

# Octadecanoid Precursors of Jasmonic Acid Activate the Synthesis of Wound-Inducible Proteinase Inhibitors

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**Jasmonic acid and methyl jasmonate have been shown previously to be powerful inducers of proteinase inhibitors in tomato, tobacco, and alfalfa leaves. We show here that when proposed octadecanoid precursors of jasmonic acid, i.e., linolenic acid, 13(S)-hydroperoxylinolenic acid, and phytodienoic acid, were applied to the surfaces of tomato leaves, these compounds also served as powerful inducers of proteinase inhibitor I and II synthesis, a simulation of a wound response. By contrast, compounds closely related to the precursors but which are not intermediates in the jasmonic acid biosynthetic pathway did not induce proteinase inhibitor synthesis. These results suggest that the octadecanoid intermediates may participate in a lipid-based signaling system that activates proteinase inhibitor synthesis in response to insect and pathogen attack.**

## INTRODUCTION

Wound damage to the leaves of plants from a number of families results in the synthesis of proteinase inhibitor proteins at the wound sites as well as in distal leaves (Green and Ryan, 1972; Brown and Ryan, 1984; Roby et al., 1987; Bradshaw et al., 1989). Several plant-derived chemicals that can regulate the expression of wound-inducible proteinase inhibitor genes have been identified. These include oligouronides arising from the plant cell wall (Bishop et al., 1981; Ryan, 1987), the growth regulators abscisic acid and auxin (Peña-Cortes et al., 1989; Kernan and Thornburg, 1989), methyl jasmonate and jasmonic acid (Farmer and Ryan, 1990; Farmer et al., 1992), and an 18-amino acid polypeptide, systemin (Pearce et al., 1991).

Our recent observation that methyl jasmonate, a volatile fatty acid-derived compound, is a potent inducer of proteinase inhibitors in tomato, tobacco, and alfalfa leaves raised the possibility that jasmonates could be components of the signaling pathway for proteinase inhibitor gene expression (Farmer and Ryan, 1990). The biosynthesis of jasmonic acid has been proposed to originate from linolenic acid through the introduction of oxygen to the carbon chain, followed by a dehydration, a reduction, and a series of  $\beta$ -oxidations (Vick and Zimmerman, 1984).

In this report, we demonstrate that three of the octadecanoid precursors of jasmonic acid, i.e., linolenic acid, 13(S)-hydroperoxylinolenic acid, and phytodienoic acid, can act as signals for proteinase inhibitor induction in tomato leaves when applied to leaf surfaces. These results suggest that wound-inducible proteinase inhibitor synthesis may be regulated by a lipid-based signaling pathway activated by wounding.

A model is proposed in which systemin and oligouronides produced by insect or pathogen attack interact with receptors in target cells to initiate the octadecanoid-based signaling pathway.

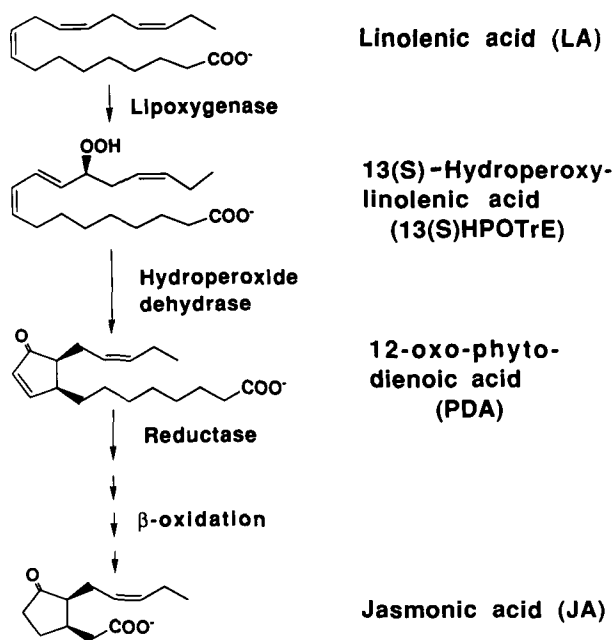
## RESULTS

Our previous results showed that jasmonates, whether as airborne signals or applied in solution to the surface of leaves, were readily taken up by tomato, tobacco, or alfalfa plants, where they powerfully activated the synthesis of proteinase inhibitor proteins (Farmer and Ryan, 1990; Farmer et al., 1992). This property led us to suspect that jasmonic acid (or a closely related molecule) may be part of the intracellular signaling pathway for regulating proteinase inhibitor synthesis in response to insect and/or pathogen attack.

