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Gene-Expression Patterns and Levels of Jasmonic Acid in Rice Treated with the Resistance Inducer 2,6-Dichloroisonicotinic Acid[1]

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Acquired disease resistance can be induced in rice (*Oryza sativa*) by a number of synthetic or natural compounds, but the molecular mechanisms behind the phenomenon are poorly understood. One of the synthetic inducers of resistance, 2,6-dichloroisonicotinic acid (INA), efficiently protected rice leaves from infection by the rice blast fungus *Magnaporthe grisea* (Hebert) Barr. A comparison of gene-expression patterns in plants treated with INA versus plants inoculated with the compatible pathogen *M. grisea* or the incompatible pathogen *Pseudomonas syringae* pv *syringae* revealed only a marginal overlap: 6 gene products, including pathogenesis-related proteins (PR1-PR9), accumulated in both INA-treated and pathogen-attacked leaves, whereas 26 other gene products accumulated only in INA-treated or only in pathogen-attacked leaves. Lipoxygenase enzyme activity and levels of nonconjugated jasmonic acid (JA) were enhanced in leaves of plants treated with a high dose of INA (100 ppm). Exogenously applied JA enhanced the gene induction and plant protection caused by lower doses of INA (0.1 to 10 ppm) that by themselves did not give rise to enhanced levels of endogenous (-)-JA. These data suggest that INA, aside from activating a pathogen-induced signaling pathway, also induces events that are not related to pathogenesis. JA acts as an enhancer of both types of INA-induced reactions in rice.

Acquired resistance or SAR of plants against pathogenic organisms probably constitutes a naturally occurring defense strategy that contributes to limiting the damage caused by virulent pathogens (for review, see Ryals et al., 1996, and Schneider et al., 1996). In the laboratory SAR can be induced by a primary inoculation with pathogenic or nonpathogenic viruses, bacteria, or fungi (Ross, 1961; Caruso and Kuc, 1979; Cohen and Kuc, 1981; Ward et al., 1991; Uknes et al., 1992). Acquired resistance can also be triggered by natural or synthetic compounds (for review, see Kessmann et al., 1994, and Schneider et al., 1996). At present the best characterized synthetic inducer of resistance is INA (Métraux et al., 1991).

INA was found to protect cucumber (*Cucumis sativus*) (Métraux et al., 1991), tobacco (*Nicotiana tabacum*) (Ward et al., 1991), Arabidopsis (Uknes et al., 1992), sugar beet (*Beta vulgaris*) (Nielsen et al., 1994), bean (*Phaseolus vulgaris*) (Dann and Deverall, 1995), rose (*Rosa* sp.) (Hijwegen et al., 1996), barley (*Hordeum vulgare*) (Kogel et al., 1994), and rice (*Oryza sativa*) (Métraux et al., 1991) from a number of pathogens. Two synthetic compounds structurally related to INA were also reported to induce resistance in rice (Yoshida et al., 1990; Seguchi et al., 1992).

The broad-spectrum activity of INA, conferring protection against viral, bacterial, and fungal diseases, strongly suggests an indirect mode of action via activation of plant defense mechanisms. This hypothesis was recently proven to be correct by the identification of *A. thaliana* mutant plants that are no longer protected by INA, in contrast to the corresponding wild-type plants (Cao et al., 1994; Delaney et al., 1995).

In tobacco the coordinated induction by INA of a set of so-called SAR genes was reported and included PR1 to PR9 proteins plus a gene called *SAR8.2* (Ward et al., 1991). "SAR gene" relates to the fact that these genes are activated in plants expressing biologically induced "natural" SAR. In other plants at least some of these genes are activated by INA (Uknes et al., 1992; Kogel et al., 1994). In sugar beet INA does not induce β-1,3 glucanase or chitinase, but conditions the plant to induce these PR proteins faster upon attack by *Cercospora beticola* (Nielsen et al., 1994). Similar results were found in INA-treated cucumber hypocotyls upon challenge by *Colletotrichum lagenarium* (Siegrist et al., 1994). Cytological investigation in barley (Kogel et al., 1994) and Arabidopsis (Uknes et al., 1992) revealed phenocopies of genetically determined incompatible interactions induced by INA, characterized by hypersensitively dying plant cells in the vicinity of fungal hyphae. These examples suggest a dual mode of action of INA: induction of defense responses prior to infection, as well as potentiation of defense responses postinoculation. It remains to be rigorously tested for each plant-pathogen interaction which of these INA-induced mechanisms finally determines the outcome of infection, a difficult task that may require the use of transgenic or mutant plants that are suppressed or defective in some defense reactions.

As far as host-gene expression in INA-treated plants is concerned, it is not known if INA induces genes other than

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Abbreviations: ASP, acid-soluble protein; INA, 2,6-dichloroisonicotinic acid; ITP, in vitro translation product; JA, (±)-jasmonic acid; LOX, lipoxygenase; PR, pathogenesis-related; SAR, systemic acquired resistance.

the known SAR genes. At least in plants that show the "conditioning" phenomenon (Nielsen et al., 1994; Siegrist et al., 1994), INA might activate additional regulatory genes responsible for the accelerated defense responses upon subsequent pathogen challenge. To better understand the mechanism of acquired resistance in plants, more detailed information about gene-expression patterns that are correlated with the buildup and maintenance of acquired resistance are required.

It was recently reported that the effect of INA on tobacco plants is not mediated by salicylic acid (Vernooij et al., 1995). One possible explanation for this observation is that INA acts downstream of salicylic acid in the same pathway. However, it cannot be excluded that INA activates another, independent signaling pathway. One possible INA-activated signaling pathway is the octadecanoid pathway with (-)-JA as a central component (Sembdner and Partier, 1993; Creelman and Mullet, 1995; Doares et al., 1995), since INA was found to be a strong inducer of LOX enzyme activity (Schaffrath, 1994).

