Plant Physiol. (1997) 114: 79–88

Jasmonate-Inducible Genes Are Activated in Rice by Pathogen Attack without a Concomitant Increase in Endogenous Jasmonic Acid Levels[1]

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The possible role of the octadecanoid signaling pathway with jasmonic acid (JA) as the central component in defense-gene regulation of pathogen-attacked rice was studied. Rice (*Oryza sativa* L.) seedlings were treated with JA or inoculated with the rice blast fungus *Magnaporthe grisea* (Hebert) Barr., and gene-expression patterns were compared between the two treatments. JA application induced the accumulation of a number of pathogenesis-related (PR) gene products at the mRNA and protein levels, but pathogen attack did not enhance the levels of (−)-JA during the time required for PR gene expression. Pathogen-induced accumulation of PR1-like proteins was reduced in plants treated with tetcyclacis, a novel inhibitor of jasmonate biosynthesis. There was an additive and negative interaction between JA and an elicitor from *M. grisea* with respect to induction of PR1-like proteins and of an abundant JA- and wound-induced protein of 26 kD, respectively. Finally, activation of the octadecanoid signaling pathway and induction of a number of PR genes by exogenous application of JA did not confer local acquired resistance to rice. The data suggest that accumulation of nonconjugated (−)-JA is not necessary for induction of PR genes and that JA does not orchestrate localized defense responses in pathogen-attacked rice. Instead, JA appears to be embedded in a signaling network with another pathogen-induced pathway(s) and may be required at a certain minimal level for induction of some PR genes.

The plant-growth regulator JA, first described as the methyl ester MeJA in the essential oils of *Jasminum* (Demole et al., 1962), has been attributed to a number of regulating functions in plant development (for review, see Sembdner and Parthier [1993]). Only recently has attention been paid to jasmonates as the key molecules of the octadecanoid signaling pathway mediating the activation of defense responses in herbivore- or pathogen-attacked plants (for review, see Sembdner and Parthier [1993], Farmer [1994], and Creelman and Mullet [1995]). The wound and pathogen responses induced by JA include the accumulation of the Ser proteinase inhibitors (Farmer and Ryan, 1990); papain inhibitors (Bolter, 1993); Leu aminopeptidase and Thr deaminase (Hildmann et al., 1992); a ribosome-inactivating protein (Chaudhry et al., 1994); Phe ammonia lyase (Gundlach et al., 1992); chalcone synthase, a vegetative storage protein and a Pro-rich cell wall protein (Creelman et al., 1992); leaf thionine (Andresen et al., 1992); acyclic homoterpenes (Hopke et al., 1994); and a range of secondary metabolites in cultured cells from a number of plant species (Gundlach et al., 1992). The proposed model for the stress-induced octadecanoid signaling pathway includes the release of α-linolenic acid from membrane lipids by activated phospholipase A (Chandra et al., 1996) and feeding into the pathway by lipoxygenase with regioselectivity for carbon atom 13 (Farmer and Ryan, 1992). However, the proposed signaling function of JA in pathogen-attacked plants is based exclusively on model systems, i.e. elicitor-treated, detached leaves or suspension-cultured cells (Mueller et al., 1993; Blechert et al., 1995; Doares ain et al., 1995), and the role of jasmonates in true pathosystems is still elusive. With a very limited number of experimental approaches has the effect of JA or MeJA on plant-pathogen interactions been examined. In potato and tomato plants, exogenously applied JA and MeJA induced systemic acquired resistance against *Phytophthora infestans* (Cohen et al., 1993). On the other hand, no evidence for the implication of jasmonates in the local pathogen response of barley was found (Schweizer et al., 1993; Kogel et al., 1995).

Lipoxygenase (EC 1.13.11.12), which catalyzes the first biosynthetic step from α-linolenic acid (C18:3) to JA, has been described as a defense-gene product in the leaves of pathogen-attacked rice (*Oryza sativa* L.), in which the expression was correlated with resistance to *Magnaporthe grisea* (Hebert) Barr., the causal agent of rice blast disease (Ohta et al., 1991; Peng et al., 1994). Moreover, 13-hydroperoxylinolenic acid, which is a metabolic precursor of JA, accumulated in *M. grisea*-infected rice and exogenous application of this precursor led to the accumulation of the rice phytoalexin momilactone A (Li et al., 1991). Recently,

[1] This work was supported in part by the Rockefeller Foundation and in part by Swiss National Science Foundation grant no. 31-34098.

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Abbreviations: ITP, in vitro translation product; JA, (±)-jasmonic acid; MeJA, methyl jasmonate; PR, pathogenesis-related.

JA accumulation in rice suspension-cultured cells treated with an elicitor was reported, as well as momilactone A accumulation in JA-treated rice cells (Nojiri et al., 1996). These observations suggest a role for the octadecanoid signaling pathway and for JA in the defense reaction of rice. On the other hand, salicylic acid, which is an important signal transduction compound in pathogen-attacked tobacco and other plants (Dempsey and Klessig, 1994), was recently suggested to have a function in preformed resistance, rather than defense gene activation, in rice (Silverman et al., 1995).

In the present study we investigated the role of JA in rice plants attacked by the rice-blast fungus *M. grisea*. Emphasis was placed on whether defense-gene induction by pathogen signals is mediated by the octadecanoid signaling pathway and whether the pathway is activated by pathogen attack.

MATERIALS AND METHODS

Plant Treatments

Japonica rice (*Oryza sativa* L. cv Nohrin 29) plants were grown in pots containing a clay:humus mixture (2:1, v/v) in a growth chamber (16 h of light, 265 μmol s^{-1} cm^{-2}; 21°C [dark period] to 27°C [light period]; 70% [light period] to 95% [dark period] RH). The pots were soaked with a solution of iron fertilizer (GESAL Pflanzen Tonic, CIBA-Geigy, Basel, Switzerland) and kept at 100% RH during the first 7 d after sowing. Each pot containing approximately 25 to 35 plants was sprayed with 2.5 mL of the JA solution in ethanol (Sandoz Agro, Basel, Switzerland). Nine- to 11-d-old plants were treated with JA. At this age leaf 3, which was the first true leaf more than 2 cm in length, had emerged 50 to 100%. Treated plants were not separated from control plants, since control plants, even when placed in the immediate vicinity to JA-sprayed plants, were never observed to be induced. MeJA (Serva Biochemicals, Heidelberg, Germany) treatment was carried out by placing plants under a tight plexiglass container, followed by pipetting pure MeJA onto a cotton ball inside of the container (0.25 μL L^{-1} air). Tetcyclacis (BASF, Limburgerhof, Germany) was dissolved in DMSO at a concentration of 100 mM. This stock solution was diluted to 0.1 mM in deionized water of 60°C with strong stirring. After cooling down to room temperature, the solution was injected into the lower part of the pots (30 mL per pot), and plants were watered with tetcyclacis from below until challenge inoculation with *Magnaporthe grisea* (Hebert) Barr. 2 d later. Wounds were applied to leaves every 3 to 5 mm by press injury using forceps.

