

# Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves

(jasmonic acid/pathogen/wound-inducible genes/localized/systemic defense responses)

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**ABSTRACT** Inducible defensive responses in plants are known to be activated locally and systemically by signaling molecules that are produced at sites of pathogen or insect attacks, but only one chemical signal, ethylene, is known to travel through the atmosphere to activate plant defensive genes. Methyl jasmonate, a common plant secondary compound, when applied to surfaces of tomato plants, induces the synthesis of defensive proteinase inhibitor proteins in the treated plants and in nearby plants as well. The presence of methyl jasmonate in the atmosphere of chambers containing plants from three species of two families, Solanaceae and Fabaceae, results in the accumulation of proteinase inhibitors in leaves of all three species. When sagebrush, *Artemisia tridentata*, a plant shown to possess methyl jasmonate in leaf surface structures, is incubated in chambers with tomato plants, proteinase inhibitor accumulation is induced in the tomato leaves, demonstrating that interplant communication can occur from leaves of one species of plant to leaves of another species to activate the expression of defensive genes.

Activation of defensive genes in plants by pathogen and herbivore attacks, or by other mechanical wounding, can result from the action of a variety of signaling molecules that are released in complex temporal patterns following the initial invasion of the tissues (1–3). These signals are transported locally by diffusion through intercellular and extracellular fluids that permeate wound or infection sites or systemically through the vascular system of the plants (4–7). Limited indirect evidence has indicated that defense responses may also be mediated by signals transported through the atmosphere (8–10). Whereas several chemicals, including ethylene, have been identified as candidate extracellular signaling molecules for inducible defense genes, no direct biochemical evidence has been presented that would implicate any volatile chemicals aside from ethylene as signals that can activate plant defensive genes.

Among the defensive chemicals that are synthesized in response to either herbivore or pathogen attacks are proteinase inhibitor proteins. Members of three families of wound-inducible proteinase inhibitors, inhibitors I and II from Solanaceae and alfalfa trypsin inhibitor from Fabaceae, and their cDNAs and genes, have been extensively characterized (11–18). Proteinase inhibitors I and II are regulated at the transcriptional level in response to wounding (19). We have been investigating the signal transduction pathways that regulate the synthesis of these inhibitors in response to herbivore attacks and have found that the inhibitor genes are induced by oligosaccharide fragments from plant (oligosaccharides) and pathogen (chitosan) cell walls (3). The mechanism by which oligosaccharide molecules activate the proteinase inhibitor genes is not known, but oligosaccharide

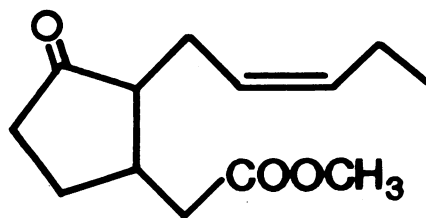


FIG. 1. Structure of methyl jasmonate.

elicitors have been associated with membrane receptors (20) and with changes in protein phosphorylation patterns of membranes (21) and cellular proteins (22) during the induction process.

In this communication we report that a lipid-derived molecule, methyl jasmonate (Fig. 1), can act as a volatile signal that induces the accumulation of proteinase inhibitor proteins to even higher levels than can be induced by wounding. We also report that the presence of methyl jasmonate from sagebrush leaves can induce proteinase inhibitor I and II accumulation in leaves of nearby tomato plants through the atmosphere. Methyl jasmonate or its deesterified derivative, jasmonic acid, is hypothesized to be a possible key component of intracellular signaling in response to wounding or pathogenic attacks.

## METHODS AND MATERIALS

**Exposure of Plants to Methyl Jasmonate.** Tomato (*Lycopersicon esculentum* cultivar Castlemart II) plants, 13–15 days after planting, were sprayed with solutions of 0.125% (vol/vol) Triton X-100 with or without ( $\pm$ )-methyl jasmonate (Bedoukian Research, Danbury, CT) or exposed to methyl jasmonate vapor in 1250-ml air-tight glass chambers by incubating plants together with cotton-tipped wooden dowels to which had been applied 1  $\mu$ l of dilutions of ( $\pm$ )-methyl jasmonate in ethanol or ethanol alone as a control. The cotton tip was placed  $\approx$ 4–6 cm from the plant leaves. The chambers were incubated in constant light (300 microeinsteins $\cdot$ m<sup>-2</sup> $\cdot$ sec<sup>-1</sup>) at 28°C for 24 h. Cuttings, in 10 ml of water, from 2-month-old tobacco plants (cultivar Xanthi) and from 1-month-old alfalfa plants (line RA3) were exposed to methyl jasmonate vapor in air-tight glass chambers in a similar way. Leaf juice was expressed from the leaves and assayed for proteinase inhibitors I and II by radial immunodiffusion (23, 24).