In the present study we compared gene expression patterns in INA-treated versus pathogen-attacked plants and measured JA levels in INA-treated plants to gain information about molecular events associated with acquired resistance in rice.

MATERIALS AND METHODS

Plants and Pathogens

Japonica rice (*Oryza sativa* cv Nohrin 29) plants were grown as described previously (Schweizer et al., 1997). Nine- to 11-d-old plants were treated with INA or JA. At this age, leaf 3, which is the first true leaf more than 2 cm in length, had emerged 50 to 100% from the leaf sheath. At the time of treatment, leaf 4 was still completely covered by the sheath of leaf 3. INA (CGA 41396, Novartis Co., Basel, Switzerland; formulated as 25% [w/w] wettable powder) was applied as a soil drench by watering plants with an INA suspension in water during the entire induction period until harvest of leaf material or challenge inoculation.

The INA concentration ranged from 0.1 to 100 ppm (mg L^{-1}) active ingredient, which corresponds theoretically to 0.53 to 530 μM. However, the INA concentration will be given in ppm throughout this paper, because the actual concentration in the soil solution was not known due to poor solubility in water and due to the possible sorption to soil particles. For each type of experiment the effect of the wettable powder without active ingredient was tested at least once and was found to be negligible. JA was applied by spraying plants with solutions in ethanol (2.5 mL per pot of approximately 30 plants) at concentrations ranging from 10 to 1000 ppm (0.01–1 mg mL^{-1}). As a control, other plants were sprayed with ethanol only.

The rice blast fungus, *Magnaporthe grisea* (Hebert) Barr (race 283; Novartis), was used for plant inoculations, as described previously (Schweizer et al., 1997). Disease was quantified by counting the number of susceptible-type 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.

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 were 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 INA-treated plants.

Stress Treatments

Leaves were wounded by squeezing with a blunt-ended, ribbed forceps every 3 to 5 mm. For heat-shock treatment, plants were incubated at 100% RH and 40°C in the dark for 18 h. $CdCl_2$ (1 mM in water) was applied as soil drench (one drench with 100 mL of solution at the beginning of the experiment).

RNA Blotting and Translation in Vitro

Total RNA and poly(A^+)-RNA were isolated from entire rice shoots (leaves 3 and 4), as described previously (Schweizer et al., 1995). Poly(A^+)-RNA was 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) or poly(A^+)-enriched RNA (1 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 the pathogen-inducible rice mRNAs PIR2 (PR5-type protein; Reimmann and Dudler, 1993) and PIR3 (PR9-type protein; Reimmann et al., 1992).

LOX Enzyme Activity

Fresh leaf material (0.1 g fresh weight) was homogenized with a glass homogenizer on ice in 1 mL of 10 mM Na_3PO_4 buffer, pH 6.5, containing 1% (v/v) Tween 20. The extracts were incubated on ice for 60 min and centrifuged (13,000 rpm at 4°C). LOX activity was measured at room temperature in 1 mL (final volume) of 100 mM sodium phosphate, pH 6.5, by adding 100 μL of 10 mM linoleic acid and 10 μL of extract supernatant. The increase of A_{234} was recorded for 2 min. The slope of the linear part of the plot was taken for the calculation of LOX enzyme activity. Enzyme activity was expressed as ΔExt_{234} per minute per gram fresh weight. Controls without the addition of plant extracts were recorded as described above and subtracted from the values obtained with plant extracts. The substrate was prepared by homogenizing 70 mg of Tween 20 (Fluka), 70 mg of linoleic acid (Sigma), and 4 mL of degassed water. The milky solution was cleared with 0.55 mL of 0.5 M NaOH and made up to 25 mL with degassed water. Aliquots were stored at −20°C.

PR Proteins and Levels of JA

PR proteins and JA were extracted and assayed as described previously (Schweizer et al., 1997).

RESULTS

Plant Protection by INA

The race of *M. grisea* used here was very virulent on rice cv Nohrin 29, causing large lesions 4 to 5 d postinoculation and collapse of heavily infected leaves 6 to 7 d postinoculation. Soil-drench application of INA protected the leaves from infection by *M. grisea*, producing a resistance response similar to genetically determined, intermediate resistance (Ou, 1965) (Fig. 1). This type of resistance is characterized by the appearance of many small (<1 mm), dark-brown lesions 2 to 3 d postinoculation. On nontreated control leaves, large susceptible-type lesions appeared that were 3 to 6 mm in length, with a gray center and often surrounded by chlorotic or necrotic tissue (Fig. 1). The susceptible-type lesions appeared no earlier than 4 d postinoculation.

In accordance with the macroscopic phenotype, INA did not induce phenocopy hypersensitive response in cv Nohrin 29, as reflected by the absence of trypan-blue staining or enhanced epifluorescence of attacked epidermal cells 24 to 72 h postinoculation (data not shown). Maximum protection (90% for leaf 3 and 79% for leaf 4) was obtained at 1 ppm INA and decreased with increasing concentrations of INA (Table I). INA had no detectable antifungal effect on monoconidial colony formation in vitro, in contrast to the fungicide standards Benomyl (Riedel-de Haën, Seelze, Germany) and Prochloraz (Riedel-de Haën) (data not shown).

Defense Gene Expression

INA induced the accumulation of PR5 (thaumatin-like) and PR9 (peroxidase) mRNA (Fig. 2). A concentration of 1 ppm active ingredient was sufficient for full induction. At 100 ppm active ingredient, PR5 and PR9 induction was attenuated, indicating that the elevated concentration was supraoptimal for induction of these mRNAs. INA also induced the accumulation of PR1 and PR3 proteins (Fig. 2B). The multiple PR1 bands detected by the antiserum suggests the existence of a gene family, as has been demonstrated for other plants (Niderman et al., 1995). In contrast to PR5 and PR9 mRNAs, PR1 and PR3 proteins accumulated only in plants treated with 100 ppm INA. All PR proteins and mRNAs analyzed also accumulated during the compatible interaction with *M. grisea* or the incompatible interaction with *P. syringae* (Figs. 2 and 3).

