaf A) and in the leaf located above the treated one (leaf B).

lation) respond with a time course which resembles that of locally wounded leaves. However, a delay of the fast time constant is observed in the systemic tissues (Fig. 6, systemic, mechanical, or electrical stimulation).

Heat-induced *Pin2* gene expression has been correlated with characteristic gas exchange relaxation kinetics, which exhibit significant modifications in comparison to those due to mechanical wounding or current injection. In particular, the first response that is usually observed within 2–3 min upon mechanical injury or current treatment is absent (Fig. 6, local stimulation by heat). Nevertheless, the assimilation and transpiration rate start to decline at 10 min. This decrease resembles the slow kinetics induced by mechanical injury or electrical current stimulation.

In general, gas exchange measurements are offset by a tremendous scatter. For these reasons, we did not attribute any significance to the background levels in the assimilation and transpiration rates. A similar argument holds for the scatter in the time constants controlling assimilation and transpiration rates (43). On the basis of these fluctuations in the time constants, we were reluctant to compare the signal propagation velocities involved among the various responses. However, the results clearly indicate the involvement of two distinct time constants in response to mechanical and electrical stimulation. In contrast, heat stimulation is characterized by one major component exclusively.

Wound-Induced *Pin2* Gene Expression and Signal Transduction Pathway

ABA appears not to be the only regulator involved in the control of changes in gene expression that occur in response to wounding. Although water stress promotes an increase of endogenous ABA levels by 8- to 10-fold, this does not lead to any accumulation of *Pin2* mRNA or any of the other wound-inducible genes from potato (10, 27). In agreement with these results, accumulation of water stress-responsive genes appears to be independent of *de novo* protein synthesis, whereas accumulation of *Pin2* mRNA is not (10). These results indicate that different transduction mechanisms regulate these two ABA-mediated responses. Whereas ABA could directly mediate responses to osmotic stress, a more complex signaling pathway leads to transcriptional activation of the defense-related genes as the final result of the increased levels of ABA caused by wounding. The fatty acid derivative JA has been hypothesized to be a key component of intracellular signaling in response to wounding or pathogen attack. MeJA was shown to stimulate the accumulation of wound-inducible vegetative storage proteins in soybean plants and suspension culture (37, 44). In addition, JA and MeJA induced the expression of phenylalanine ammonia lyase genes that are known to be involved in the chemical defense mechanism of plants against pathogens (45). As mentioned above, MeJA and intermediates of the JA biosynthetic pathway lead to an accumulation of *Pin2* mRNA in both tomato and potato leaves (13, 14, 33) as well as all the known ABA-responsive/wound-induced genes (27). More interestingly, treatment of potato leaves with JA results in similar levels of mRNA accumulation in both wild-type and ABA-deficient mutants. These data suggest that the step involving JA is located downstream of the ABA requirement in the pathway that links wounding to *Pin2* gene activation. This is consistent with the association often found in plant responses to JA and ABA treatments (Fig. 7). In addition, JA can bypass the initial recognition events requiring ABA and thus trigger the induction of genes even in the absence of ABA. The fact that ABA appears not to be able to overcome the inhibitory

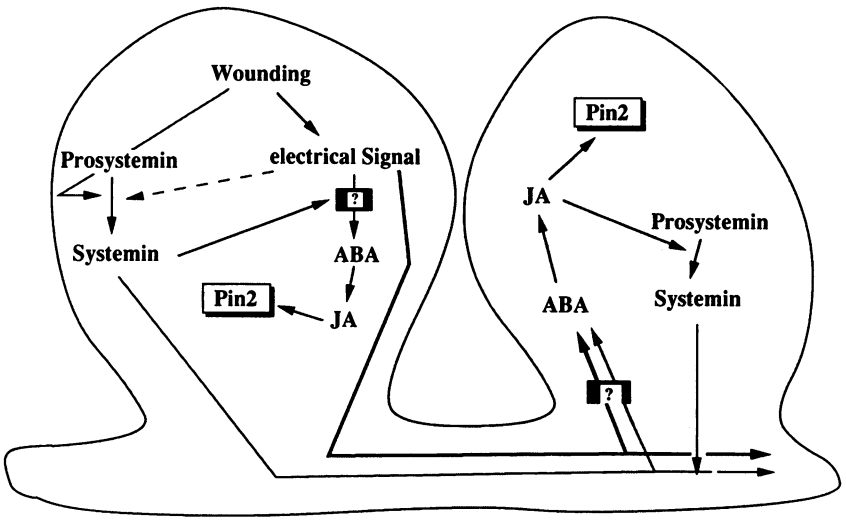

FIG. 7. Signals and predictable location of the steps mediating wound response in potato and tomato plants.

effect of aspirin in tomato and that JA suppresses the inhibition by all inhibitors further indicates that the step in the signal transduction chain in which JA is involved is located downstream of ABA (35). However, JA-induced *Pin2* gene activation can also be blocked by cycloheximide. This suggests that *de novo* protein biosynthesis is essential to produce the required factors involved in the final steps mediating *Pin2* gene activation.