**Purification and Characterization of Methyl Jasmonate from *Artemisia tridentata* Nutt. ssp. *tridentata*.** The terminal 15 cm of *A. tridentata* branches were collected from a natural population growing at Lyons Ferry, WA. Branches (1 kg) containing several hundred small leaves were agitated in ethanol (1 liter) for 10 sec. The resultant mixture was concentrated to a brown oil (2.4 ml) that was extracted into pentane (75 ml). The pentane was removed by rotary evaporation and the resultant yellow oil (1.2 ml) was fractionated on a silica gel

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(Silicar, 60A; Mallinckrodt) column (1.2 × 20 cm). The column was first washed in pentane. The ethyl acetate content of the pentane was increased in 5% (vol/vol) steps to 10% (vol/vol) and then increased in 10% steps to 100% ethyl acetate. A fraction containing volatile proteinase inhibitor-inducing activity eluted in 30% (vol/vol) ethyl acetate. This fraction was further resolved by preparative thin-layer chromatography on silica gel, developed in benzene/ethyl acetate, 10:1 (vol/vol). Compounds migrating with an  $R_f$  of 0.38 exhibited proteinase inhibitor-inducing activity when exposed to young tomato plants. This active material was further chromatographed by analytical silica gel thin layer (Kodak) chromatography. The thin layer sheet was pre-soaked in 5% (wt/vol) silver nitrate in 75% (vol/vol) methanol. Before chromatography the plate was allowed to dry for 20 h at 28°C. The plate was chromatographed in 30% (vol/vol) ethyl acetate in hexane. Proteinase inhibitor-inducing activity was found associated with components that migrated at an  $R_f$  of 0.17. This material was further resolved isocratically by reverse-phase HPLC on a Beckman Ultrasphere C<sub>18</sub> ion pair column (4.6 × 250 mm, 5 μm) in 50% acetonitrile/0.1% trifluoroacetic acid in water. A single fraction containing a partially resolved double peak copurified with the proteinase inhibitor-inducing activity. Gas chromatography/mass spectroscopy was carried out on a Hewlett-Packard 5985 GC/MS system. The column was a Superox FA (30 m × 0.25 mm; Alltech Associates) with He carrier gas at 1.4 kg·cm<sup>-2</sup>. The column temperature was 45°C for 5 min and then was increased at 10°C/min to 220°C, which was held for 10 min.

## RESULTS AND DISCUSSION

Methyl jasmonate is a naturally occurring compound that has been identified in plants from at least nine families (25) and has been utilized as a perfume fragrance for decades. This compound has recently been the subject of considerable interest because of its biological activities in plants in inducing senescence and in regulating vegetative storage proteins in plant leaves (25). The proteinase inhibitor-inducing ability of methyl jasmonate was first noted when, upon spraying the compound on leaves of tomato plants, it powerfully induced the synthesis and accumulation of proteinase inhibitor I protein (Table 1). The chemical induced the accumulation of inhibitor I to levels higher than could be induced by wounding. It was also noted during these experiments that control plants that had not been sprayed with methyl jasmonate, but incubated in the same chambers with the sprayed plants, accumulated low levels of proteinase inhibitor I protein (Table 1). Control plants incubated in separate chambers did not accumulate inhibitor I at all. These reproducible results

Table 1. Accumulation of proteinase inhibitor I in tomato leaves induced by methyl jasmonate or wounding

Plants	n	Proteinase inhibitor I, μg/g of tissue
Chamber A*		
Methyl jasmonate-sprayed	6	210 ± 7
Control	6	28 ± 3
Chamber B†		
Wounded	4	86 ± 8
Control	6	0

Proteinase inhibitor I levels were assayed by radial immunodiffusion (22, 23). Values are reported as mean ± SEM.

\*Sprayed and control plants were allowed to dry in the open at room temperature for 30 min and then placed in large sealed Plexiglas boxes (capacity, 11.34 liters) and incubated under constant light (300 microeinsteins·m<sup>-2</sup>·sec<sup>-1</sup>) for 24 h.