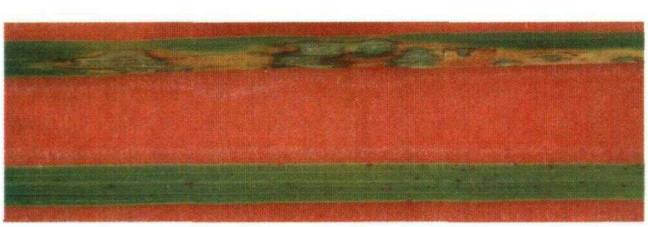

Figure 1. INA induces a phenocopy of intermediate resistance in rice. Top, Rice leaf from control plant; bottom, leaf from plant treated with 1 ppm (5.3 μM) INA by soil-drench application 3 d prior to challenge inoculation with *M. grisea*. The photograph was taken 5 d postinoculation.

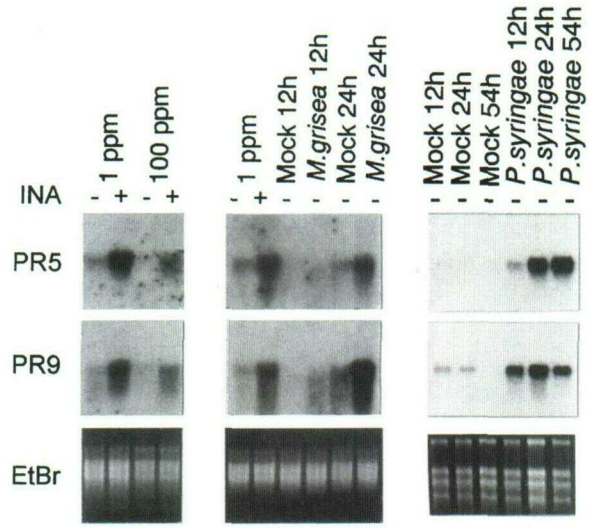

Figure 2. Accumulation of PR5 and PR9 mRNA in INA-treated and pathogen-attacked leaves. Ten micrograms of total rice RNA or 1 μg of poly(A^+)-enriched RNA (*P. syringae*) was transferred to nylon membranes and hybridized with ^{32}P-labeled cDNA of rice PR5 and rice PR9, as specified in "Materials and Methods." RNA was extracted 48 h after the onset of INA treatment. EtBr, Loading control stained with ethidium bromide. For total RNA (left gel), one-tenth of each RNA sample used for blotting was run on a separate, nondenaturing gel containing ethidium bromide and photographed. For poly(A^+)-enriched RNA (right gel), ethidium bromide was added to the sample buffer, and a photograph of the gel was taken prior to blotting. Ribosomal bands are still visible in poly(A^+)-enriched samples, since the RNA was passed only once over oligo(dT)-cellulose. For INA concentrations, see "Materials and Methods."

General Gene-Expression Patterns

General patterns of gene expression in leaves of INA-treated plants and of plants inoculated with *M. grisea* or *P. syringae* were analyzed by one-dimensional SDS-PAGE of ASPs stained with Coomassie blue and by two-dimensional display of ITPs. The one-dimensional display of acid-soluble proteins showed an abundant, INA-induced protein of 31 kD apparent molecular mass, ASP31 (Fig. 3A). This protein did not accumulate in pathogen-attacked, wounded, or otherwise stressed leaves (Fig. 3A), whereas PR1 and PR3 proteins did (Fig. 3B).

The two-dimensional-display method of ITPs gave reproducible results, as shown in Figure 4, where the patterns of INA-induced ITPs are compared between two independent experiments. Forty-eight hours after a treatment with 100 ppm INA, 11 ITPs were enhanced in abundance (Fig. 4); 48 or 86 h after treatment with 1 ppm INA, a subset of these 11 ITPs was enhanced in abundance (Figs. 5 and 6). The control plants used for the 86-h time point strongly expressed mRNAs corresponding to ITP26, ITP66,

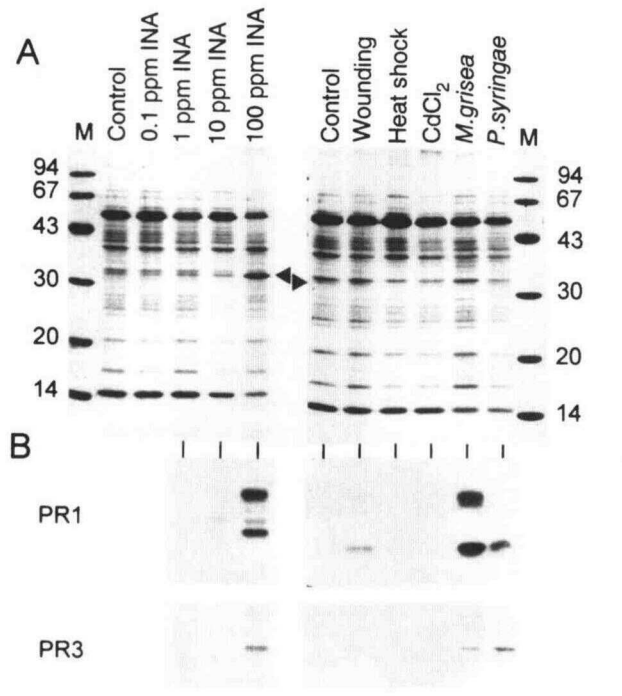

Figure 3. Accumulation of acid-soluble proteins in INA-treated and stressed leaves. A, Acid-soluble proteins were extracted 3 d after the treatments or inoculations or at the end of an 18-h heat-shock period at 40°C, separated on SDS gels, and stained with Coomassie blue (7.5 μg protein lane^{-1}). The stress treatments were applied as described in "Materials and Methods." The arrowheads indicate ASP31. B, The same acid-soluble proteins as in A were transferred to nitrocellulose and detected using antisera against the major basic PR1 protein of tomato (Fischer et al., 1989; 1000-fold dilution) and against a barley PR3 protein (Swegle et al., 1992; 500-fold dilution), respectively. For INA concentrations, see "Materials and Methods."