Fungal and Bacterial Inoculations

M. grisea (race 283 from CIBA-Geigy) was maintained on autoclaved, soaked rice seed and plated on rice-polish agar for spore production, as described previously (Smith and Métraux, 1991). The inoculum was sprayed onto leaves in 0.05% (v/v) Tween 20, 0.25% (w/v) gelatin until leaves were covered with fine droplets. From the first 20 h in darkness followed by continuous light, inoculated plants were kept at 100% RH until the end of the experiment. Disease was quantified by counting the number of acute lesions with a gray, sporulating center 5 d postinoculation. Small, dark-brown, incompatible-type lesions became visible 2 to 3 d postinoculation and were not counted. For extraction of acid-soluble proteins, mRNA or JA plants were inoculated with 5×10^5 conidia mL^{-1}. For determination of local acquired resistance plants were inoculated with 2×10^5 conidia mL^{-1}.

Pseudomonas syringae pv *syringae* (Smith and Métraux, 1991) was grown overnight in Luria broth at 28°C. For inoculation, 1×10^9 colony-forming units mL^{-1} in water was injected at six locations into rice leaves using a 1-mL syringe without a needle. Mock-inoculated leaves were injected with water alone. Inoculated plants were kept in a growth chamber under the same conditions as noninoculated or JA-treated plants.

A rifampicin-resistant strain of *Xanthomonas campestris* pv. *oryzae* derived from strain 698 (CIBA-Geigy) was selected in Luria broth containing 100 μg mL^{-1} rifampicin at 28°C during 6 d. The resistant strain was maintained on YDC (1% w/v yeast extract, 2% w/v Glc, and 2% w/v calcium carbonate) agar containing 50 μg mL^{-1} rifampicin. For plant inoculations, bacteria were suspended in water and adjusted to 10^8 colony-forming units mL^{-1}. Leaves were cut at the tip with scissors dipped into the bacterial suspension. Inoculated plants were incubated as *P. syringae*-inoculated plants.

Elicitor Preparation

Crude elicitor from *M. grisea* was prepared by homogenizing 5 g wet weight of fungal mycelium, grown on rice-polish agar, in 50 mL of water in a glass homogenizer and autoclaving the homogenate at 121°C for 20 min. The autoclaved slurry was filtered and dilutions of the resulting suspension were sprayed in 0.05% (v/v) Tween 20 onto rice plants (3 mL per pot of approximately 25 plants).

PR Proteins

Six to 10 leaves were homogenized in 3 mL of 84 mM citric acid and 32 mM Na_2HPO_4 (pH 2.8) containing a spatule tip of polyvinylpolypyrrolidone. Homogenates were passed through Miracloth (Calbiochem) and centrifuged at 13,000 rpm for 15 min, and proteins in 1 mL of supernatant were precipitated with 0.5 g of solid ammonium sulfate. After the sample was centrifuged and the supernatant quantitatively removed, proteins were resuspended in 50 μL of 50 mM Tris-HCl, pH 7.5, and 20% (v/v) glycerol, and the protein concentration was adjusted to 1 μg μL^{-1} before the SDS-sample buffer was added. Electrophoresis in SDS-15% polyacrylamide gels and immunoblotting were carried out according to the methods of Laemmli (1970) and Towbin and Gordon (1984), respectively. The amount of 2.5 μg of protein was loaded per lane. The same samples were loaded on gels (7.5 μg per lane) stained with Coomassie brilliant blue R-250 as the loading controls. Immunoblots were chal-

lenged with antisera raised against the major basic PR1 of tomato (Fischer et al., 1989; 1,000× dilution), against a class I PR2 of tobacco (Felix and Meins, 1985; 2,000× dilution), or against a basic PR3 from barley seeds (Swegle et al., 1991; 2,000× dilution). Immunoreactive bands were visualized by goat anti-rabbit secondary antibody coupled to alkaline phosphatase.

RNA Blotting and Translation in Vitro

Total RNA and poly(A)$^+$ RNA were isolated from entire rice shoots (leaf 3 and leaf 4), as described previously (Schweizer et al., 1995). Poly(A)$^+$ RNA translated in vitro by a rabbit reticulocyte lysate and the [^{35}S]Met-/Cys-labeled ITPs were displayed by two-dimensional PAGE, as described previously (Schweizer et al., 1995). Total RNA (10 μg per lane) was separated on formaldehyde-containing agarose gels, transferred onto nylon membranes (Hybond, Amersham), and hybridized with ^{32}P-labeled cDNA probes of pathogen-inducible rice mRNAs PIR2 (PR5-type protein, Reimmann and Dudler, 1993) and PIR3 (PR9-type protein, Reimmann et al., 1992).

JA Levels

JA was extracted from leaf 4 by homogenizing 0.5 to 1 g fresh weight in 5 mL of ethanol using a polytron (Kinematica, Kriens-Luzern, Switzerland). Before homogenization, 0.5 μg of 9,10-dihydro-JA was added as an internal standard. The extracts were processed as described previously (Gundlach et al., 1992), except for the incubation of the crude homogenate in ethanol on a rotating incubator for 2 h at room temperature. The final diazoethane-methylated residues were redissolved in 20 μL of *n*-hexane for quantitative analysis by GC/MS. Separations were carried out on a capillary column (25 m × 0.2 mm) of BP-225 (Scientific Glass Engineering, Melbourne, Australia) fitted to a Hewlett-Packard 5980 GC coupled to a 5970 mass-specific detector. The methyl esters of JA and 9,10-dihydro-JA were detected and quantified by selective ion monitoring (electron impact 70 eV) at m/z 224 and 156, respectively. The detection limit for JA was approximately 0.5 ng g^{-1} fresh weight. The identity of endogenous rice MeJA with authentic MeJA was confirmed by monitoring m/z 109, 135, 151, 156, 193, and 224. The relative abundance of these diagnostic ions was 100 (100), 77 (62), 93 (78), 28 (42), 28 (33), and 18% (52%), respectively (values in parentheses are for authentic MeJA).

RESULTS

Gene Induction by JA and *M. grisea*

Both JA and *M. grisea* induced the accumulation of PR1 (unknown enzymatic activity, antifungal), PR2 (β-1,3-glucanase), PR3 (chitinase), PR5 (thaumatin-like), and PR9 (peroxidase) mRNA or protein (Figs. 1 and 2). The antisera detected several induced bands of PR1- and PR2-like proteins of approximately 18 to 20 kD and approximately 29 to 36 kD, respectively, but only one 29-kD band of a PR3-like protein (Fig. 1). In some experiments the anti-PR3 antiserum reacted with a constitutive protein of approximately 35 kD. In general, the molecular masses of these PR proteins were slightly overestimated, since the prestained markers did not always migrate accurately. The isoforms of the PR proteins induced by JA and *M. grisea* had the same migration characteristics, suggesting their identity. One PR2-like protein of approximately 29 kD was induced only by JA. The PR5 and PR9 cDNA probes detected multigene families each, which means that no conclusion about induction of the same genes by JA and *M. grisea* can be made. The mock inoculation also weakly induced several PR proteins and mRNAs. We assume that this effect was due to either gelatin that was present in the spraying solution or the incubation conditions (see "Materials and Methods") since Tween 20, when applied in water, was inactive (Fig. 7). The incompatible pathogen *P. syringae* induced the same PR proteins and mRNAs (Smith and Métraux, 1991; P. Schweizer and J.-P. Metraux, unpublished data).

