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Induction of bacterial blight (*Xanthomonas oryzae* pv. *oryzae*) resistance in rice by treatment with acibenzolar-S-methyl

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Summary

The role of the plant defence activator, acibenzolar-S-methyl (ASM), in inducing resistance in rice against bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was studied. Application of ASM induced resistance in rice to infection by *Xoo*. When the pathogen was clip-inoculated to the rice plants, it caused bacterial leaf blight symptoms in the untreated control. However, in the rice plants pretreated with ASM, infection was significantly reduced. Induced systemic resistance was found to persist for up to 3 days in the pretreated rice plants. Increased phenolic content and accumulation of pathogenesis-related (PR) proteins, *viz*. chitinase, β-1,3-glucanase and thaumatin-like protein (TLP; PR 5) were observed in rice plants pretreated with ASM followed by inoculation with *Xoo*. Immunoblot analysis using rice TLP and tobacco chitinase antiserum revealed rapid induction and over-expression of 25 and 35 kDa TLP and chitinase, respectively, in rice in response to pretreatment with ASM followed by *Xoo* inoculation. Based on these experiments, it is evident that induction of disease resistance in rice was accelerated following treatment with ASM.

Key words: Acibenzolar-S-methyl (ASM), bacterial leaf blight, pathogenesis-related (PR) proteins, rice, *Xanthomonas oryzae* pv. *oryzae*

Introduction

Rice (*Oryza sativa* L.) bacterial leaf blight (BLB) caused by *Xanthomonas oryzae* pv. *oryzae* Ishiyama (*Xoo*) is one of the most important and destructive diseases affecting rice production world wide (Adhikari *et al.,* 1995). Effective chemical control measures against the disease are lacking and breeding for disease resistance is the most important approach to its management. Disease-resistant cultivars with one or two major resistance genes are unsustainable in the field because of high pathogenic variability. Development of rice cultivars with durable resistance is ideal but success in this regard is limited. However, it is well known that plants have evolved an array of defence mechanisms to combat invasion by plant pathogens. Besides pre-existing physical and chemical barriers, a variety of defence mechanisms are activated upon pathogen infection (Kessmann *et al.,* 1994; Chen *et al.,* 1999; Hong *et al.,* 1999). This induced resistance is at first localised around the point of pathogen infection. Subsequently, the resistance spreads systemically and develops in distal, uninfected parts of the plant, thereby conferring an elevated level of protection (Siegrist *et al.,* 1997; Sticher *et al.,* 1997). The use of induced resistance in plants is a promising, environment-friendly strategy for controlling plant diseases, including those caused by bacteria.

Induced resistance, which is expressed systemically and/or locally, is biologically activated in response to necrotising pathogens (Ryals *et al.,* 1996; Anfoka & Buchenauer, 1997) or root-colonising soil bacteria (Hammerschmidt, 1999). The best-characterised systemic resistance is systemic acquired resistance (SAR), which is an inducible defence mechanism that plays a central role in disease resistance (Hammerschmidt & Kuc, 1995). In SAR, a number of defence pathways are stimulated and diverse defence products are synthesised, including lignin, pathogenesis-related proteins, phytoalexins, thionins and defensins (Kessmann *et al.,* 1994; Tosi *et al.,* 1999; Epple *et al.,* 1997; Sticher *et al.,* 1997; Ziadi *et al*., 2001). A unique feature of SAR is that an invading pathogen is not needed for induction. Certain chemicals such as benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester, salicylic acid (SA), 2,6-

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dichloroisonicotinic acid (INA) and DL-β-aminobutyric acid (BABA) can induce SAR without the plant-pathogen interaction (Uknes *et al.,* 1992; Friedrich *et al.,* 1996; Smith-Becker *et al.,* 1998; Chen *et al.,* 1999; Hong *et al.,* 1999).