ITP91, and ITP134; the expressions of ITP26 and ITP66 were further stimulated by INA. These ITPs were also JA-inducible (Fig. 6), and it may be speculated that, for unknown reasons, endogenous JA levels were high in these plants. We observed considerable variations of basal JA levels in nontreated rice leaves between independent experiments (P. Schweizer, unpublished data). The compatible and incompatible pathogens *M. grisea* and *P. syringae*, respectively, induced a set of ITPs that differed markedly from the set induced by INA, suggesting the existence of INA-specific mRNAs (Figs. 5 and 6).

LOX Enzyme Activity and JA Levels

Induction of LOX enzyme activity in rice leaves treated with 100 ppm INA by soil-drench application was reported (Schaffrath, 1994). Thus, one possible INA-induced LOX pathway is the octadecanoid-signaling pathway, which has (-)-JA as its central component. We therefore measured LOX activity and levels of nonconjugated (-)-JA in leaves of INA-treated plants. At 100 ppm, INA induced LOX activity and enhanced (-)-JA levels (Fig. 7). In contrast, no significant induction of LOX activity or JA level was observed in plants treated with 1 ppm INA. These results suggest that, although INA can activate the octadecanoid-signaling pathway, an activation of the pathway is not required for resistance induction, as good protection was obtained with 1 ppm INA (see above).

Synergistic Effects of INA and JA

JA appears to play a role as an enhancer of defense reactions in pathogen-attacked rice (Schweizer et al., 1997). The possibility that JA also enhances the effect of INA was tested by applying both INA and JA to rice plants, followed by analysis of the accumulation of acid-soluble proteins (Fig. 8) and induction of resistance (Table I). Simultaneous application of INA and JA synergistically induced the accumulation of ASP31, which became the most abundant ASP at the highest dose of INA and JA (Fig. 8, top panel, compare samples 3, 4, 7, 15, and 16). Both INA and JA caused accumulation of PR1 proteins (Fig. 8, bottom panel, samples 4 and 7). Simultaneous application of INA and JA had a weak synergistic effect on PR1 accumulation (compare samples 3, 6, and 12). However, in some of the independent repetitions of this experiment, the effect was only additive (data not shown). INA repressed the accumulation of ASP26, another JA-induced, ASP of 26 kD (Fig. 8, top panel, samples 7 and 14–16). The accumulation of ASP13, a third JA-induced protein of 13 kD, was not influenced by INA (Fig. 8, top panel, samples 14–16).

Topical spray treatment of plants at the 3-leaf stage with JA enhanced protection of leaves 3 and 4 obtained by low doses of INA, although JA alone did not protect leaf 3 (Table I). When taking the results for leaves 3 and 4 together, JA enhanced protection caused by 0.1 ppm INA treatment in five out of five experiments, by 1 ppm INA in 5 out of 6 experiments, and by 10 ppm in four out of six experiments. At 100 ppm INA, no enhancement of protection by JA was observed. One experiment was designed to allow statistical analysis (series B, leaf 3, experiment II; 5 parallel pots per treatment): In this experiment 0.1 ppm INA did not provide significant protection, whereas the combination of 0.1 ppm INA with JA significantly protected the leaves ($P < 0.0005$).

DISCUSSION

Biological, chemical, and physical induction of resistance to pathogens have been described in cereals (Ouchi et al., 1974; Schweizer et al., 1989, 1995, 1996; Cantone and Dunkle, 1990; Kogel et al., 1994; Görlach et al., 1996). However, the molecular events associated with the establishment of acquired resistance in cereals are less well understood, and information about signals involved in acquired resistance or existence of SAR in cereals is scarce (Smith and Métraux, 1991; Schweizer et al., 1993; Kogel et al., 1995; Silverman et al., 1995). In the present work we have used INA as chemical inducer of acquired resistance to study gene-expression patterns and signaling events associated with acquired resistance in the economically important cereal crop, rice.

INA is a powerful inducer of acquired resistance in rice and protected the plants at a dose (1 ppm) that was much

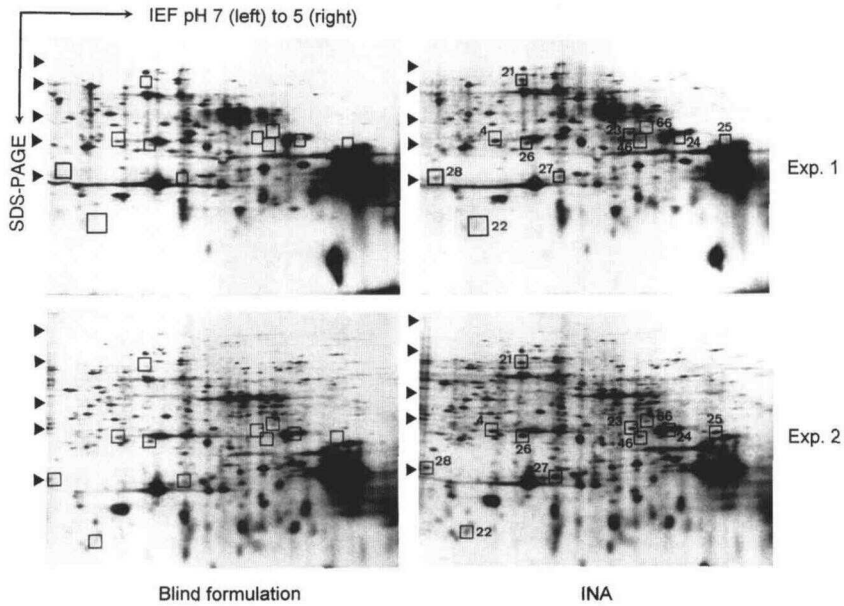

Figure 4. Reproducible in vitro-translation patterns induced by INA. mRNA was isolated 48 h after soil-drench application of 100 ppm (530 μM) INA and translated in vitro into ^{35}S-labeled ITPs by rabbit reticulocyte lysate, as specified in "Materials and Methods." mRNA from two independent experiments was used in the repeated in vitro translations. ITPs with enhanced abundance in INA-treated plants are framed and provided with identifier numbers, based on their migration characteristics relative to neighboring landmark spots. Arrowheads at the left border of the gels indicate positions of molecular mass standards (87, 48, 33, 29, and 21 kD, from top to bottom).

