Different patterns of host genes are induced in rice by *Pseudomonas syringae*, a biological inducer of resistance, and the chemical inducer benzothiadiazole (BTH)

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Abstract

Rice seedlings treated with the synthetic compound benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) acquired resistance to subsequent attack by the rice blast fungus *Magnaporthe grisea* (Hebert) Barr. BTH (trade name Bion™) has been released to the market as a plant protecting agent for rice. Here, we analysed the pattern of expressed genes in rice plants treated with BTH, and compared this pattern with those induced by the formerly discovered resistance inducer 2,6-dichloroisonicotinic acid (INA) and by *Pseudomonas syringae* pv. *syringae*, a non-host pathogen inducing a hypersensitive response. Both INA and BTH induced similar patterns of genes, suggesting that these compounds are functional analogues. In contrast, the patterns induced by the chemical inducers and by *P. syringae* were clearly dissimilar.

Abbreviations: BTH – benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester; 2D – two dimensional; INA – 2,6-dichloroisonicotinic acid; ITP – *in vitro* translation product; SA – salicylic acid; SAR – systemic acquired resistance; PR – pathogenesis-related.

Introduction

Acquired resistance to pathogens has been described both in dicotyledonous and monocotyledonous plant species that were attacked by compatible or incompatible pathogens (Kuc, 1982; Ouchi et al., 1974; Ryals et al., 1996; Schweizer et al., 1989; Sticher et al., 1997). In rice, resistance induced by *Pseudomonas syringae* pv. *syringae* has been reported (Smith and Métraux, 1991; Reimmann et al., 1995). The recent discovery and broad use of synthetic inducers of resistance, especially 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) represented a milestone in the research of (systemic) acquired resistance (SAR) (Görlach et al.,1996; Métraux et al., 1991). BTH was reported to induce resistance in *Arabidopsis* (Lawton et al., 1996), bean (Siegrist et al., 1997), *Brassica* (Jensen et al., 1998), cucumber (Benhamou and Bèlanger, 1998), tobacco (Friedrich et al., 1996) and wheat (Goŕlach et al., 1996). While INA was not further developed due to phytotoxic side effects, BTH has been released to the market.

In several dicotyledonous systems, INA and BTH induced a set of 'SAR genes' including members of the pathgenesis-related (PR) families 1–5 (Friedrich et al., 1996; Lawton et al., 1996; Uknes et al., 1992; Ward et al., 1991). The same genes were also induced by pathogen attack and by biological inducers of SAR. In wheat, a number of cDNAs corresponding to BTH-induced genes had been isolated and these appear to belong to different classes of genes than the 'SAR genes' of tobacco and *Arabidopsis* (Görlach et al., 1996). Similarly, a rice gene induced by the

resistance inducer probenazole also does not belong to the classical 'SAR genes' (Midoh and Iwata, 1996). The BTH-induced wheat genes were not inducible by the biological inducer of resistance *Erysiphe graminis* f.sp. *hordei*, whereas a set of pathogen-induced wheat genes was not inducible by BTH (Schaffrath et al., 1997). This contrasts to the situation in *Arabidopsis*, tobacco and cucumber where the 'SAR genes' were both inducible by pathogens that induce SAR as well as by INA and BTH. The non-overlapping sets of induced wheat genes in plants treated with either the biological or the chemical inducer of resistance suggests the co-existence of several resistance mechanisms in cereals.

Recent analysis of gene-expression patterns in INA-treated rice appeared to indicate a similar situation to that in wheat (Schweizer et al., 1997a). Since no marker genes of BTH-induced resistance in rice are presently available, we decided to study the gene-expression pattern in BTH-treated rice plants and to compare it to patterns obtained in INA-treated plants as well as in rice attacked by the non-host pathogen *Pseudomonas syringae* pv. *syringae* (Reimmann et al., 1995).