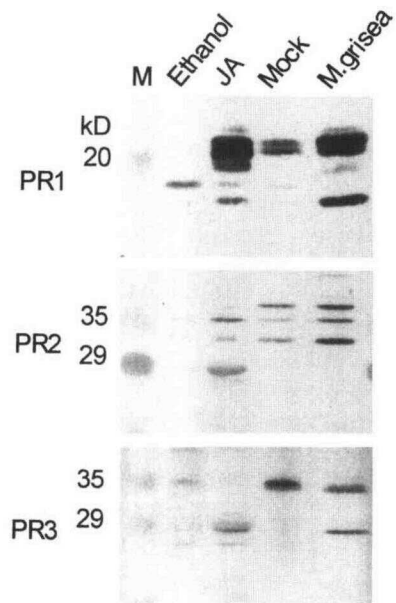

Figure 1. Induction of PR proteins by JA and *M. grisea*. Rice leaves were sprayed with 1 mg mL^{-1} JA in ethanol or with a spore suspension of *M. grisea*, and proteins were extracted 3 d later. Mock, Spray with 0.25% (w/v) gelatin, and 0.05% (v/v) Tween 20, without spores. Acid-soluble (pH 2.8) proteins from rice leaves were separated by SDS-PAGE (2.5 μg lane^{-1}) and blotted onto nitrocellulose, and PR proteins were detected with antisera against PR1, PR2, and PR3, as specified in "Materials and Methods." M, Molecular mass marker.

General gene expression patterns were analyzed by a two-dimensional display of ITPs with a slightly acidic pI, which represents the majority of ITPs. Choosing a wider pH range (3.0–10.0) resulted in the display of a few additional alkaline ITPs but the resolution of the slightly alkaline ITPs was poorer. This method is sensitive and allows differentiation between individual products of multigene families. The two-dimensional display of ITPs revealed 17 pathogen-induced gene products (Fig. 3; Table I). The data shown in Table I are a compilation of ITPs induced in four independent experiments at several times postinoculation.

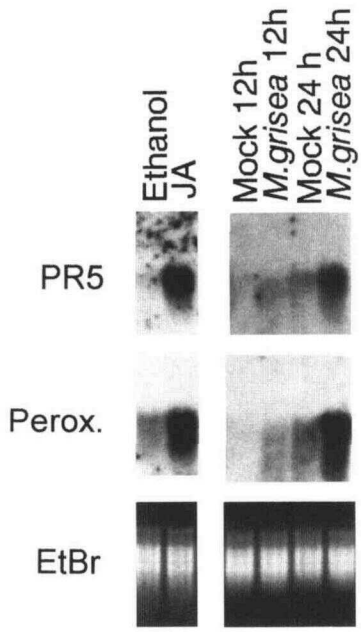

Figure 2. *Induction of mRNA for PR proteins by JA and by M. grisea.* Rice leaves were sprayed with 1 mg mL^{-1} JA in ethanol or with a spore suspension of *M. grisea*, and RNA was extracted 12 h after JA treatment or 12 to 24 h postinoculation. Total RNA (10 μg $lane^{-1}$) was separated on formaldehyde-containing agarose gels, blotted to nylon membranes, and hybridized with ^{32}P-labeled cDNA probes for rice PR5 and peroxidase (Perox., PR9), as specified in "Materials and Methods." EtBr, Ethidium-bromide stained RNA (1 μg $lane^{-1}$); Mock, See Figure 1.

ITPs induced in at least two independent experiments were taken into consideration. This explains why not all pathogen-induced ITPs are visible in Figure 3. Of the 17 pathogen-induced ITPs, 9 were also induced by JA, which had a pleiotropic effect on gene expression and induced at least 29 additional ITPs. Similarly to that found for barley leaves floating on MeJA solutions (Mueller-Uri et al., 1988), we did not find evidence for massive repression of gene expression by JA at the transcriptional level. However, unlike the situation in barley (Roloff et al., 1994), translation and, as a consequence, Rubisco protein were not decreased 3 d after the JA treatment of rice leaves (data not shown). The difference might be due to the different modes of JA or MeJA application: continuous treatment of barley for 3 d versus one spray of rice, with the sprayed JA being rapidly conjugated or degraded (data not shown).

JA Levels

The levels of nonconjugated (−)-JA in pathogen-attacked leaves were determined by combined GC-MS (Fig. 4). The compatible pathogen *M. grisea* did not give rise to a significant increase of (−)-JA (Fig. 4A). Inoculation with the incompatible pathogen *P. syringae* gave rise to a slow, 4-fold increase of (−)-JA, compared with the nontreated leaves, but this increase was obviously due to the injection procedure, since it was also observed in the water-injected "mock" leaves (Fig. 4B). If *P. syringae* was a real inducer of the JA levels, then this JA accumulation should not have been masked by the mock treatment, since the later represents only a weak effect, compared with, for example, wounding that enhanced (−)-JA levels to approximately 40 ng g^{-1} fresh weight (P. Schweizer, A. Buchala, and J.-P. Metraux, unpublished data). The difference in the basal (−)-JA levels (between Fig. 4, A and B) was due to the use of leaves 4 and 3, respectively. At 48 h postinoculation with *P. syringae* dark brown, incompatible-type lesions were clearly visible, demonstrating that symptom development was not associated with enhanced (−)-JA levels, compared

Figure 3. *Two-dimensional display of ITPs from* JA-treated and *M. grisea*-infected leaves. Rice leaves were sprayed with 1 mg mL^{-1} JA in ethanol or with a spore suspension of *M. grisea*. mRNA was extracted 12 h after JA treatment or 12 h postinoculation with *M. grisea*, translated in vitro by a rabbit reticulocyte lysate into ^{35}S-labeled ITPs that were separated by two-dimensional PAGE. *ITPs inside triangles or a* rectangle are enhanced in response to JA or *M. grisea*. The pathogen-induced ITPs are identified by numbers, and JA-induced ITPs with identical migration characteristics are given the same numbers. Mock, See Figure 1. Molecular mass is indicated in kD on the right of the figure.