The pathway for the synthesis of jasmonic acid in plants, proposed by Vick and Zimmerman (1984), is shown in Figure 1. The availability of linolenic acid, 13(S)-hydroperoxylinolenic acid, phytodienoic acid, and some closely related compounds that are not jasmonate intermediates provided the opportunity to investigate whether these compounds might behave as precursors of jasmonic acid in tomato leaves and therefore act as signaling molecules that activate proteinase inhibitor synthesis.

Figure 2 shows that suspensions of linolenic acid (18:3) sprayed on tomato leaves strongly induced inhibitor I synthesis in tomato plants. The levels of inhibitor I that accumulated in response to linolenic acid were much higher than those induced by wounding. Linoleic acid (18:2), sprayed at 10 mM

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**Figure 1.** Proposed Scheme for Jasmonic Acid Biosynthesis in Angiosperms (Vick and Zimmerman, 1984).

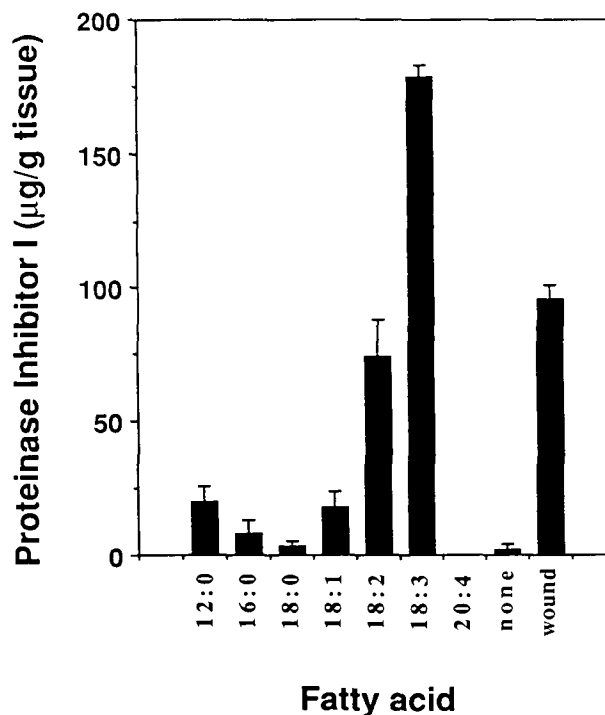
concentration, also induced the accumulation of inhibitor I in tomato leaves, but the levels of inhibitor induced were less than with linolenic acid (Figure 2). Several other fatty acids (12:0, 16:0, 18:0, 18:1, and 20:4) were essentially inactive inducers of the synthesis of inhibitor I (Figure 2). Thus, the induction of inhibitor I in leaves by 18:3 and 18:2 appears to be specific and is not a general response to fatty acids.

To further examine the specificities of 18:3 in inducing proteinase inhibitor synthesis, a closely related isomer of linolenic acid,  $\gamma$ -linolenic acid, with double bonds at positions 6, 9, and 12 instead of at positions 9, 12, and 15, was similarly assayed for proteinase inhibitor-inducing activity when applied to leaves. In this case, fatty acid suspensions were applied to the adaxial leaf surfaces in 5- $\mu$ L droplets containing 0.1% Triton X-100. Results of a typical experiment are shown in Figure 3. Within 90 min after applying radiolabeled linolenic acid on tomato leaf surfaces,  $\sim$ 5% of the radioactivity was converted to other compounds resembling 13(S)-hydroperoxylinolenic acid and its metabolites. The identity of these compounds is under investigation (E.E. Farmer and C.A. Ryan, unpublished data).  $\gamma$ -Linolenic acid was shown to be essentially inactive over a wide range of concentrations in which linolenic acid showed high proteinase inhibitor-inducing activity.