Materials and methods

Plant and pathogens

Rice seedlings, cv Nohrin 29 at the four-leaf stage, were challenge-inoculated with *Magnaporthe grisea* (Hebert) Barr. (strain 238 from Novartis Crop Protection Co.) as described (Schweizer et al., 1997b). The inoculum density was 2×10^5 conidia ml^{-1}. Five days after inoculation, disease was estimated by counting the number of spindle-shaped lesions of at least 2 mm length per leaf (see Schweizer et al. (1997a) for an example of disease symptoms). Leaf 3 and leaf 4 were infiltrated with *Pseudomonas syringae* pv. *syringae* (1×10^9 cfu ml^{-1}; strain 548, pathogenic on pear) as specified in the figure legends and as described by Wäspi et al., 1998.

Inducer treatment

At the emergence of leaf 4, plants were treated with INA or BTH by soil-drench application (Schweizer et al., 1997a). INA and BTH were applied as a 1 : 3 (w/w) and 1 : 1 (w/w) mixture of active ingredient with wettable powder, respectively. Control plants were treated with wettable powder alone. INA and BTH concentrations are indicated as ppm (mg active ingredient l^{-1} drench solution). Four days after the onset of the BTH treatment, plants were challenge-inoculated with *M. grisea*.

RNA blotting and translation *in vitro*

Total RNA and poly(A)$^+$-RNA was isolated from leaves of young rice seedlings (Schweizer et al., 1995). Poly(A)$^+$-RNA was translated *in vitro* by a rabbit reticulocyte lysate, and the ^{35}S-methionine/cysteine-labelled *in vitro* translation products (ITPs) were displayed by two dimensional (2D) polyacrylamide gel electrophoresis (Schweizer et al., 1995). Total RNA (10 μg per lane) was separated on formaldehyde-containing agarose gels, transferred onto nylon membranes (Hybond™, Amersham, UK) and hybridised with ^{32}P-labelled cDNA probes of pathogen-related rice proteins PIR2 (PR5-type protein, Reimmann and Dudler, 1993), PIR3 (PR9-type protein, Reimmann et al., 1992) and PIR7b (esterase, Wäspi et al., 1998). The accumulation of the INA- and BTH-induced mRNA RCI1 was detected using a ^{32}P-labelled PCR fragment that was differentially amplified from cDNA of INA-treated plants (U. Schaffrath and R. Dudler, to be published elsewhere).

Results

Induced resistance

Soil-drench application of BTH induced resistance in rice seedlings against *M. grisea* (Table 1). Best protection was observed at the lowest dose (1 ppm). The inverse correlation between BTH dose and protection supports the view that the compound acts indirectly by inducing resistance in the host rather than by being directly fungicidal, in agreement with earlier reports (Friedrich et al., 1996; Görlach et al., 1996). A trend to reduced protection with high doses of INA was also observed (Schweizer et al., 1997a).

Gene-expression patterns

The expression of several defence-related genes in BTH-treated or pathogen-attacked rice plants was examined by Northern blot experiments (Figure 1). The mRNAs of *PIR2, PIR3* and *PIR7b* accumulated strongly in plants treated with the biological inducer *P. syringae*. No significant accumulation of these RNAs was observed in BTH-treated plants. By contrast, the

Table 1. Protection of rice against *M. grisea* by soil-drench application of BTH

Treatment	Conc. (ppm)	Lesions per leaf[a]	Rel. infection (%)	p (t-test)
Control[b]	—	11.3 ± 1.2	100	—
BTH	1	2.1 ± 0.3	19	<0.0005
BTH	10	4.3 ± 0.6	38	<0.0005
BTH	100	7.1 ± 0.8	66	<0.005