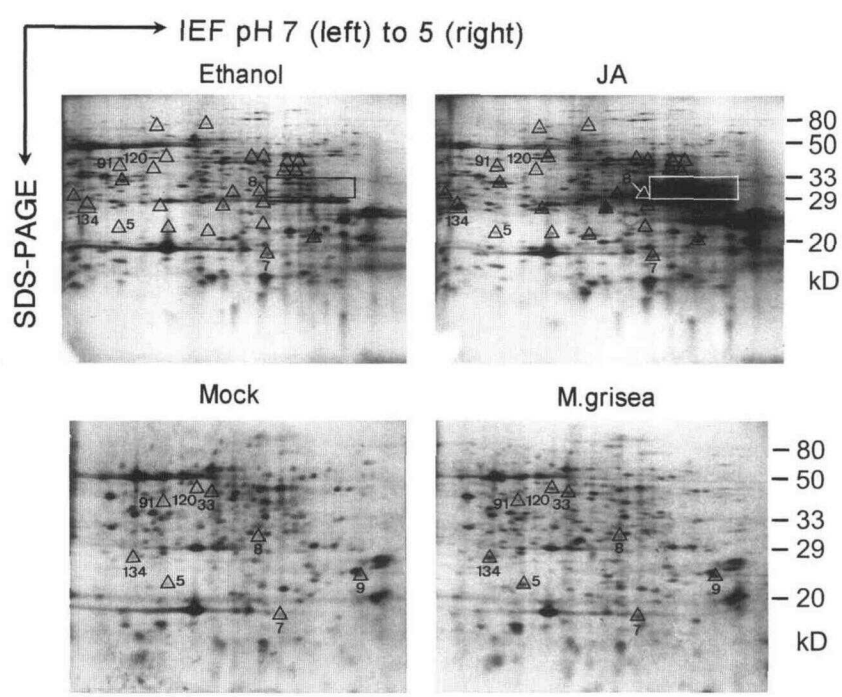

Table I. *Summary of gene expression data in rice*

Gene Product	*M. grisea*	JA
PR1 protein	++[a]	++
PR2 protein	++	+[b] to ++
PR3 protein	++	++
PR5 mRNA	++	++
PR9 mRNA	++	++
ITP4	+	+
ITP5	+ to ++	+ to ++
ITP7	++	+
ITP8	++	++
ITP9	+	−[c]
ITP10	++	++
ITP11	++	−
ITP17	++	−
ITP18	++	−
ITP33	++	−
ITP51	+	+
ITP72	+	+
ITP86	+	−
ITP91	++	++
ITP120	++	++
ITP131	++	−
ITP134	++	++
29 ITPs	−	++
ASP26	−	++

[a] ++, Strong induction. [b] +, Weak induction (usually above basal level of expression). [c] −, No induction.

with the mock treatment that did not induce any visible symptoms. The 4-fold increase in the (−)-JA levels in the mock-injected leaves did not induce PR gene expression (P. Schweizer and J.-P. Metraux, unpublished data). At 12 to 48 h postinoculation with *M. grisea*, the latest time analyzed for (−)-JA accumulation, PR genes and proteins were clearly induced (Figs. 2, 3, and 5). This demonstrates that (−)-JA accumulation is not required for induction of these jasmonate-inducible PR genes.

Evidence for a Signaling Network

Tetcyclacis is a growth retardant that inhibits the enzymes of the Cyt P450 superfamily (Rademacher, 1991). Tetcyclacis was recently reported to block jasmonate biosynthesis in barley, probably by inhibiting allene oxide synthase, a P450 enzyme (Wasternack et al., 1995). We used this novel inhibitor, which blocks downstream of lipoxygenases and might therefore be more specific than the known lipoxygenase inhibitors, in an attempt to lower jasmonate levels in rice. Tetcyclacis caused the stunting of rice plants without any signs of phytotoxicity and did not affect disease development (data not shown). Levels of (−)-JA in inhibitor-treated plants were lower than in control plants (Table II). We then addressed the question of whether these lower JA levels influenced the up-regulation of PR1 and PR2 by *M. grisea*. The accumulation of the PR1-like proteins was reduced in tetcyclacis-treated, infected plants, and PR1 induction was rescued by treating plants with gaseous MeJA (Fig. 6A). Gaseous MeJA was used here because JA was reported to be antifungal when applied to leaf surfaces immediately before challenge inoculation (Schweizer et al., 1993). The PR1-like proteins of a higher molecular mass were more sensitive to tetcyclacis inhibition and were also the ones that were most efficiently induced by JA (see also Fig. 1). The accumulation of PR2-like proteins was apparently not influenced by tetcyclacis. Two independent repetitions of the experiment gave similar results (Fig. 6, B and C). The effect of tetcyclacis on JA levels and PR1 accumulation was partial, possibly due to an insufficient concentration of the inhibitor. However, because of the very limited solubility in water, the concentration could not be increased further. Another inhibitor of jasmonate biosynthesis, diethyldithiocarbamic acid (Farmer et al., 1994), also prevented elicitor-induced PR1, but not PR2, induction when sprayed together with the elicitor onto leaves (data not shown). PR1 induction could be rescued by exogenous JA. Surprisingly, this inhibitor enhanced JA levels in treated leaves. We speculate that this may have been due to the visible, phytotoxic effect of diethyldithiocarbamic acid and that JA increased in those cells or tissues that were not reached by the inhibitor but perceived stress-related signals from the cells suffering

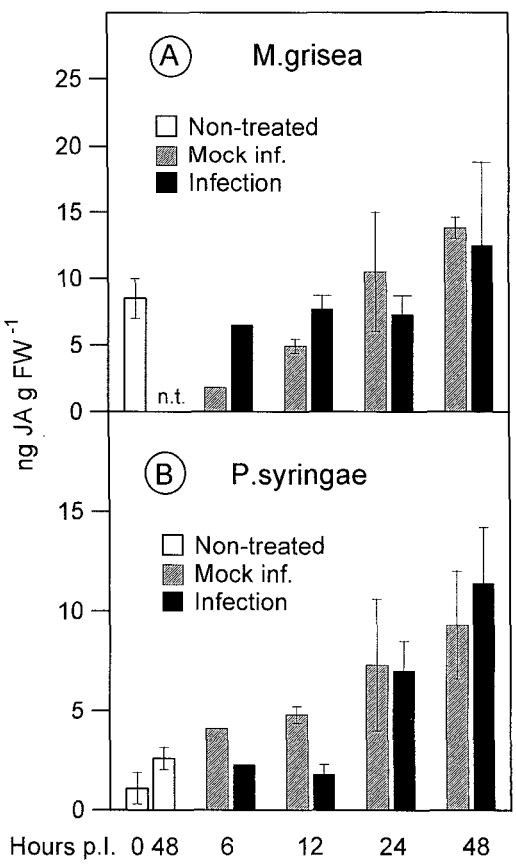

Figure 4. Levels of JA are not enhanced in response to pathogen attack. Leaves were inoculated with either *M. grisea* (A) or *P. syringae* pv *syringae* (B), and levels of nonconjugated (−)-JA were detected by combined GC-MS at the times indicated postinoculation (p.l.). Means ± SE are from several independent experiments. Thirty to 50 plants were used per sample. Mock infection (Mock inf.) for *M. grisea* and *P. syringae* refers to a spray with gelatin/Tween 20 solution and water injection, respectively. FW, Fresh weight; n.t., Not tested.