The first step in the proposed pathway to jasmonic acid (Figure 1) is the conversion of linolenic acid to 13(S)-hydroperoxylinolenic acid via lipoxygenase. Figure 4 shows that this hydroperoxide induced proteinase inhibitor I synthesis

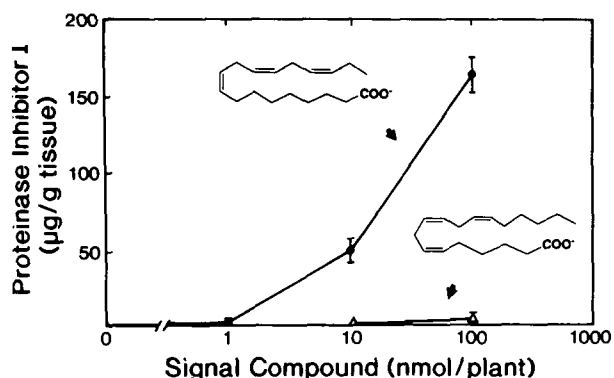
when applied to leaves. A closely related compound that is not an intermediate leading to jasmonic acid, 13(S)-hydroperoxylinoleic acid, was unable to induce inhibitor I synthesis (Figure 4), again indicating that the specificity of induction by 13(S)-hydroperoxylinolenic acid and its precursor are consistent with their proposed roles as jasmonate precursors.

When phytodienoic acid, the cyclic product derived from 13(S)-hydroperoxylinolenic acid (Figure 1), was applied to tomato leaves, it was nearly as active as jasmonic acid in inducing proteinase inhibitor I synthesis. Figure 5 compares the amounts of phytodienoic acid that induced inhibitor I in leaves to the levels of linolenic acid, 13(S)-hydroperoxylinolenic acid, and jasmonic acid required for activity when applied to tomato leaf surfaces. Whereas the stability of each precursor, the amounts of the compound that are taken up by the plant, and the ease of conversion to a biologically active signal (viz. jasmonic acid) would all be factors in their activities, it is clear that all of the precursors are potent inducers



**Figure 2.** Accumulation of Proteinase Inhibitor I in Tomato Leaves in Response to Various Fatty Acids and to Wounding.

Fourteen-day-old tomato plants were sprayed with 10 mM suspensions of various fatty acids in a carrier solution consisting of 0.1% Triton X-100 (v/v). Plants were then incubated in constant light at 28°C for 24 hr; then the juice was expressed from the leaves and assayed for proteinase inhibitor I by radial immunodiffusion. Fatty acids are as follows: 12:0, lauric acid; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 20:4, arachidonic acid. Data are for six plants  $\pm$  SE.



**Figure 3.** Structural Specificity of Linolenic Acid Derivatives as Signals for Proteinase Inhibitor I Induction in Tomato.

Each compound was applied to the adaxial surfaces of tomato leaves as a 5- $\mu$ L droplet. Plants were then incubated in constant light at 28°C for 24 hr before assaying for proteinase inhibitor I. Inhibitor I levels are shown  $\pm$  SE,  $n = 6$ . ●, linolenic acid;  $\Delta$ ,  $\gamma$ -linolenic acid.

of proteinase inhibitor I synthesis and that the induction is highly specific for precursors of jasmonic acid.

In tomato leaves, two proteinase inhibitors are induced either by wounding, by systemin, by oligouronides, or by jasmonic acid. To establish whether both inhibitors I and II were induced by the various jasmonic acid precursors, the induction of inhibitor II by these precursors was compared with induction by jasmonic acid. Table 1 shows that inhibitor II synthesis was induced by all of the precursors, with the potency of each compound in inducing inhibitor II synthesis exactly paralleling its activity in inducing inhibitor I synthesis (Figure 5). This result demonstrated that these compounds are interacting with a signaling pathway that leads to the induction of synthesis of both inhibitor I and II and that they are likely doing so through the synthesis of jasmonic acid.