[a]Mean $\pm$ SE of 63–79 plants per treatment.
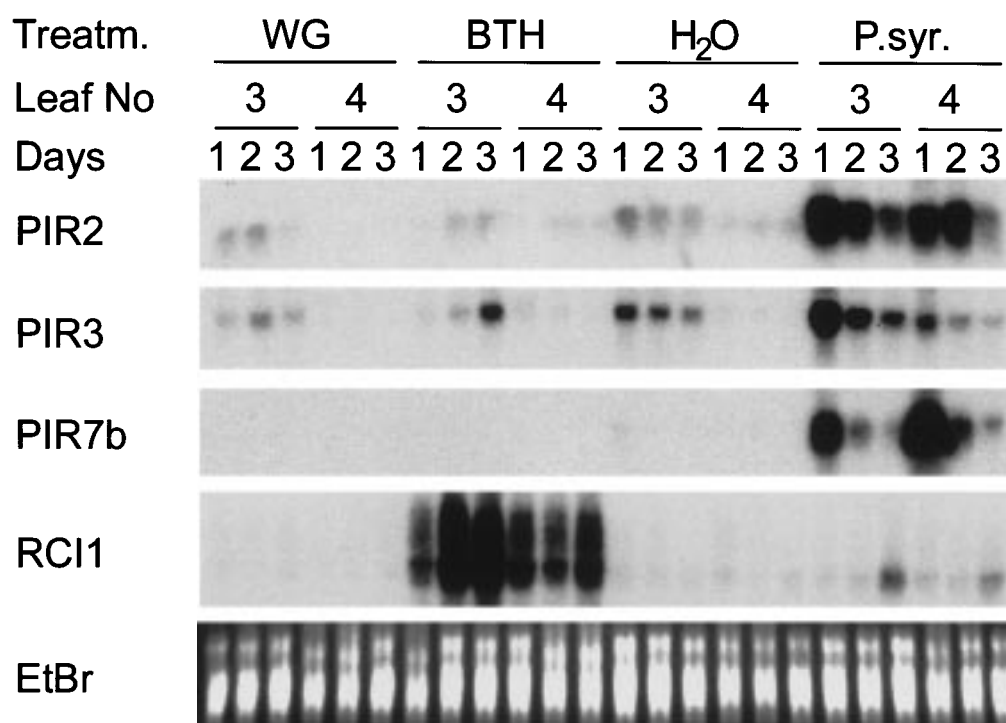[b]Treated with wettable powder corresponding to 100 ppm BTH.

Figure 1. Non-overlapping set of genes are induced by BTH and *P. syringae*. Young rice plants at the four-leaf stage were treated with BTH by soil drench (10 ppm active ingredient) or inoculated with *P. syringae* on leaf 3 and leaf 4. At the indicated times after treatment, RNA was extracted from leaf 3 and leaf 4, separated on formaldehyde-containing agarose gels and blotted onto a nylon membrane. The blots were hybridised with ^{32}P-labelled DNA fragments corresponding to defence-related rice RNAs as indicated on the left side. Control plants for BTH were treated with wettable granulate (WG) without active ingredient. Control plants for *P. syringae* were injected with H_2O. PIR2, thaumatin-like protein (Reimann and Dudler, 1993); PIR3, putative peroxidase (Reimann et a., 1992); PIR7b, α/β hydrolase fold esterase (Wäspi et al., 1998); *RCI1*, INA-induced gene (see 'Materials and methods'). EtBr, the gel stained with ethidium bromide served as loading control.

RCI1 RNA accumulated only in BTH-treated plants to any significant amount. The *RCI1* gene was found in a PCR-based, differential approach with INA-treated plants. No significant difference in the response of leaf 3 and leaf 4 to BTH or *P. syringae* was observed. In summary, the analysed sets of expressed genes were different in BTH- and *P. syringae*-treated rice seedlings. We also compared gene expression in plants treated with BTH and the related compound INA (Figure 2). The expression of the *PIR2, PIR3, PIR7b* and *RCI1* genes was similar between BTH and INA, except for a weak trend for enhanced levels of PIR2 and PIR3 RNA in INA-treated plants, as reported earlier (Schweizer et al., 1997a).