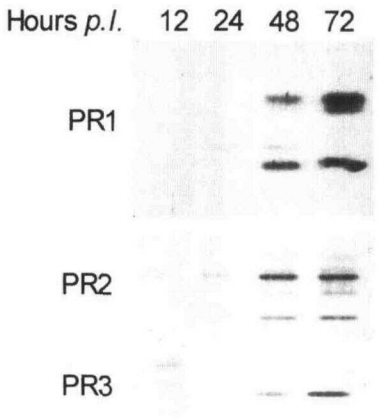

Figure 5. Kinetics of PR protein accumulation in *M. grisea*-infected leaves. Acid-soluble proteins were extracted at the times indicated postinoculation (p.l.) and separated by SDS-PAGE (2.5 μg lane^{-1}), and PR proteins on nitrocellulose were detected by antisera against PR1, PR2, and PR3. For details, see Figure 1 and "Materials and Methods."

from the phytotoxic effect. Two other inhibitors of jasmonate biosynthesis, salicylic acid and ursolic acid, were ineffective in rice (data not shown).

The second approach consisted of the addition of increasing amounts of JA to dilutions of a crude elicitor of *M. grisea* with subsequent monitoring of PR1 induction, to look for additive or synergistic interactions between the octadecanoid and another hypothetical, pathogen-induced pathway. The elicitor and JA induced accumulation of PR1-like proteins in a weakly synergistic and additive manner at low and high concentrations of JA, respectively (Fig. 7). In conclusion, JA appears to enhance, in a signaling network, induction of PR1-like proteins by pathogen-derived signals.

Wounding and JA induced the accumulation of an abundant, acid-soluble protein of apparently 26 kD named ASP26 (Fig. 8). ASP26 was not induced by the elicitor or by *M. grisea* (Fig. 8; data not shown). The elicitor repressed the induction of ASP26 by JA, demonstrating the existence of negative cross-talk between the octadecanoid-activated and another, elicitor-activated signaling pathway.

Plant Protection

Plants were sprayed with increasing concentrations of JA and challenge-inoculated 2 or 4 d later with either *M. grisea* or *X. campestris* (Fig. 9). The strain of *M. grisea* that was used was very virulent on cv Nohrin 29, causing large lesions on the youngest leaf within 4 to 5 d postinoculation and often collapse of the entire leaf 6 to 7 d postinoculation (data not shown). Because of considerable variations of disease severity between individual inoculation experiments, relative values of infection are given. JA did not protect the leaf from infection by *M. grisea* (Fig. 9A). The strain of *X. campestris* that we used grew abundantly in the leaves of cv Nohrin 29, and JA did not protect the leaf from infection by this bacterial pathogen either (Fig. 9B).

DISCUSSION

The implication of the octadecanoid pathway in defense reactions against pathogens is mainly derived from model systems (Gundlach et al., 1992; Mueller et al., 1993; Blechert et al., 1995; Nojiri et al., 1996), and only in a few cases was the model tested in true plant-pathogen interactions (Cohen et al., 1993; Schweizer et al., 1993; Kogel et al., 1995). Here we tested the hypothetical role of JA in the interaction of rice with fungal and bacterial pathogens.

Exogenous application of JA induced the accumulation of mRNA or protein of PR1, PR2, PR3, PR5, and PR9 in rice, plus 50% of pathogen-induced mRNAs of unknown identity, reflected by their respective ITPs. This result suggests a role for JA in mediating defense-related gene induction in rice. We therefore expected to find an activation of the octadecanoid pathway by a pathogen attack in rice, reflected by an accumulation of (−)-JA. To our surprise, (−)-JA did not accumulate upon inoculation with either the compatible fungal pathogen *M. grisea* or the incompatible bacterial pathogen *P. syringae*, although both pathogens induced the same PR1-like and PR2-like proteins, plus PR5 and PR9 mRNAs (this report; Smith and Métraux, 1991; P. Schweizer and J.-P. Metraux, unpublished data). This negative result is reminiscent of the situation in pathogen-attacked barley (Kogel et al., 1995). JA was reported to induce some momilactone A accumulation in rice cells (Nojiri et al., 1996). Since momilactone A was found to accumulate not before 60 h postinoculation in a compatible interaction with *M. grisea* (Cartwright and Langcake, 1980), it cannot be excluded that (−)-JA levels would increase in infected rice leaves later than 48 h postinoculation, which is the latest time measured here. However, such a late accumulation of (−)-JA would not be relevant for induction of the PR genes discussed here. It is theoretically possible that

Table II. *Tetcyclacis reduces JA levels in infected rice leaves*

Treatment[a]	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Mean[b]	P[c]	%
			ng JA g^{-1} fresh wt				
Control[d]	7.9	6.6	9.9	3.9	7.1 ± 1.3	–	100
Tetcyclacis	5.2	4.1	4.4	2.9	4.2 ± 0.5	0.05	62

[a] Plants were treated with tetcyclacis and inoculated with *M. grisea* 48 h later. Nonconjugated (−)-JA was extracted 12 h postinoculation from the young, expanding leaf 4 and analyzed by combined GC-MS. [b] Means ± SD from experiments 1 to 4. Each independent experiment was performed in triplicate, using approximately 90 plants per treatment. [c] A Student's *t* test was used for statistical significance of the difference between control and tetcyclacis. [d] Control plants were treated with the same amount of DMSO as tetcyclacis-treated plants.

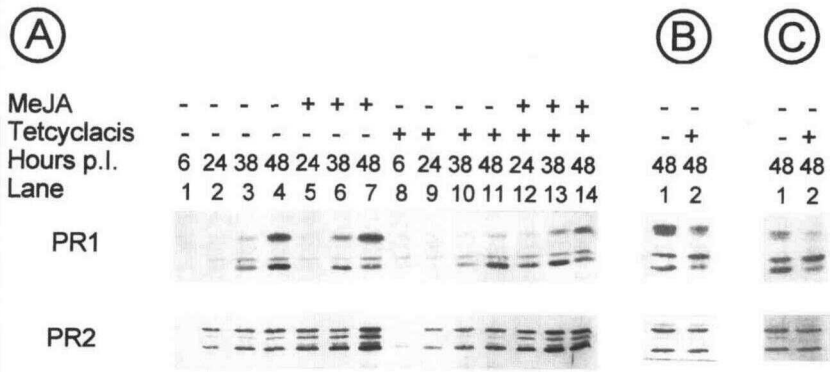

Figure 6. Tetcyclacis reduces the accumulation of PR1-like proteins in infected leaves. Plants were treated with tetcyclacis and inoculated with *M. grisea* 48 h later. A, Acid-soluble proteins were extracted from the young, expanding leaf 4 at the times indicated postinoculation (p.l.) and separated by SDS-PAGE (2.5 μg lane^{-1}), and PR proteins on nitrocellulose were detected by antisera against PR1 and PR2. Where indicated, inoculated plants were incubated in a MeJA atmosphere from 6 h postinoculation until harvest of leaf material. B and C, Independent repetitions of the experiment.

in rice an activation of the octadecanoid pathway is reflected by an accumulation of jasmonate conjugates (Sembdner and Parthier, 1993) instead of nonconjugated (−)-JA or by altered compartmentation of (−)-JA. However, we found a strong accumulation of nonconjugated (−)-JA upon wounding or chemical treatment of rice (P. Schweizer, unpublished data). Therefore, there is no reason to assume that in rice an activation of the octadecanoid pathway would not be reflected by an accumulation of nonconjugated (−)-JA. Upon sink limitation in soybean, multiple lipoxygenase mRNAs are induced, which are also strongly up-regulated by JA, but JA levels remained low (Bunker et al., 1995). That report, together with the results presented here, demonstrate that great care has to be taken when drawing conclusions about the implication of signaling pathways in physiological responses solely from results about inducibility of any such response by signaling compounds.