The benzothiadiazole (BTH) compound, benzo (1, 2, 3) thiadiazole-7-carbothioic acid S-methyl ester, also known as acibenzolar-S-methyl (ASM; CGA-245704;) or Bion®, has been developed as one of a novel class of crop protection agents which do not themselves have anti-microbial properties, but instead increase crop resistance to disease (Kessmann *et al.,* 1994; Ruess *et al.,* 1995; Friedrich *et al.,* 1996; Gorlach *et al.,* 1996; Lawton *et al.,* 1996; Morris *et al.,* 1998; Jensen *et al.,* 1998; Beber *et al.,* 2000; Sauerborn *et al.,* 2002) by activating the SAR signal transduction pathway (Schweizer *et al.,* 1999; Hammerschmidt, 1999). The broad-spectrum activity of BTH compounds has been reported to protect dicotyledonous and monocotyledonous plant species against a number of bacterial, fungal and viral diseases, strongly suggesting an indirect mode of action via activation of plant defence mechanisms (Friedrich *et al.,* 1996; Gorlach *et al.,* 1996; Lawton *et al.,* 1996; Benhamou & Belanger, 1998; Cole, 1999; Godard *et al.,* 1999; Buonaurio *et al*., 2002). According to Gorlach *et al.* (1996), the induction of SAR in wheat plants against powdery mildew (*Blumeria (Erysiphe) graminis* f.sp. *tritici*) by BTH treatment is accompanied by the induction of a well characterised set of genes (SAR genes). Moreover, Schweizer *et al.* (1999) reported that rice seedlings treated with BTH acquired resistance to subsequent attack by the rice blast fungus, *Magnaporthe grisea*. Recently, it has been demonstrated that BTH treatment of tomato plants protected them against the root rot disease caused by *Fusarium oxysporum* f.sp. *radicis-lycoperisici* (Benhamou & Bélanger, 1998). Little attention has been given to induced resistance in plants against bacterial pathogens of the *Xanthomonas* genus (Siegrist *et al.,* 1997). Furthermore, in the Poaceae family, very few studies have been made on induced resistance in rice plants.

To assess the possibility of using ASM as a SAR inducer in rice crops, its efficacy in controlling BLB disease caused by *Xoo* was investigated. The aim of this study was to investigate whether some defence-related compounds such as phenolics and PR proteins accumulate in rice seedlings exhibiting systemic resistance against *Xoo* following treatment with ASM.

Materials and Methods

Plant material

Seeds of the susceptible rice cultivar IR 50 were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. This cultivar was chosen for the current study because of its proven susceptibility to BLB disease. Seeds were sown in 25 cm-diameter earthenware pots containing rice-field soil and kept in a greenhouse chamber.

Pathogen isolation and maintenance

Xanthomonas oryzae pv. *oryzae* (*Xoo*) was isolated from rice plants with bacterial leaf blight (cv. IR 50) and maintained on Wakimoto's semi-synthetic potato sucrose agar (PSA) (Wakimoto, 1960). After 48 h of incubation in slants at room temperature (28 ± 2°C), the yellowish bacterial growth was purified by the dilution plate technique (Waksman, 1952). Pure culture was maintained on agar slants of PSA and stored at -10°C. To prepare inoculum, bacteria were grown on PSA at 28 ± 2°C for 48 h and suspended in deionised water. The suspension was adjusted to 10^9 colony forming units (cfu) ml^{-1} from absorbance measurements (A_{600} = 0.3). The virulence of the isolate was confirmed by subjecting the rice leaves to pathogenicity tests with clip-inoculation.

Application of ASM

Acibenzolar-S-methyl (ASM; CGA-245704, 50% WP, Novartis, Basel, Switzerland) was obtained from Novartis India Ltd. Three concentrations of ASM, *viz*. 1, 10 and 100 µg a.i. ml^{-1}, were prepared and used to drench the potted plants (500 ml pot^{-1}) containing 25 day-old rice seedlings. To standardise the optimum resistance induction period, the chemical was applied through soil at 1, 2 and 3 days prior to inoculation with *Xoo*. After the addition of the inducer on 1, 2 and 3 days earlier, leaves of the rice plants were clip-inoculated with the bacterial (*Xoo*) suspension. The severity of bacterial blight disease was recorded 7 days after each inoculation and expressed as lesion length. A set of uninoculated plants and untreated controls were maintained for comparison purposes. All data are the means of 25 leaves per treatment. All experiments were performed three times with similar results; the data presented are from one experiment only.

Induction of defence mechanisms

The changes in phenolics, chitinase and β-1,3-glucanase activities in leaves of susceptible rice cultivar (IR 50) were assessed in 21 day-old rice plants that were: (a) pretreated with 100 µg a.i. ml^{-1} of ASM with a waiting period of 3 days prior to inoculation with *Xoo*; (b) ASM treated (100 µg a.i. ml^{-1}) without *Xoo* inoculation; (c) *Xoo*-inoculated and without ASM treatment; and (d) uninoculated and treated with sterile water. Leaf samples (1 g) were obtained at 0, 12, 24, 48 and 72 h after treatment and three independent samples were collected and assayed for the changes in phenolics and defence related enzymes.