†Plants were wounded once across their lower leaves with a hemostat and assayed 24 h later.

suggested that volatile methyl jasmonate was inducing the synthesis of proteinase inhibitors in the nearby untreated control plants. To further test this possibility, tomato plants were placed in air-tight chambers together with cotton-tipped wooden dowels onto which various dilutions of methyl jasmonate in ethanol had been applied to the cotton. The dowels were placed so that no interaction was possible between the methyl jasmonate and the plants except through the atmosphere. After incubating the plants in light for 24 h following the introduction of methyl jasmonate to the chambers, leaf juice from the plants was assayed for inhibitor I and II protein levels. The presence of increasing levels of methyl jasmonate in the different chambers resulted in the synthesis and accumulation of proteinase inhibitors I and II in a dose-dependent manner (Fig. 2). Below about 10 nl of methyl jasmonate per chamber the response was dose dependent. The results in Fig. 2 suggested that at low concentrations of methyl jasmonate on the cotton wicks the volatile levels were limiting and that leaves were not able to assimilate enough of the molecules to maximize the signaling response. Above 10 nl per chamber, volatile methyl jasmonate appeared to be at high enough concentrations in the chambers to be assimilated at levels that could maximally induce the inhibitors.

When enough methyl jasmonate to cause near-maximal induction of proteinase inhibitors was present in the chambers (100 nl per chamber), tomato plants began to accumulate proteinase inhibitors I and II at about 5 h after the initial exposure to the volatile compound and continued to accumulate the proteins at linear rates for nearly 20 h (Fig. 3). After about 20 h, however, the rates of accumulation of the two inhibitors declined. The reasons for this decline are unclear. In wounded plants the rates of accumulation of the proteinase inhibitors declined about 10 h after an initial wound, but the wound induction could be reinforced by a wound administered about 12 h later (19). The kinetics of uptake of methyl jasmonate and its fate in the leaves await further investigation.

Exposure of tomato plants for only 30 min to gaseous methyl jasmonate originating from 100 nl of the chemical per chamber was sufficient to induce a moderate level of accumulation of proteinase inhibitor protein (Fig. 4). With increasing times of exposure to the volatile methyl jasmonate the accumulation of inhibitor protein increased and approached a maximum at about 8 h.

To determine if methyl jasmonate could activate proteinase inhibitor synthesis in other plant genera and another plant

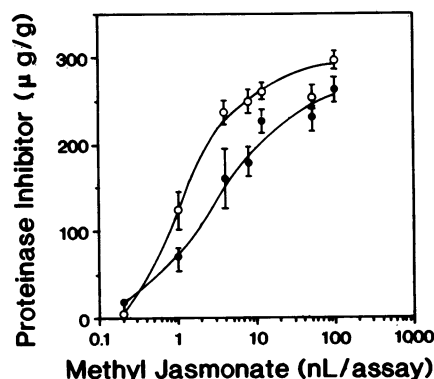


Fig. 2. Induction of proteinase inhibitor I and II proteins in the leaves of intact tomato plants after exposure to airborne methyl jasmonate for 24 h. Two-week-old tomato plants were placed in 1250-ml air-tight glass chambers together with cotton-tipped wooden dowels to which had been applied 1 μl of dilutions of (±)-methyl jasmonate in ethanol. The cotton tip was placed ≈4–6 cm from the plant leaves. The jars were incubated in constant light at 28°C for 24 h. Leaf juice was analyzed for proteinase inhibitors I and II. Bars indicate the SEM (n = 4). ○, Inhibitor I; ●, inhibitor II.

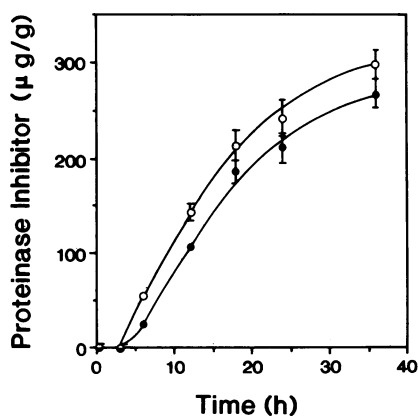


FIG. 3. Time course of induction of proteinase inhibitor I and II proteins in the leaves of tomato plants in response to airborne methyl jasmonate. Tomato plants were incubated in air-tight glass jars together with a cotton tipped dowel on which 1  $\mu$ l of a solution of 100 nl of methyl jasmonate in 1  $\mu$ l ethanol had been applied. At various times after the initial application of methyl jasmonate, levels of proteinase inhibitors I and II were determined. Bars show the SEM ( $n = 4$ ).  $\circ$ , Inhibitor I;  $\bullet$ , inhibitor II.