The octadecanoid pathway was not activated in rice attacked by either *M. grisea* or *P. syringae*, a compatible and an incompatible pathogen, respectively. Why then are so many PR genes induced by JA? One possible explanation is that defense responses in rice can be activated either by the octadecanoid or another pathway, depending on the pathogen. This would imply the existence of other rice pathogens that activate the octadecanoid pathway. Another explanation may be that some PR genes also play a role in stress situations other than pathogen attack, e.g. wounding. Indeed, we found a sharp increase of JA levels in wounded leaves and induction of most of the PR genes analyzed (P. Schweizer, A. Chala, and J.-P. Metraux, unpublished data). Finally, there exists the possibility that JA plays the role as a partner in a signaling network with other pathogen-induced pathways, resulting in the cooperative induction of PR genes upon pathogen attack. In this model JA may not need to accumulate but be available at a certain minimal concentration. The higher the JA levels, e.g. brought about by other forms of stress, the stronger the PR response may be. We set out to test this last hypothesis by lowering endogenous JA levels with inhibitors of jasmonate biosynthesis and by enhancing the levels with an exogenous application of JA. PR1 and PR2 accumulation in response to *M. grisea* or the elicitor were then analyzed. Reducing endogenous JA levels also reduced the accumulation of PR1-like proteins. Enhancing JA levels resulted in synergistic induction of PR1-like proteins. Therefore, although (−)-JA did not accumulate upon pathogen attack, it appeared to be required for PR1 accumulation and had an enhancer effect on the accumulation of this family of proteins. This proposed role of JA in signaling does not apply to all JA-inducible genes, since JA seemed to be redundant for PR2 induction by the pathogen. In accordance with the

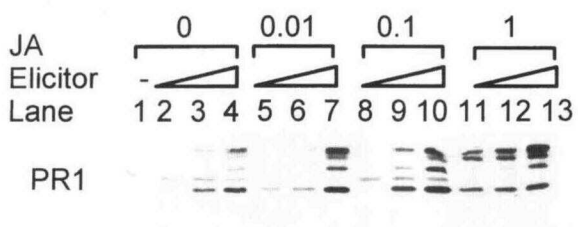

Figure 7. Synergistic induction of PR1-like proteins by JA and the elicitor. Leaves were sprayed simultaneously with various concentrations of JA (in mg mL^{-1}) and crude elicitor from *M. grisea*, as indicated above the upper blot. Acid-soluble proteins were extracted 3 d after the treatment and separated by SDS-PAGE (2.5 μg lane^{-1}), and PR proteins on nitrocellulose were detected by antisera against PR1 and PR2. The elicitor was applied at dilutions of 1:100, 1:10, and 1:0, symbolized by triangles above the lanes. In lane 1 plants were sprayed with 0.05% Tween 20.

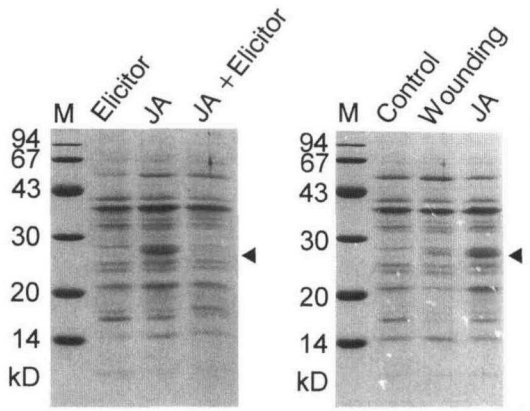

Figure 8. Antagonistic regulation of an abundant protein by JA and fungal elicitor. Leaves were sprayed with 1 mg mL^{-1} JA, with or without undiluted elicitor, or wounded. Acid-soluble proteins were extracted 3 d after the treatment, separated on SDS-PAGE (7.5 μg lane^{-1}), and stained with Coomassie blue. The arrowheads point to the induced ASP26. Lanes M, Molecular mass marker.

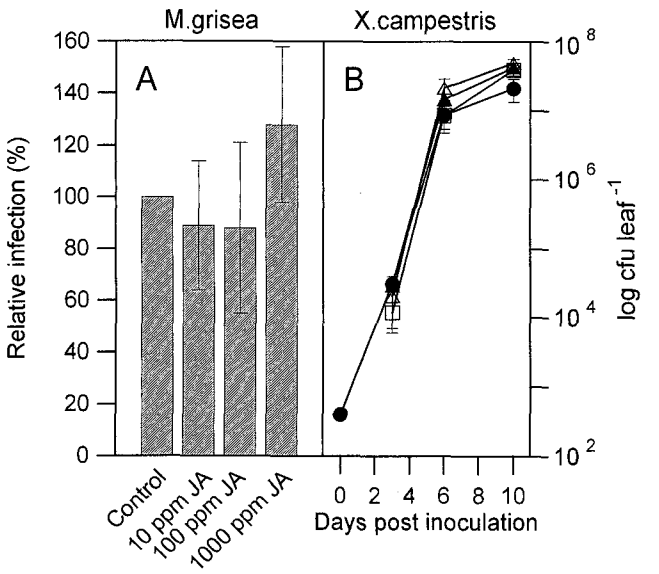

Figure 9. JA does not induce local pathogen resistance. A, Leaf 3 was sprayed with JA and challenge-inoculated 2 d later with *M. grisea*. Results are means ± SD from three independent experiments with approximately 40 to 50 plants per treatment and experiment. Control, Sprayed with ethanol. B, Plants were left nontreated (●), sprayed with ethanol (□), or sprayed with 0.1 mg mL^{-1} (△) or 1 mg mL^{-1} (▲) JA, and challenge-inoculated with *X. campestris* 4 d later. Bacterial titer in leaves was determined at the times indicated. Results are means ± SD from one typical experiment (n = 4). cfu, Colony-forming unit.

view of the octadecanoid pathway as an enhancer of defense reactions is the finding that exogenously applied JA potentiated gene and resistance induction in rice by low doses of the synthetic inducer 2,6-dichloroisonicotinic acid (P. Schweizer and J.-P. Metraux, unpublished data). It may be speculated about the cooperation that exists between the wound-induced octadecanoid pathway and pathogen-induced pathways in rice exposed simultaneously to both pathogens and herbivores, resulting in an enhanced, broad-spectrum resistance against pathogens and herbivores. Positive interaction between MeJA and ethylene and between MeJA and fungal elicitor have also been reported for tobacco (Rickauer et al., 1992; Xu et al., 1994).