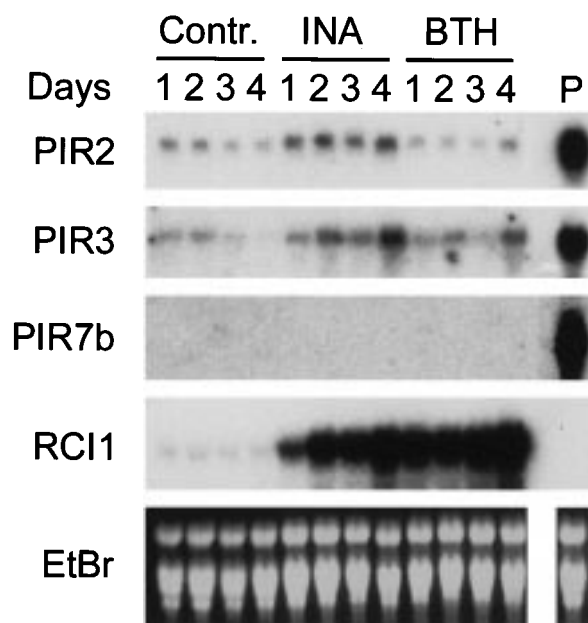The study of gene expression was extended to 2D display of ITPs. This method is sensitive and gives

Figure 2. Similar patterns of expressed genes in rice treated with INA and BTH. RNA from leaf 4 of INA- or BTH-treated plants was isolated 1–4 days after onset of the treatment (soil-drench application with 10 ppm active ingredient). Control plants were treated with the corresponding dummy formulation (wettable powder). Lane 'P', rice RNA isolated 24 h post inoculation with *P. syringae* strain 548 (Wäspi et al., 1998) served as positive control for the defence-related transcripts. For details see Figure 1.

reproducible results (Schweizer et al., 1997a). In general, only ITPs that accumulated in at least two independent experiments per treatment were considered. The ITP display patterns of INA- or BTH-treated plants were derived from leaf 4, whereas the ITP display patterns of *P. syringae* attacked plants were derived from leaf 3. However, the results shown in Figure 1 suggest that the patterns of induced genes are similar in both leaves. No attention was paid here to ITPs with reduced abundance in samples from treated plants. Figure 3 shows an example of the 2D display of ITPs obtained with mRNA from control, INA-, BTH-, or *P. syringae*-treated plants. The RNA was extracted 48 h after onset of the chemical treatments or 54 h post inoculation with *P. syringae*. A number of ITPs accumulated in response to the inducers (inside squares). Several of these ITPs were also detectable with mRNA from control plants suggesting that the corresponding genes had a basal level of expression. The patterns of ITPs with increased appearance in RNA from INA- and BTH-treated plants were identical, except for ITP4 and ITP5 which did

not accumulate with RNA from INA-treated and BTH-treated plants, respectively. The pattern of accumulating ITPs obtained with RNA from *P. syringae*-attacked plants (inside circles) showed no overlap with the INA- or BTH-induced patterns, except for ITP18, which accumulated in all tested inducing treatments by an estimated factor of 2–3 above a considerable basal level of expression. The overall ITP patterns, corresponding to non-induced RNAs, of plants treated with the chemical inducers versus plants inoculated with *P. syringae* differed to some extent (compare the left-hand side to the right-hand side of Figure 2). This reflects the fact that the corresponding RNAs were obtained in two different experiments and the leaf material of chemically treated versus *P. syringae*-inoculated plants was harvested at different times of the day. The abundance of several ITPs was found to oscillate in a circadian manner (P. Schweizer, unpublished). However, the patterns of accumulating ITPs from INA-, BTH- as well as *P. syringae*-treated plants, which were obtained by comparison with the corresponding, stringent controls harvested at exactly the same time as the treated leaves, were reproducibly found (see also Schweizer et al., 1997a).