family, small tobacco plants (Xanthi) and small alfalfa plants (line RA3) were exposed to volatile methyl jasmonate (100 nl of methyl jasmonate per 1250-ml chamber) and leaf juice was assayed for the presence of tobacco trypsin inhibitor and alfalfa trypsin inhibitor. In the presence of methyl jasmonate, tobacco trypsin inhibitor levels were elevated from  $7 \pm 3$   $\mu$ g/g of tissue ( $n = 4$ ) in leaves of control plants to  $116 \pm 37$   $\mu$ g/g of tissue ( $n = 4$ ) in leaves of plants exposed to methyl jasmonate. Alfalfa trypsin inhibitor levels were elevated from  $33 \pm 7.3$   $\mu$ g/g of tissue ( $n = 4$ ) in control plants to  $385 \pm 26.5$   $\mu$ g/g of tissue ( $n = 4$ ) in exposed plants. Thus airborne methyl jasmonate induced the expression of several proteinase inhibitor genes representing three inhibitor families in leaves of plants from the Solanaceae and Fabaceae families.

Because of the widespread occurrence of methyl jasmonate in the plant kingdom, we investigated the possibility that a species of plant that contained methyl jasmonate in its leaves could induce expression of proteinase inhibitor genes in nearby tomato plants. If so, it would establish a biochemical basis for a previously unrecognized form of defense gene

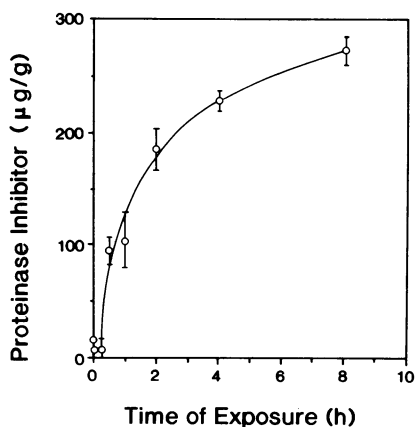


FIG. 4. Synthesis of proteinase inhibitor I protein in response to increasing times of exposure of tomato plants to airborne methyl jasmonate. Tomato plants were incubated in air-tight 1250-ml glass chambers in the presence of volatile methyl jasmonate (100 nl dissolved in 1  $\mu$ l of ethanol) pipetted onto cotton swabs. After exposure to gaseous methyl jasmonate, the plants were transferred to chambers free of methyl jasmonate and further incubated for a total of 24 h after the initial exposure, when leaf juice was assayed for proteinase inhibitor I content. Bars show the SEM ( $n = 4$ ).

regulation involving interplant communication through the atmosphere. At least one member of the genus *Artemisia*, *Artemisia absinthium*, is known to contain methyl jasmonate in its leaves (26). We therefore chose a related species, the sagebrush *A. tridentata* Nutt. ssp. *tridentata*, an ecologically dominant species in the western United States, as a possible donor of volatile methyl jasmonate. Small tomato plants were incubated in air-tight chambers in the absence or presence of 5 g of leafy branches of *A. tridentata*, with no direct physical contact at any time between the plants. Within 2 days the leaves of the tomato plants exhibited elevated levels of proteinase inhibitors I and II (Table 2). These experiments (assaying the induction of inhibitor I) have been repeated numerous times. Although individual experiments vary somewhat, the results—i.e., the induction of inhibitor I—was always noted.

To identify the volatile signaling molecule, cotton swabs were brushed across the leaf surfaces of *A. tridentata* leaves and placed in glass chambers with tomato plants, similar to the experiments described previously with methyl jasmonate. A volatile component was present that induced the accumulation of proteinase inhibitors in the leaves (data not shown). To determine if methyl jasmonate was actually present in the leaf surface trichomes of *A. tridentata*, an ethanolic fraction from the leaf surface was obtained and fractionated into its components by silica gel column chromatography, thin-layer chromatography, and reverse-phase HPLC. At each step of purification, components were assayed for volatile proteinase inhibitor-inducing activity. Active fractions were further purified. Reverse-phase HPLC of a biologically active fraction from silver nitrate thin-layer chromatography produced two overlapping peaks with retention times similar to two methyl jasmonate isomers. The mass spectrum of one of the isomers isolated from *A. tridentata* is shown in Fig. 5 to be virtually identical to a sample of chemically synthesized methyl jasmonate isomer. These results strongly suggest that the airborne chemical signal from *A. tridentata* leaves that induces the expression of the proteinase inhibitor genes is methyl jasmonate.