There also exists negative cross-talk between the two pathways, exemplified by the repression of the wound- and JA-inducible protein ASP26 in the presence of the elicitor. The block of ASP26 induction by pathogen-derived signals is located after the formation of JA. Repression of the octadecanoid pathway by salicylic acid as a central molecule of a pathogen-induced signaling pathway was found in potato, but in this system, the block appears to be located upstream from the formation of jasmonate (Pena-Cortes et al., 1993).

Treatment of rice leaves with JA induced a number of defense-related genes but not local acquired resistance to a fungal or a bacterial pathogen. This strongly suggests that JA did not induce the whole set of defense reaction(s), some of which are crucial for warding off these pathogens. There exist yet unknown defense mechanisms in rice cv Nohrin 29 that are efficient against at least *M. grisea* and that can be induced by preinoculation with *P. syringae* (Reimmann et al., 1995) or 2,6-dichloroisonicotinic acid (P. Schweizer and J.-P. Metraux, unpublished data). It is interesting that resistance induction in rice cv Nohrin 29 by 2,6-dichloroisonicotinic acid was not strictly correlated with the accumulation of PR1-, PR2-, and PR3-like proteins (P. Schweizer and J.-P. Metraux, unpublished data). Therefore, the two approaches complement each other, arguing against a role of these PR proteins in acquired resistance of rice to *M. grisea*. However, it would be interesting to test the resistance-inducing potential of JA to other rice pathogens such as *Pythium* spp. This oomycete might by susceptible to the JA-induced, PR1-like proteins of rice (Niderman et al., 1995). Our results concerning the lack of local resistance induction in rice by JA are in accordance with earlier reports of the lack of local acquired resistance in JA-treated barley (Schweizer et al., 1993; Kogel et al., 1995). This finding, together with the fact that JA could not induce the full set of PR genes, leads to the conclusion that jasmonates do not orchestrate localized defense responses in rice.

We have found examples of positive (PR1) and negative (ASP26) interaction between JA and pathogen-derived signals. These data are the first indication for subtle regulation of defense responses in rice by a signaling network comprising the octadecanoid pathway. The network may be operating at the promoter level of the different PR or wound-related genes rather than at the level of activation or repression of entire pathways. This model is corroborated by recent reports that specific isoforms of rice PR genes or proteins are inducible by wounding, as well as by pathogens or elicitors (Simmons et al., 1992; Zhu et al., 1993, 1995; Anarutha et al., 1996; Xu et al., 1996). Taken together, a picture emerges of multiple jasmonate-, wound-, and pathogen-inducibility of many defense-related genes in rice. This is different from what was found in several dicotyledonous species in which defense-related genes are usually induced either by wounding and jasmonates or by pathogen attack and salicylic acid (Pena-Cortes et al., 1993; Choi et al., 1994; Doares et al., 1995; Seo et al., 1995). In rice salicylic acid did not accumulate upon pathogen attack. Instead, the leaves contained high basal levels of nonconjugated salicylic acid (Silverman et al., 1995). Exogenously applied salicylic acid did not induce the accumulation of PR proteins in rice (P. Schweizer and J.-P. Métraux, unpublished data). Based on this evidence, we believe that salicylic acid does not play an important role as the PR signal in rice, which raises the question about other signaling compounds, besides JA and salicylic acid, mediating the PR response.

ACKNOWLEDGMENTS

We would like to thank Luc Vincent and Ruth Bosshard for taking care of the rice culture and Drs. Egon Mösinger, Fred Meins, and Subbaratnam Muthukrishnan for the kind gift of antisera against tomato PR1, tobacco PR2, and barley PR3, respectively. We are thankful to CIBA-Geigy for the kind gift of the rice seed and pathogens and to Dr. W. Rademacher (BASF) for the kind gift of tetcyclacis. Furthermore, assistance by Jean-Jaques Pittet with the electronic image processing is acknowledged.

Received September 24, 1996; accepted January 24, 1997.

LITERATURE CITED

Anarutha CS, Zen K-C, Cole KC, Mew T, Muthukrishnan S (1996) Induction of chitinases and β-1,3-glucanases in *Rhizoctonia solani*-infected rice plants: isolation of an infection-related chitinase cDNA clone. Physiol Plant **97:** 39–46

Andresen I, Becker W, Schlüter K, Burges J, Parthier B, Apel K (1992) The identification of leaf thionin as one of the main jasmonate-induced proteins of barley (*Hordeum vulgare*). Plant Mol Biol **19:** 193–204

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

Blechert S, Brodschelm W, Hölder S, Kammerer L, Kutchan TM, Mueller MJ, Xia Z-Q, Zenk MH (1995) The octadecanoic pathway: signal molecules for the regulation of secondary pathways. Proc Natl Acad Sci USA **92:** 4099–4105

Bolter CJ (1993) Methyl jasmonate induces papain inhibitor(s) in tomato leaves. Plant Physiol **103:** 1347–1353

Bunker TW, Koetje DS, Stephenson LC, Creelman RA, Mullet JE, Grimes HD (1995) Sink limitation induces the expression of multiple soybean vegetative lipoxygenase mRNAs while the endogenous jasmonic acid level remains low. Plant Cell **7:** 1319–1331

Cartwright W, Langcake P (1980) Phytoalexin production in rice and its enhancement by a chlorocyclopropane fungicide. Physiol Plant Pathol **17:** 259–267

Chandra S, Heinstein PF, Low PS (1996) Activation of phospholipase A by plant defense elicitors. Plant Physiol **110:** 979–986

Chaudhry B, Müller-Uri F, Cameron-Mills V, Gough S, Simpson D, Skriver K, Mundy J (1994) The barley 60 kDa jasmonate-induced protein (JIP60) is a novel ribosome-inactivating protein. Plant J **6:** 815–824

Choi D, Bostock RM, Avdiushko S, Hildebrand DF (1994) Lipid-derived signals that discriminate wound- and pathogen-responsive isoprenoid pathways in plants: methyl jasmonate and the fungal elicitor arachidonic acid induce different 3-hydroxy-3-methylglutaryl-coenzyme A reductase genes and antimicrobial isoprenoids in *Solanum tuberosum* L. Proc Natl Acad Sci USA **91:** 2329–2333

Cohen Y, Gisi U, Niderman T (1993) Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic-methyl-ester. Phytopathology **83:** 1054–1062

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

Demole E, Lederer E, Mercier D (1962) Isolement et détermination de la structure du jasmonate de méthyle, constituant odorant characteristique de l'essence de jasmin. Helv Chim Acta **45:** 675–685

Dempsey D'MA, Klessig DF (1994) Salicylic acid, active oxygen species and acquired resistance in plants. Trends Cell Biol **4:** 334–338

Doares SH, Syrovets T, Weiler EW, Ryan CA (1995) Oligogalacturonides and chitosan activate plant defensive genes through the octadecanoid pathway. Proc Natl Acad Sci USA **92:** 4095–4098

Farmer EE (1994) Fatty acid signalling in plants and their associated microorganisms. Plant Mol Biol **26:** 1423–1437

Farmer EE, Caldelari D, Pearce G, Walker-Simmons MK, Ryan CA (1994) Diethyl dithiocarbamic acid inhibits the octadecanoid signalling pathway for the wound induction of proteinase inhibitors in tomato leaves. Plant Physiol **106:** 337–342