lower than the threshold concentration of phytotoxicity of the compound (around 100 ppm). INA exerted no detectable fungicidal activity against *M. grisea* in vitro. Moreover, plant protection was weaker at 100 ppm INA, compared with 1 ppm, which is a strong indication for an indirect mode of action, i.e. acquired resistance. We compared plant protection by INA and by the novel resistance-inducing compound benzo(1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (benzothiadiazole; Görlach et al., 1996) and found that, under laboratory conditions, INA was at least as effective as benzothiadiazole (data not shown).

Molecular events associated with INA-induced resistance in tobacco and Arabidopsis comprise the coordinate induction of a set of PR genes that were termed SAR genes due to the fact that their mRNAs also accumulated systemically during biologically induced SAR (Keller et al., 1996; Ward et al., 1991; Uknes et al., 1992). These results indicated that INA feeds into the SAR pathway that is activated in pathogen-attacked plants. In rice INA also induced the accumulation of PR gene products belonging to the same families as SAR genes, thus providing further support for the above-mentioned model. However, since systemic SAR gene expression has not been found in rice (Smith and Métraux, 1991) and INA is highly mobile in plants (Métraux et al., 1991), it remains unclear if the effect of INA on rice corresponds to localized responses or to SAR.

The study of gene expression in INA-treated rice was extended to major, Coomassie blue-stained proteins and to mRNAs that were translated in vitro into ITPs with slightly acidic pIs. INA induced the massive accumulation of an ASP of 31 kD (ASP31) that was not inducible by other stress treatments, including inoculation with compatible or incompatible pathogens. ASP31 was not induced by the putative stress signals salicylic acid, JA, or ethylene, or by the phytohormones auxin, kinetin, ABA, or GA_3 (data not shown).

Two-dimensional analysis of ASPs showed that ASP31 is not identical to the weak, constitutive protein band of the same molecular mass (data not shown). Therefore, ASP31 appears to be a specifically INA-induced protein. Moreover, a number of ITPs accumulated only in INA-treated plants and not in plants inoculated with either *M. grisea*, a highly compatible pathogen, or with *P. syringae*, an incompatible pathogen and a biological inducer of resistance in rice (Smith and Métraux, 1991; Reimmann et al., 1995).

The gene-expression patterns from inoculated leaves, as shown in Figure 6, represents compiled data from in vitro translations with mRNA isolated 12, 24, and 72 h postinoculation with *M. grisea*, or 12, 24, and 54 h postinoculation with *P. syringae*. No additional, INA-induced ITPs were detected using mRNA isolated 24 h, instead of 48 h, after INA treatment (data not shown). It therefore seems unlikely that the differences in the gene-expression patterns between INA-treated and inoculated leaves were due to the selection of inappropriate time points for the two-dimensional display.

We cannot exclude the possibility that some pathogen-induced ITPs were in fact derived from *M. grisea* mRNA. However, this is unlikely for three reasons: (a) most pathogen-induced ITPs were also detectable at low levels with mRNA from mock-inoculated leaves, and several also accumulated in *P. syringae*-inoculated leaves; (b) most ITPs were already detectable with mRNA isolated 14 h postinoculation, a time when conidia were just about to penetrate