Discussion

In tobacco and *Arabidopsis*, two model plants for studying SAR, INA and BTH induced the same set of genes as did biological inducers of SAR like tobacco mosaic virus or *P. syringae* (Friedrich et al., 1996; Lawton et al., 1996; Uknes et al., 1992; Ward et al., 1991). Therefore, the two compounds were assumed to trigger the same SAR pathway as the biological inducers and to be functional analogues of the SAR-signalling compound salicylic acid (SA). Support for this hypothesis came from studies with mutant *Arabidopsis* plants that were impaired both in their response to biological inducers of resistance as well as to SA, INA and BTH (Cao et al., 1994; Delaney et al., 1995). However, it became increasingly clear in recent years that there exist other signalling pathways, besides the SAR pathway, which also lead to induced resistance (Epple et al., 1995; Penninckx et al., 1996; Pieterse et al., 1998). Activation of these alternative pathways by biological inducers was found to be SA independent, but required ethylene and jasmonic acid and was associated with induction of genes that are different from the SAR genes (Epple et al., 1995; Penninckx et al., 1996; Pieterse et al., 1998).
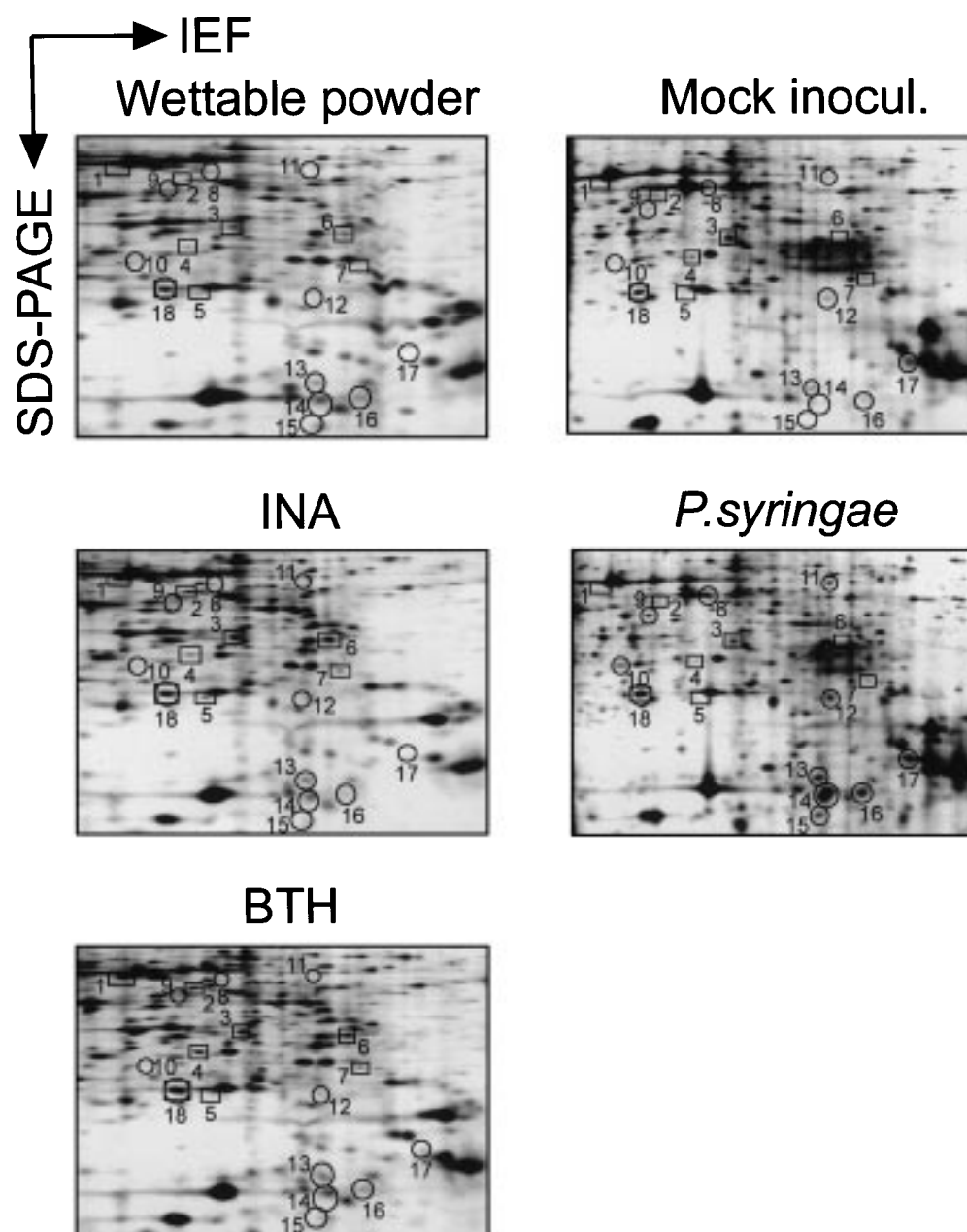
Figure 3. Two-dimensional display of ITPs of inducer-treated plants. For analysis of INA/BTH-induced RNAs, plants were treated with 10 ppm INA or 10 ppm BTH by soil-drench application, and RNA was extracted from leaf 4 48 h after onset of the treatments. For analysis of pathogen-induced RNAs, plants were infiltrated with H_2O ('Mock inocul.') or *P. syringae*, and RNA was extracted 54 h post inoculation. Poly(A)[+]-enriched RNA was translated *in vitro* by a rabbit reticulocyte lysate and the ^{35}S-methionine/cysteine-labelled ITPs were separated by 2D polyacrylamide electrophoresis. First dimension, isoelectric focussing (IEF) pH 5–7; second dimension, 15% SDS-PAGE. The ITPs corresponding to RNAs with enhanced abundance in INA- or BTH-treated plants are inside squares and numbered consecutively. The ITPs corresponding to *P. syringae* induced RNAs are inside circles and numbered consecutively. Only the central part of the 2D gels is shown for better clarity. For high-abundant landmark spots see upper left corner (next to ITP1), bottom, and lower right edge (next to ITP17).