Mechanical damage and the peptide systemin induce *Pin2* expression and lead to an increase of endogenous ABA and JA levels in wild-type plants. According to the model proposed (33), increased levels of ABA as a result of tissue injury might lead to the activation of a lipase in the plasma membrane and the release of LA or, alternatively, to the activation of specific LOXs that, when acting upon LA, could produce a rapid accumulation of JA. Both mechanical wounding and systemin neither induce the gene in ABA-deficient mutant plants nor increase endogenous ABA or JA levels. Their inability to activate gene expression is not due to the lack of JA production in ABA-deficient plants. Tomato leaves of ABA-deficient plants show increased levels of JA, similar to those found in wild-type plants, after treatment with LA. Therefore, the different steps involved in JA biosynthesis located downstream from LA are fully functional and allow JA biosynthesis in ABA-deficient plants. Whether mechanical damage or systemin acts by directly promoting ABA biosynthesis is still an open question. Although the process(es) occurring immediately after wounding remains unknown (Fig. 7), it can be assumed that either stimulus generates a response, involving a differential regulation of several systems that might be activated at the same time. For instance, endogenous auxin levels decline after wounding in tobacco plants (8). When transgenic tobacco plants that contain the CAT reporter gene under the control of a *Pin2* promoter were pretreated with increasing amounts of auxins, they showed reduced CAT activity upon wounding (7). Therefore, changes in the internal concentration of auxins are required for modulating wound-induced gene expression in plants.

The observations that both potato and tomato (wild type and ABA deficient) leaves show an increase of endogenous levels of JA upon ABA treatment but no ABA increase following JA application again suggest (illustrated in Fig. 7) (*i*) that the site of systemin action is located upstream of the site of ABA action, (*ii*) that the site of JA action is located downstream of ABA and systemin, and (*iii*) that ABA activates the JA biosynthetic pathway by some still unknown mechanism. Whatever the exact mechanism of ABA action, it is important to note that high levels of this phytohormone are required to trigger the JA signaling pathway.

Furthermore, tomato plants treated with electrical current accumulate *Pin2* mRNA in both the treated leaves and in the systemic untreated tissues. In addition, current application leads to an increase of ABA levels similar to those found upon wounding or heat treatment. Therefore, electrical current stimulation activates both the local and systemic signaling pathways mediating the wound response. Conversely, electrical current stimulation is unable to induce *Pin2* gene expression in ABA-deficient sitiens mutants (unpublished data). These results suggest that electrical current-induced steps, like systemin, are more likely located before the site of action of ABA.

In summary (see Fig. 7), mechanical injury (current application) may generate changes in plasma membrane potential, creating electrical signals that may propagate throughout the plant acting as the *Pin2* systemic inducing signal. The wound (electrical current)-induced action potential may cause variation of ion concentration, leading to the activation of a mechanism that elicits the release of active systemin. We cannot exclude that both stimuli generate both a chemical (systemin) and an electrical signal simultaneously. The next step in the signal transduction pathway involves an increase of ABA, which may turn on the biosynthesis of JA. Whether JA acts as a systemic signal is still unknown. However, it was demonstrated that ABA moves to the systemic tissues, inducing *Pin2* gene expression.

Gas exchange experiments show that electrical current application and mechanical wounding result in two major time constants in the assimilation and transpiration rates, indicating a transient stomatal closure within 2–3 min and a more pronounced closure at 10 min. The rapid fast time constant resembles a fast action potential-like signal, and it could be the same signal already reported by Wildon *et al.* (17). It has been demonstrated in various studies that stomatal movements are due to ionic flux across the plasmalemma of the guard cells (41, 46). For this reason, these gas exchange measurements clearly indicate the involvement of an ionic component in the signal transduction pathway induced by mechanical or electrical current stimulation. However, our results clearly indicate the presence of a second component 10 min after stimulus application. This second, slower time constant may represent a variation potential that might be generated by chemical signals. Further signals known to be involved in stomatal closure are ABA (47) and JA (48). Both substances have also been shown to induce *Pin2* gene expression in the absence of any wounding (3, 35). In addition, mechanical damage increases the endog-

enous levels of ABA and JA in tomato leaves (38). Since we observe *Pin2* gene expression and stomatal closure under these conditions, we propose that ABA and/or JA are involved in generation of the second component of gas relaxation kinetics (Fig. 6).

Our results support and extend previous reports on the participation of electrical signals in long-distance information transfer within plants described as fast electrical signal after mechanical wounding (17). Whatever the systemic signal, some evidence suggests that this systemic signal might be propagated through the phloem or related structures. Indeed, using transgenic potato plants, consistent with the idea of a propagation of the wound signal via the phloem, the *Pin2* promoter activity in systemically induced leaves was highest in the tissue surrounding the vascular bundles (23).

Further experiments will be required to elucidate the propagation of the putative systemic signal as well as the modes of action of ABA, systemin, JA, and the electrical signal in order to further our understanding of how wound-induced gene expression is regulated.

We are indebted to Dr. C. Wasternack and Dr. R. Atzorn (Institute of Plant Biochemistry, Halle, Germany) for ABA and JA quantitation. We also thank R. Breitfield for taking care of the plants in the greenhouse and A. Voigts for the photographic work.

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