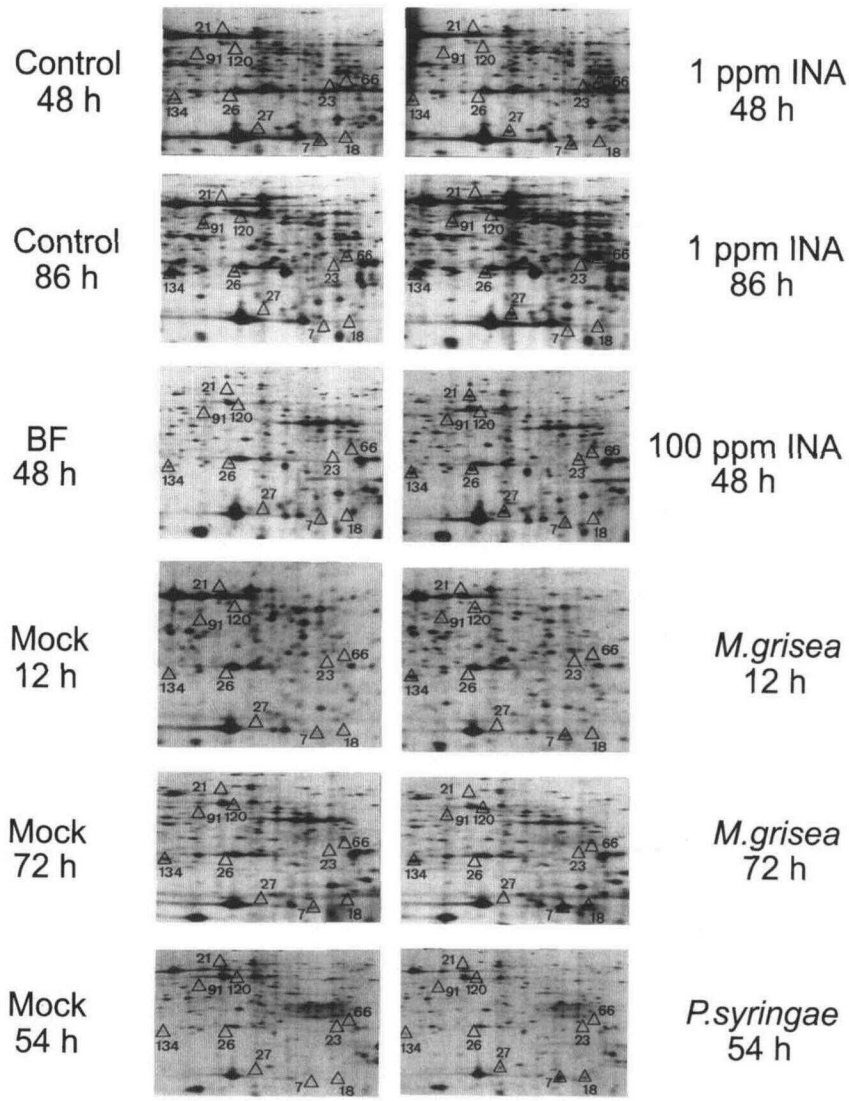

Figure 5. Different sets of ITPs are induced by INA treatment and pathogen attack. Only the central part of the two-dimensional gels (see Fig. 4) containing some easily detectable ITPs is shown. ITPs with enhanced abundance in either INA-treated or inoculated plants are framed and provided with identifier numbers, based on their migration characteristics relative to neighboring landmark spots. For simplicity, only ITPs induced by both pathogens are marked here. For INA concentrations, see "Materials and Methods."

the leaf epidermis and when the amount of fungal biomass was negligible (Talbot et al., 1993); and (c) the in vitro translation system displayed only ITPs from abundant mRNAs, which means that fungal mRNAs detectable 14 h postinoculation would have to be extremely abundant.

Since bacterial RNAs are not polyadenylated and have a poor translation-initiation consensus for eukaryotic systems, contamination of the two-dimensional display with ITPs from *P. syringae* RNA is unlikely. A particularly interesting gene product is ITP27. This ITP was found in reactions with mRNA from plants treated with 1 ppm as well as with 100 ppm INA. Moreover, ITP27 was only found in reactions with mRNA from *P. syringae*-inoculated leaves, not with that from *M. grisea*-inoculated leaves. Therefore, ITP27 was correlated with chemically as well as biologically induced resistance (Reimmann et al., 1995), but not with disease caused by *M. grisea*.

In summary, INA induced the accumulation of a number of gene products. The gene products accumulating in plants treated with 1 ppm INA (e.g. PR5 and PR9 mRNA or ITP27) may serve as markers for acquired resistance, whereas those gene products accumulating only at 100 ppm INA (e.g. PR1, PR3, ASP31, or ITP21) may serve as markers for INA toxicity or INA detoxification. The regulation of the latter class of INA-induced genes by xenobiotics and their potential role in xenobiotic detoxification remains to be examined. However, the accumulation of INA-specific gene products in rice may also indicate that INA, in addition to activating a pathogen-inducible signaling pathway, activates another pathway for which the nat-

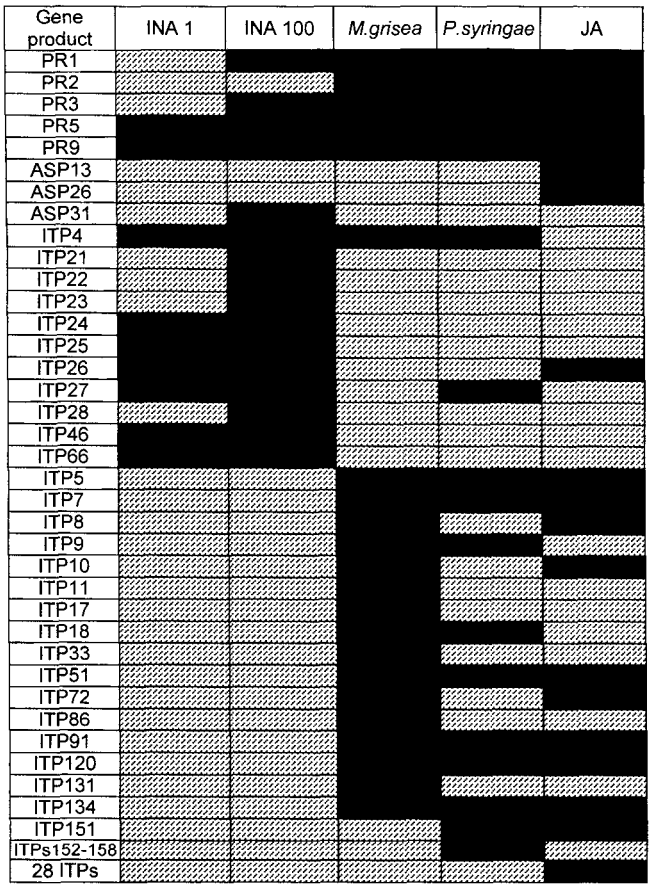

Figure 6. Gene-expression patterns in INA-treated versus pathogen-attacked leaves. Summary of Figures 2 to 5, plus Figure 8. The data of PR2 expression in *P. syringae*-attacked leaves and of gene expression in JA-treated leaves are derived from Smith and Métraux (1991) and from Schweizer et al. (1997), respectively. Shaded field, No induction; black field, induction. INA1, 1 ppm INA; INA 100, 100 ppm INA (for INA concentrations, see "Materials and Methods").

ural signal is as yet unknown (Fig. 9). The challenge, therefore, is to identify the natural signal(s) that activate the INA-specific genes in rice, and to elucidate the biological relevance of these genes.