## DISCUSSION

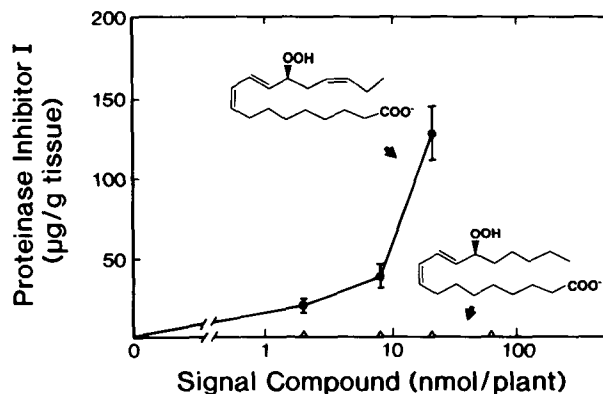
In tomato leaves, wounding resulting from herbivorous insect attacks leads to the release of signals that can travel from the wounded leaf to nearby and distal leaf tissues to activate proteinase inhibitor synthesis (Green and Ryan, 1972). A candidate for long-distance signaling was recently reported to be an 18-amino acid polypeptide, systemin (Pearce et al., 1991). The recent observation that methyl jasmonate and jasmonic acid are also powerful inducers of proteinase inhibitor synthesis (Farmer and Ryan, 1990) raised the possibility that jasmonates could be intracellular signal intermediates in the induction of proteinase inhibitor gene expression by wounding or through the action of systemin.

Several intermediates in the pathway from linolenic acid to jasmonic acid (Figure 1) were assayed for their ability to activate proteinase inhibitor synthesis when applied to surfaces of tomato leaves. As with jasmonic acid, these intermediates could be applied to the plants at low concentrations in a non-destructive manner. In addition to the jasmonic acid intermediates, several compounds similar in structure that were not intermediates in jasmonic acid synthesis were tested for proteinase inhibitor-inducing activities.

Linolenic acid, hydroperoxylinolenic acid, and phytydienoic acid were all found to be powerful inducers of inhibitor I and II synthesis when applied to tomato leaf surfaces (Figures 1 to 5, Table 1). This result indicated that these compounds were being actively converted to jasmonic acid in the plants and were activating the genes via a jasmonic acid biosynthetic pathway.

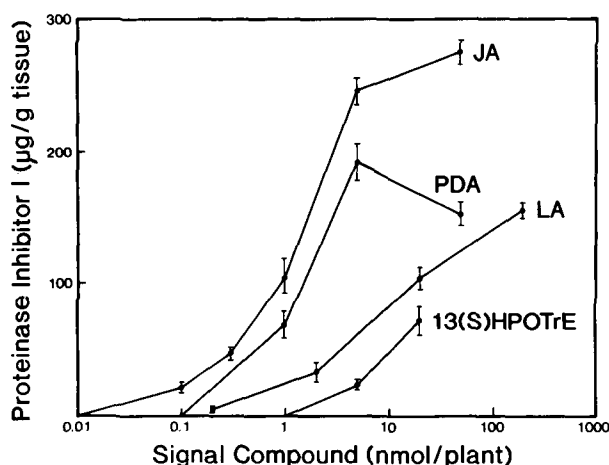
The specificity of the activation of the inhibitor genes by the jasmonic acid intermediates appeared to be absolute, as several closely related fatty acids (Figures 2 and 3), except linoleic acid (which can be converted by the plant to linolenic acid), were inactive as inducers of proteinase inhibitor synthesis. Also, hydroperoxylinoleic acid, differing from hydroperoxylinolenic acid only by the lack of a double bond at carbon 15, was inactive (Figure 4). Cyclization of 13(S)-hydroperoxylinolenic acid to phytydienoic acid was previously shown (Vick et al., 1980) to require a double bond at carbon 15.

Taken together, these observations demonstrated that several octadecanoids of the jasmonic acid biosynthetic pathway signal cellular events in tomato plants, leading to the expression of proteinase inhibitor genes. It is possible, therefore, that octadecanoids as well as jasmonate may be involved in complex signaling networks that regulate defensive or developmental genes in plants. Octadecanoid signaling in plants shows some interesting parallels to eicosanoid signaling in animals. Eicosanoids comprise a diverse class of compounds,



**Figure 4.** Structural Specificity of Hydroperoxides as Signals for Proteinase Inhibitor I Induction in Tomato Leaves.

$\Delta$ , 13(S)HPODE, 13(S)-hydroperoxylinoleic acid; ●, 13(S)HPOTrE, 13(S)-hydroperoxylinolenic acid. Error bars show SE ( $n = 6$ ).



**Figure 5.** Relative Activities of Octadecanoid and Octadecanoid-Derived Signals in Inducing Proteinase Inhibitor I in Tomato Leaves.