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

Farmer EE, Ryan CA (1992) Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell **4:** 129–134

Felix G, Meins F Jr (1985) Purification, immunoassay and characterization of an abundant, cytokinin-regulated polypeptide in cultured tobacco tissues. Planta **164:** 423–428

Fischer W, Christ U, Baumgartner M, Erismann KH, Mösinger E (1989) Pathogenesis-related proteins of tomato. II. Biochemical and immunological characterization. Physiol Mol Plant Pathol **35:** 67–83

Gundlach H, Müller MJ, Kutchan TM, Zenk MH (1992) Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA **89:** 2389–2393

Hildmann T, Ebneth M, Pena-Cortés H, Sanchez-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

Hopke J, Donath J, Blechert S, Boland W (1994) Herbivore-induced volatiles: the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by β-glucosidase and jasmonic acid. FEBS Lett **352:** 146–150

Kogel K-H, Ortel B, Jarosch B, Atzorn R, Schiffer R, Wasternack C (1995) Resistance in barley against the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*) is not associated with enhanced levels of endogenous jasmonates. Eur J Plant Pathol **101:** 319–332

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680–685

Li WX, Kodama O, Akatsuka T (1991) Role of oxygenated fatty acids in rice phytoalexin production. Agric Biol Chem **55:** 1041–1047

Mueller MJ, Brodschelm W, Spannagl E, Zenk MH (1993) Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc Natl Acad Sci USA **90:** 7490–7494

Mueller-Uri F, Parthier B, Nover L (1988) Jasmonate-induced alteration of gene expression in barley leaf segments analyzed by in-vivo and in-vitro protein synthesis. Planta **176:** 241–247

Niderman T, Genetet I, Bruyere T, Gees R, Stinzi A, Legrand M, Fritig B, Moesinger E (1995) Pathogenesis-related PR-1 proteins are antifungal. Isolation and characterization of three 14-kilodalton proteins of tomato and of a basic PR-1 of tobacco with inhibitory activity against *Phytophthora infestans*. Plant Physiol **108:** 17–27

Nojiri H, Sugimori M, Yamane H, Nishimura Y, Yamada A, Shibuya N, Kodama O, Murofushi N, Omori T (1996) Involvement of jasmonic acid in elicitor-induced phytoalexin production in suspension-cultured rice cells. Plant Physiol **110:** 387–392

Ohta H, Shida K, Peng Y-L, Furusawa I, Shishiyama J, Aibara S, Morita Y (1991) A lipoxygenase pathway is activated in rice after infection with the rice blast fungus *Magnaporthe grisea*. Plant Physiol **9:** 94–98

Pena-Cortes 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

Peng YL, Shirano Y, Ohta H, Hibino T, Tanaka K, Shibata D (1994) A novel lipoxygenase from rice. Primary structure and specific expression upon incompatible infection with rice blast fungus. J Biol Chem **269:** 3755–3761

Rademacher W (1991) Biochemical effects of plant growth retardants. *In* Gausman HW, ed, Plant Biochemical Regulators. Marcel Dekker, New York, pp 169–200

Reimmann C, Dudler R (1993) cDNA cloning and sequence analysis of a pathogen-induced thaumatin-like protein from rice (*Oryza sativa*). Plant Physiol **101:** 1113–1114

Reimmann C, Hofmann C, Mauch F, Dudler R (1995) Characterization of a rice gene induced by *Pseudomonas syringae* pv *syringae*: requirement for the bacterial *lemA* gene function. Physiol Mol Plant Pathol **46:** 71–81

Reimmann C, Ringli C, Dudler R (1992) Complementary cDNA cloning and sequence analysis of a pathogen-induced putative peroxidase from rice. Plant Physiol **100:** 1611–1612

Rickauer M, Bottin A, Esquerré-Tugayé M-T (1992) Regulation of proteinase inhibitor production in tobacco cells by fungal elicitors, hormonal factors and methyl jasmonate. Plant Physiol Biochem **30:** 579–584

Roloff A, Parthier B, Wasternack C (1994) Relationship between degradation of ribulose-bisphosphate carboxylase/oxygenase and synthesis of an abundant protein of 23 kDa of barley leaves (*Hordeum vulgare* cv. Salome) induced by jasmonates. J Plant Physiol **143:** 39–46

Schweizer P, Gees R, Mösinger E (1993) Effect of jasmonic acid on the interaction of barley (*Hordeum vulgare* L.) with the powdery mildew *Erysiphe graminis* f.sp. *hordei*. Plant Physiol **10:** 503–511

Schweizer P, Vallélian-Bindschedler L, Mösinger E (1995) Heat-induced resistance in barley to the powdery mildew fungus *Erysiphe graminis* f.sp. *hordei*. Physiol Mol Plant Pathol **47:** 51–66

Sembdner G, Parthier B (1993) The biochemistry and the physiological and molecular actions of jasmonates. Annu Rev Plant Physiol Plant Mol Biol **44:** 569–589

Seo S, Okamoto M, Seto H, Ishizuka K, Sano H, Ohashi Y (1995) Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. Science **270:** 1988–1992

Silverman P, Seskar M, Kanter D, Schweizer P, Métraux J-P, Raskin I (1995) Salicylic acid in rice: biosynthesis, conjugation, and possible role. Plant Physiol **108:** 633–639

Simmons CR, Litts JC, Huang N, Rodriguez RL (1992) Structure of a rice-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors. Plant Mol Biol **18:** 33–45

Smith J, Métraux J-P (1991) *Pseudomonas syringae* pv. *syringae* induces systemic resistance to *Pyricularia oryzae* in rice. Physiol Mol Plant Pathol **39:** 451–461

Swegle M, Kramer KJ, Muthukrishnan S (1992) Properties of barley seed chitinases and release of embryo-associated isoforms during early stages of imbibition. Plant Physiol **99:** 1009–1014

Towbin H, Gordon JC (1984) Immunoblotting and dot immunobinding: current status and outlook. J Immunol Methods **72:** 313–340.

Wasternack C, Atzorn R, Leopold J, Feussner I, Rademacher W, Parthier B (1995) Synthesis of jasmonate-induced proteins in barley (*Hordeum vulgare*) is inhibited by the growth retardant tetcyclacis. Physiol Plant **94:** 335–341

Xu Y, Chang PL, Liu D, Narasimhan ML, Raghothama KG, Hasegawa PM, Bressan RA (1994) Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6:** 1077–1085

Xu Y, Zhu Q, Panbangred W, Shirasu K, Lamb CJ (1996) Regulation, expression and function of a new basic chitinase gene in rice (*Oryza sativa* L.). Plant Mol Biol **30:** 387–401

Zhu Q, Dabi T, Beeche A, Yamamoto R, Lawton MA, Lamb CJ (1995) Cloning and properties of a rice gene encoding phenylalanine ammonia-lyase. Plant Mol Biol **29:** 535–550

Zhu Q, Doerner PW, Lamb CJ (1993) Stress induction and developmental regulation of a rice chitinase promoter in transgenic tobacco. Plant J **3:** 203–212