The existence of a novel, INA-induced signaling pathway is supported by the INA-specific gene products ITP24, ITP25, ITP46, and ITP66, all of which were induced at a dose (1 ppm) of INA beneath the threshold concentration of phytotoxicity (around 100 ppm). These results demonstrate that the two-dimensional display of ITPs, which provides a largely unbiased glance at gene expression patterns, is a useful technique for revealing hidden modes of gene regulation. Based on the induction of PR gene products we concluded that INA activates only a PR pathway. However, while this technique allows conclusions to be drawn about mRNA levels, it does not reveal effects on translational or posttranslational levels of gene regulation. The results presented here are corroborated by a recent study on gene expression patterns in wheat, in which two different sets of genes were induced upon chemical and biological induction of resistance by BTH and the powdery mildew fungus *Erysiphe graminis*, respectively (Schaffrath et al., 1997).

LOX enzyme activity was associated with pathogen resistance (Ohta et al., 1991) and was found to be induced by INA in rice (Schaffrath, 1994). Enhanced LOX activity might result in the activation of the octadecanoid signaling pathway with (-)-JA or its precursor, 12-*oxo*-phytodienoic acid, as the central component (for review, see Sembdner and Parthier, 1993). The role of this stress-induced pathway in mediating responses to pathogen attack or acquired resistance is not clear (Doares et al., 1995; Kogel et al., 1995; Schweizer et al., 1993). We found that although the octadecanoid pathway is inducible by INA in rice, resistance induction by a low dose of INA was not associated with elevated levels of endogenous (−)-JA.

Although exogenously applied JA activated a number of PR genes, it obviously did not orchestrate localized defense reactions, since it did not induce local disease resistance (Table I; Schweizer et al., 1997). Therefore, elevated LOX activity and (-)-JA levels probably reflect plant stress induced by the high dose of INA, rather than providing intermediate signals for the establishment of acquired resistance (Fig. 9). This assumption is supported by the up-

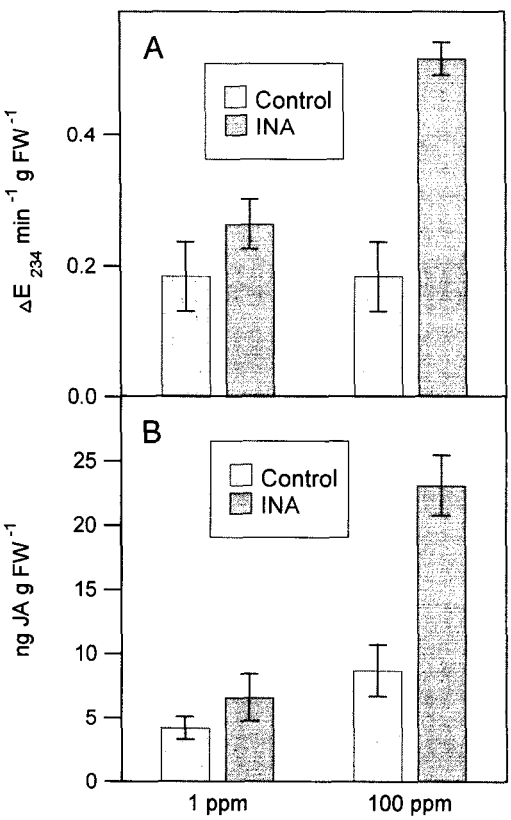

Figure 7. Activation of the octadecanoid signaling pathway by INA. A, LOX enzyme activity in leaves of plants 48 h after soil-drench application of INA. Mean ± SDM (n = 3) from one typical experiment. B, Levels of (-)-JA in leaves 48 h after soil-drench application of INA, as determined by combined GC-MS analysis. Mean ± SDM from three to four independent experiments with approximately 50 plants per experiment and treatment. FW, Fresh weight. For INA concentrations, see "Materials and Methods."

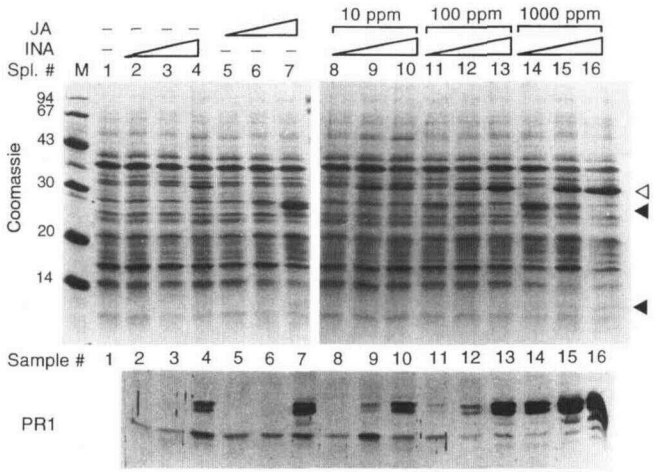

Figure 8. Synergistic induction of acid-soluble proteins by INA and JA. Plants were treated simultaneously with INA and JA, as described in "Materials and Methods," and 3 d later acid-soluble proteins were extracted and separated on SDS gels. Top panel, Acid-soluble proteins (7.5 μg lane^{-1}) were stained with Coomassie blue. INA was applied at concentrations of 1, 10, or 100 ppm, symbolized by triangles above the respective lanes (for INA concentrations, see "Materials and Methods"). JA was applied at concentrations of 10, 100, or 1000 ppm, symbolized by a triangle above samples 5 to 7. The open and closed arrowheads indicate the INA-induced protein ASP31 and the JA-induced proteins ASP26 and ASP13, respectively. Bottom panel, The same acid-soluble proteins shown in the top panel were transferred to nitrocellulose (2.5 μg lane^{-1}), and PR1 proteins were detected by an anti-PR1 antiserum (Fischer et al., 1989). Because of the limited number of lanes, sample 1 was transferred to another membrane. The PR1 signal of sample 1 was very similar to sample 2 (data not shown).