We found that INA and BTH induced a different set of genes in rice than did *P. syringae* (Figure 4). For the following reasons, it is unlikely that the observed difference in gene-expression patterns between the chemical inducers and *P. syringae* was due to selection of inappropriate time points for the 2D display: Firstly, the expression profiles of the *PIR2, PIR3, PIR7b* and *RCI1* genes remained stable during 4 days of INA or BTH treatment suggesting that the effect of these compounds on gene expression in rice was not highly transient. Secondly, all the *P. syringae*-induced transcripts revealed by the 2D display were also induced at other time points (12 and 24 h post inoculation; data not shown). The weak but reproducible induction of some pathogen-induced genes by INA might be related to the phytotoxic side effect of the compound. It is well known that several PR genes are inducible by many biotic and abiotic stresses (see e.g. Frendo et al., 1992). The absence of phytotoxic effects with BTH in rice may thus explain why it did not induce the PR genes. The high similarity of the 2D patterns of accumulating ITPs suggest that INA and BTH activate the same signalling pathway in rice and may be considered as functional analogues, as proposed earlier for wheat and tobacco (Friedrich et al., 1996; Görlach et al., 1996). In gen-

eral, the results presented here conform with our earlier results obtained with INA in rice (Schweizer et al., 1997a) and BTH in wheat (Schaffrath et al., 1997). In both cases, a set of INA- or BTH-specific genes was found that was not inducible by biological inducers of resistance.

The basically non-overlapping sets of induced genes in INA- or BTH-treated versus *P. syringae*-attacked plants suggest that there exist several pathways leading to induced resistance in rice. Since both INA and BTH are synthetic compounds, it has to be assumed that natural inducers for the INA/BTH pathway do exist. It might be speculated that there exist some particular pathogens, non-pathogens or herbivores of rice that can act as the natural inducers of this pathway. However, as long as such natural inducers are not found, it should also be considered that INA and BTH might act by conditioning potentiated defence-gene induction upon subsequent pathogen attack, as has been shown in several plants (Kastner et al., 1998; Katz et al., 1998; Nielsen et al., 1994). However, no conditioning-related genes have been reported for these systems. It appears possible that the *WCI* transcripts of wheat (Görlach et al., 1996), the *RCI1* transcript of rice as well as the INA- and BTH-specific ITPs correspond to such conditioning-related genes that allow the plants to mount a potentiated defence response upon pathogen attack. The sequences of the *WCI* transcripts do not provide any clear clues for direct antifungal activities, in contrast to the SAR genes. More efforts including better characterisation of *WCI* and *RCI* genes are required to distinguish between the two mentioned possibilities.

Acknowledgements

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Figure 4. Summary of the gene-expression data of rice treated with inducers of resistance. Black boxes, induced; grey boxes, not induced; ITP, *in vitro* translation product.

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