LA, linolenic acid; JA, jasmonic acid; 13(S)HPOTrE, 13(S)hydroperoxylinolenic acid; PDA, phytodienoic acid. Each compound was applied to the adaxial surfaces of tomato leaves as a 5- $\mu$ L droplet. Plants were then incubated in constant light at 28°C for 24 hr before assaying for proteinase inhibitor I. Data are for 12 plants  $\pm$  SE.

such as prostaglandins and leukotrienes, that often mediate localized stress responses in animals cells. Enzymes of eicosanoid biosynthesis are often constitutively present in the animal cell, and the rate-limiting step for eicosanoid synthesis can be precursor fatty acid release (Needleman et al., 1986). That exogenous precursors of jasmonic acid cause proteinase inhibitor induction suggests that all of the enzymes responsible for jasmonate synthesis from linolenic acid are probably present in tomato leaves before wounding. Thus, a key event in the cellular signaling of wound-inducible proteinase inhibitors would be the release of linolenic acid by the activation, liberation, or synthesis of a lipase.

A model for the regulation of wound-inducible proteinase inhibitor synthesis by an octadecanoid-based signaling pathway is shown in Figure 6. This model takes into account much of the previous literature on proteinase inhibitor induction. We have categorized known, plant-derived extracellular signals that activate proteinase inhibitor genes into those likely released as a consequence of wounding (herbivory), i.e., systemin (Pearce et al., 1991), and those likely to be produced during pathogenesis, i.e., oligouronides, originating from the plant cell wall (Bishop et al., 1981). Fungal oligosaccharides such as chitosan (Walker-Simmons and Ryan, 1984) and  $\beta$ -glucan-containing elicitors (Rickauer et al., 1989) that are not included in this model are also known to induce proteinase inhibitor synthesis and are likely to utilize similar pathways. In the model, the key steps involve the interaction of the signals with plasma membrane receptors, leading to the activation of lipase and the release of linolenic acid into

the cytoplasm. Linolenic acid would be readily converted through phytodienoic acid to jasmonic acid, which is proposed to interact with a receptor to activate proteinase inhibitor gene expression.

At present, there is no direct evidence for the existence of the lipase implicated in our model; however, the systemic effects of wounding on protoplast fragility were reported several years ago (Walker-Simmons et al., 1984), suggesting that a systemic wound signal was exerting a major effect on the integrity of receptor cell membranes. Protoplasts isolated from the upper leaves of tomato plants previously wounded on lower leaves were significantly more fragile to isolation than those isolated from the upper leaves of undamaged plants (Walker-Simmons et al., 1984). The time course of the loss of membrane integrity paralleled the time course of systemic signal production from wounded leaves determined independently (Nelson et al., 1983). The loss of integrity could be explained by the activation of lipase activity and release of free fatty acids changing the physical properties of the membrane.

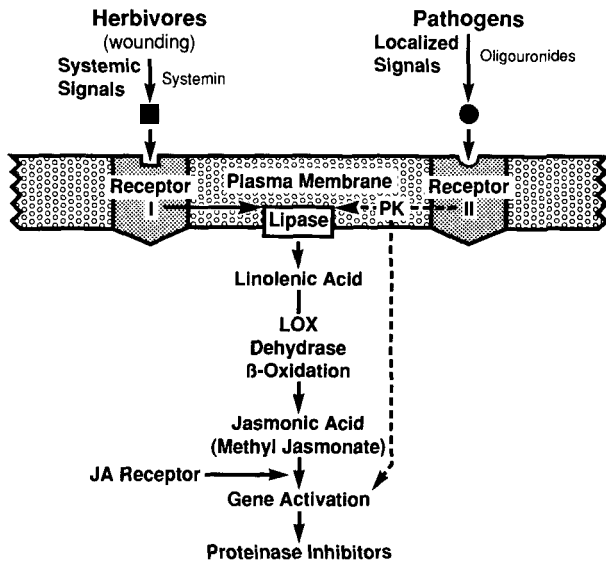
In the model in Figure 6, linolenic acid released in response to wound signals would be converted through several intermediates to jasmonate. The ability of jasmonic acid to more powerfully activate the synthesis of proteinase inhibitors in leaves as compared to wounding suggests that this compound is saturating a receptor system that regulates proteinase inhibitor gene expression. Wounds may not generate sufficient quantities of linolenic acid to synthesize enough jasmonic acid to fully saturate the receptors, which may function directly to activate the transcription of the gene.