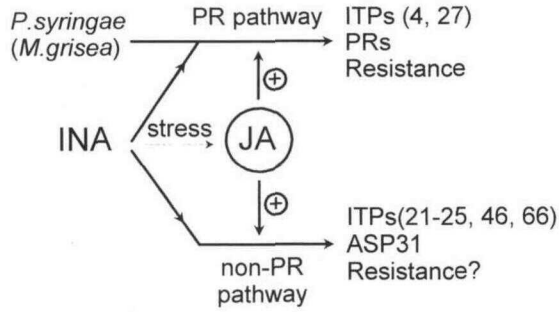

Figure 9. Model of INA- and pathogen-induced signaling in rice. *M. grisea* is indicated in parentheses, since the induction of ITP27 or resistance by this pathogen was not demonstrated. Another hypothetical PR pathway leading to the induction of pathogen-specific ITPs (e.g. ITPs 7 and 18) is not shown.

regulation of the mRNA of ITP27 by 1 ppm INA and by the biological inducer of resistance *P. syringae*, but not by exogenously applied JA (Fig. 6). It remains unknown if PR1 and PR3 induction is mediated by the observed enhanced (-)-JA levels in leaves treated with 100 ppm INA, because both PR proteins also accumulated in JA-treated leaves. The fact that many JA-induced gene products did not accumulate in plants treated with 100 ppm INA may be explained by a negative interaction between the octadecanoid pathway and another, INA-activated pathway (see below).

Exogenously applied JA enhanced INA-induced resistance and accumulation of PR1 and ASP31 in rice leaves. Therefore, INA-induced resistance and protein accumulation in rice may be regulated by a signaling network comprising the octadecanoid pathway (Fig. 9). Leaves sprayed with 100 ppm JA contained approximately 1100 ng JA g^{-1} fresh weight, as determined by combined GC-MS analysis 30 min after the spray. This value is lower than expected from the amount of JA administered (about 120 μg g^{-1} fresh weight) and suggests inefficient deposition or rapid metabolization of the sprayed JA. Indeed, 3 d after the JA spray, the levels of extractable JA were similar to those in control leaves sprayed with ethanol (data not shown). Therefore, although quite high doses of JA were applied, the amount finally reaching a putative JA receptor may have been much lower.

Table I. *Plant protection by INA and enhancement by JA*

Exp., Experiment; RI, relative infection.

Series	INA[a]	JA[b]	Leaf 3							Leaf 4						
			Exp. I		Exp. II		Exp. III		Exp. I–III Mean RI[e]	Exp. IV		Exp. V		Exp. VI		Exp. IV–VI Mean RI
			Lesions[c]	RI[d]	Lesions	RI	Lesions	RI		Lesions	RI	Lesions	RI	Lesions	RI	
				%		%		%	%		%		%		%	%
A	0	−	15.9	100	9.4	100	10.4	100	100	24.5	100	37.3	100	29.0	100	100
A	0	+	17.5	110	2.2	23	13.6	131	88 ± 33	–	–	–	–	–	–	–
A	1	−	2.1	13	1.0	11	0.54	5	10 ± 2	3.4	14	15.3	41	2.6	9	21 ± 10
A	1	+	1.2	8	0.6	6	0.47	5	6 ± 1	0.5	2	8.6	23	1.0	3	9 ± 7
A	10	−	1.8	11	1.5	16	1.2	12	13 ± 2	4.7	19	22.8	61	7.1	24	35 ± 13
A	10	+	1.5	9	0.7	8	1.1	11	9 ± 1	4.6	19	9.9	27	2.8	10	19 ± 5
A	100	−	8.7	55	3.2	34	3.6	35	41 ± 7	2.5	10	11.5	31	14.1	49	30 ± 11
A	100	+	8.5	53	3.5	37	4.5	43	44 ± 5	3.7	15	8.5	23	12.1	42	27 ± 8
B	0	−	4.7	100	1.4	100	–	–	100	8.4	100	12.3	100	59.2	100	100
B	0.1	−	3.9	83	1.2	86	–	–	85 ± 2	7.2	86	6.9	56	37.0	62	68 ± 9
B	0.1	+	2.7	58	0.3	21	–	–	40 ± 19	5.8	68	5.9	48	19.4	33	50 ± 10

[a] Drench application with 0.1 to 100 ppm (0.53–530 μM) active ingredient (see also "Materials and Methods"). [b] Spray application to leaf 3 with 100 ppm. [c] Number of acute, sporulating lesions per leaf. Mean of two to five parallel treatments (pots of approximately 30 plants) per experiment. [d] Based on the controls without INA or JA. [e] Mean ± SDM (except for series B, leaf 3: mean ± range).

A number of recent reports support the view of JA as an enhancer of defense responses (Graham and Graham, 1996; Kauss et al., 1994; Nojiri et al., 1996; Schweizer et al., 1997; Xu et al., 1994). The fact that ASP31 accumulation is enhanced by JA, although JA by itself could not induce accumulation of this protein, is a clear indication of the existence of a signaling network between the octadecanoid pathway and another, INA-induced pathway (Figs. 8 and 9). Based on the results presented here, it is possible to speculate about a positive feedback loop of gene regulation by INA and JA. Elevated JA levels in INA-treated rice further stimulate the accumulation of INA-induced gene products (Fig. 9). Such a positive feedback loop does not seem to play a role in the resistance induction that was maximal at INA doses that did not enhance JA levels. In this context, it would be interesting to test the responsiveness of stressed rice plants with enhanced endogenous JA levels to INA or other resistance inducers.

Another indication for the operation of a signaling network in INA-treated rice is the repression by INA of the accumulation of ASP26, a jasmonate-induced protein. Similar results were obtained in leaves treated with an elicitor from *M. grisea*. JA enhanced PR1 induction by the elicitor, which repressed ASP26 induction by JA (Schweizer et al., 1997). More precise information about the role of JA in INA-treated or infected rice will depend on the availability of powerful inhibitors of the octadecanoid pathway or of plants severely impaired in JA biosynthesis or with reduced sensitivity to JA.

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