The chemical structure of jasmonic acid is similar to the prostaglandins, important signaling molecules in animals (25). Jasmonic acid is apparently synthesized from linolenic acid, a fatty acid ubiquitous in plants (27). The release of linolenic acid, triggered by the activation of specific lipases in response to pest or pathogen attacks, could lead rapidly to

Table 2. Induction of proteinase inhibitors I and II in leaves of tomato plants incubated in the presence of leafy branches of the sagebrush *A. tridentata* ssp. *tridentata*

Plants	Proteinase inhibitor			
	I		II	
	$\mu$ g/g of tissue	$n$	$\mu$ g/g of tissue	$n$
Experiment 1				
Incubated with				
<i>A. tridentata</i>	$54.5 \pm 8.7$	4	ND	
Control	0	4	ND	
Experiment 2				
Incubated with				
<i>A. tridentata</i>	$71.0 \pm 16.1$	10	$50.6 \pm 13.7$	12
Control	0	10	0	12

The plants were placed so that no physical contact was possible between the sagebrush leaves and tomato leaves. The chambers (volume, 1250 ml) were sealed, incubated for 2 days under the conditions described in Table 1, and assayed. Control tomato plants were placed in separate chambers in the absence of sagebrush and incubated under identical conditions. Plants for experiment 1 were collected from 1 mile ( $\approx 1.6$  km) east of Desert Aire, WA (location I). Plants for experiment 2 were collected from Lyons Ferry, WA (location 2). ND, not determined.

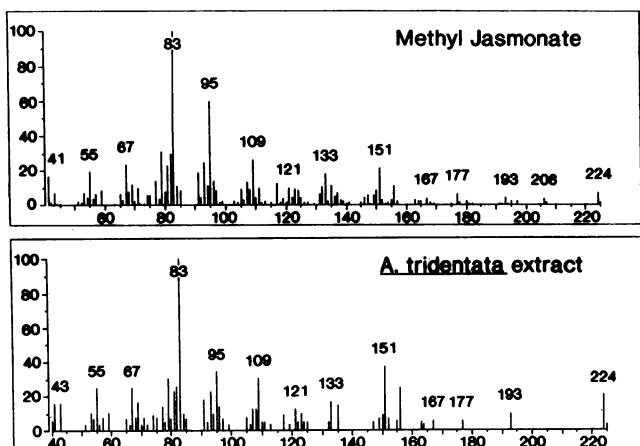


FIG. 5. Mass spectrum of authentic methyl jasmonate compared to methyl jasmonate isolated from the sagebrush *A. tridentata* ssp. *tridentata*.

the production of jasmonic acid through the action of cyclooxygenases. The possible role of jasmonic acid in signal transduction pathways leading to localized and systemic defensive gene expression needs to be investigated.

Airborne methyl jasmonate molecules may enter the vascular system by way of stomates and activate the proteinase inhibitor genes through a receptor-mediated signal transduction pathway. Alternatively, methyl jasmonate may diffuse into the leaf cell cytoplasm where it would be hydrolyzed to jasmonic acid by intracellular esterases. The free acid may, in turn, be an integral part of a general signal transduction system that regulates inducible defensive genes in plants. Jasmonic acid has a much lower vapor pressure than methyl jasmonate, but it does induce proteinase inhibitors in tomato leaves, albeit more weakly, when present in closed chambers on cotton wicks (data not shown). We have supplied solutions of jasmonic acid to young tomato plants through their cut petioles and this compound induces proteinase inhibitor synthesis, but it must be supplied at micromolar levels. It may be that the free jasmonic acid does not easily penetrate cells. This aspect of the signaling phenomenon needs further study.

Previous studies have shown that methyl jasmonate, or jasmonic acid, when applied directly to plants can produce various responses, including growth inhibition (28, 29), promotion of senescence and/or abscission (26, 30), and induction of specific leaf proteins in monocots and dicots (31–33). The data herein demonstrate that a highly sensitive mechanism is present in Solanaceae and Fabaceae families that can activate proteinase inhibitor genes in response to volatile methyl jasmonate. The mechanism may be broadly present in nature. Whether volatile methyl jasmonate can activate the other responses mentioned above is not known and should be addressed. It is possible that methyl jasmonate, or other volatile signals, released by such plants as *A. tridentata*, have multiple effects on nearby plants, either by inducing the expression of defensive genes, or genes involved in other responses such as senescence, in distal tissues of the same plants or in neighboring plant species. If such signaling is widespread in nature it could have profound ecological

significance that has been seldom considered in evaluating interactions within and between plant communities.

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