Oligouronides, derived from the plant cell wall, are well-established signals for the induction of proteinase inhibitor synthesis (Bishop et al., 1981; Farmer et al., 1991). These signals appear to be produced by pathogenesis (Pautot et al., 1991) and not by wounding because they are not readily mobile within the plant (Baydoun and Fry, 1985) and the enzymes necessary for their release from cell wall pectin have

**Table 1.** Induction of Proteinase Inhibitor II in Tomato Leaves in Response to Octadecanoids and Jasmonic Acid.

Treatment	Inhibitor II ( $\mu$ g/g tissue)
Control	0
Linolenic acid (100 nmol)	94 $\pm$ 24
13(S)HPOTrE (20 nmol)	60 $\pm$ 10
Phytodienoic acid (2 nmol)	186 $\pm$ 12
Jasmonic acid (2 nmol)	188 $\pm$ 9

The control solution was 1.0% (v/v) Triton X-100 in water. Substances tested were either dissolved in this solution (phytodienoic acid and jasmonic acid) or suspended by sonication [linolenic acid and 13(S)HPOTrE]. All solutions were added to the adaxial surfaces of tomato leaves of 14-day-old intact plants as 10- $\mu$ L droplets. Plants were then incubated in constant light for 24 hr, at which time proteinase inhibitor II was measured by radial immunodiffusion. Values are given  $\pm$  SE;  $n = 6$ .



**Figure 6.** Proposed Model for the Signaling that Leads to the Expression of Wound-Inducible Proteinase Genes in Tomato Leaves.

PK, protein kinase; LOX, lipoxygenase; JA, jasmonic acid.

not been detected in tomato leaf tissue (C.A. Ryan, unpublished data). There is no direct evidence for the existence of receptors for oligouronides in the plant plasma membrane, but oligouronides have been shown to modulate the phosphorylation of tomato leaf plasma membrane proteins *in vitro* (Farmer et al., 1989, 1991) and affect ion flux (Thain et al., 1990). The specificity of the plants' response to oligouronides as well as the *in vitro* phosphorylation data suggest that a specific receptor system is present. This mechanism may activate the octadecanoid pathway, but it is possible that an entirely different signaling mechanism may be present (dashed lines in Figure 6). The model in Figure 6 presents a framework for future research to assess the basis for octadecanoid signaling of the wound-inducible system that activates proteinase inhibitor genes and for its possible role in signaling other wound-inducible genes and/or jasmonate-activated genes that regulate both environmental and developmental responses in plants.

## METHODS

### Chemicals

13(S)-Hydroperoxylinolenic acid, (9Z,11E,15Z,13(S)) 13-hydroperoxyoctadecatrien-1-oic acid and 13(S)-hydroperoxylinoleic acid, (9Z,11E,13(S)) 13-hydroperoxyoctadecadien-1-oic acid were from Cayman Chemical Co. (Ann Arbor, MI). Methyl jasmonate was from Bedoukian Research Inc. (Danbury, CT) and was deesterified to jasmonic acid as previously detailed (Farmer et al., 1992). Methyl jasmonate was 90.6% 1*R*,2*R*-enantiomer and 8.1% 1*R*,2*S*-enantiomer according to

data supplied by the manufacturer. Phytodienoic acid (1*S*,2*S*-enantiomer) was a generous gift from Dr. B. Vick (USDA, ARS, Fargo, ND). Other fatty acids were from Sigma Chemical Co. (St. Louis, MO). All substances were applied to plants in 0.1% (v/v) Triton X-100. Jasmonic acid and phytodienoic acid were soluble in this solution. All other fatty acids and hydroperoxides were suspended in 0.1% (v/v) Triton X-100 by sonication and were used immediately.

### Biological Assay

All experiments were conducted with intact tomato plants that were 14 to 15 days old, grown under previously described conditions (Farmer et al., 1992). Chemicals were assayed for proteinase inhibitor-inducing activity by either spraying solutions directly on the plant or by applying 5- or 10- $\mu$ L droplets to the upper surfaces of each leaf. Plants treated with inducing substances and control plants were incubated in constant light (300  $\mu$ E m<sup>-2</sup> sec<sup>-1</sup>) at 28°C for 24 hr. Juice was expressed from the leaves and assayed for proteinase inhibitors I and II by radial immunodiffusion (Ryan, 1967; Trautman et al., 1971). The detection limit for the assay was  $\sim$ 2  $\mu$ g proteinase inhibitor per gram of leaf tissue.

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