Estimation of phenolic content

Phenolic content of rice leaf tissue was estimated by the procedure described by Zieslin & Ben-Zaken (1993). A tissue sample (1 g) was homogenised in 10 ml 80% methanol and agitated for 15 min at 70°C. The methanolic extract (1 ml) was added to 5 ml distilled water and 250 µl Folin-Ciocalteau reagent (1N) and the solution was kept at 25°C for 3 min. A saturated solution of Na_2CO_3 (1 ml) and distilled water (1 ml) were added and the reaction mixture was incubated for 1 h at 25°C. The absorbance of the developed blue colour was measured using a Beckman DU64 spectrophotometer at 725 nm. The content of the total soluble phenols was calculated according to a standard curve obtained from a Folin-Ciocalteau phenol reaction and expressed as phenol equivalents in µg g^{-1} fresh weight.

Chitinase assay

Samples of rice leaf tissue (1 g) were collected at various time intervals (as described above) and the samples were immediately extracted with 10 ml of 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged for 10 min at 10 000 ***g*** at 4°C and the supernatant was used as the enzyme source. Colloidal chitin was prepared from crab shell chitin (Sigma) according to Berger & Reynolds (1958). Commercial lyophilised snail gut enzyme (Helicase, obtained from Sepracor, France) was desalted as described by Boller & Mauch (1988). For the colorimetric assay of chitinase, 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme extract and 0.1 ml colloidal chitin (1 mg) were pipetted into a 1.5 ml Eppendorf tube. After 2 h at 37°C, the reaction was stopped by centrifugation at 1000 ***g*** for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.1) and incubated with 20 µl desalted snail gut enzyme for 1 h. The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to Reissig *et al*. (1959) using internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as nmol GlcNAc equivalents min^{-1} g^{-1} fresh weight.

β-1,3-glucanase assay

β-1,3-glucanase activity was assayed colorimetrically by the laminarin-dinitrosalicylate method (Pan *et al.,* 1991). Samples of rice leaf tissue (1 g) were collected at various time intervals (as described above) and the samples were extracted with 5 ml 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4°C using a pestle and mortar. The extract was then centrifuged at 10 000 ***g*** for 15 min at 4°C and the supernatant was used in the enzyme assay. The reaction mixture consisted 62.5 µl of 4% laminarin and 62.5 µl of enzyme extract. The reaction was carried out at 40°C for 10 min. The reaction was then stopped by adding 375 µl dinitrosalicylic reagent and heating for 5 min in a boiling water bath. The resulting coloured solution was diluted with 4.5 ml distilled water and vortexed, and its absorbance at 500 nm was determined. Enzyme activity was expressed as nmol min^{-1} g^{-1} fresh weight.

Induction of pathogenesis-related (PR) proteins

PR proteins were induced in plants from all treatment categories in 3-day, 2-day and 1-day ASM-treated (100 µg a.i. ml^{-1}) or pathogen-inoculated rice plants. Proteins soluble at low pH were isolated by extracting 1 g of leaf tissue in 2 ml 0.1 M potassium phosphate buffer (pH 6.5) using a pre-chilled pestle and mortar at 4°C. The homogenate was centrifuged at 10 000 ***g*** for 15 min at 4°C. Protein content of the supernatant was determined according to the method described by Bradford (1976) using bovine serum albumin as a standard. Approximately 100 mg protein per sample was added to 40 µl of a sample buffer containing 0.0625 M Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.01% bromophenol blue at pH 6.8 by vigorous vortexing and then boiled for 5 min. SDS-PAGE was carried out according to the procedure of Laemmli (1970) with 12% separating gel. The electrophoresis was performed at 40 V until the dye reached the separating gel and then increased to 100 V and continued until the blue dye reached the bottom of the gel.

After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, USA) using semi-dry "Transblot" apparatus (Bio-Rad, Richmond, CA, USA). After electroblotting, the membrane was blocked for 3 h by constant shaking at room temperature in Tris-buffered saline (TBS) (10 mm Tris-HCl, 150 mM NaCl, pH 8.0) containing 0.05% Tween 20 and 2.5% (w/v) gelatin. The blot was then incubated for 3 h at room temperature with gentle shaking in antiserum containing antibodies raised against rice thaumatin-like protein (TLP) diluted to 1:1000 in TBS containing 0.05% Tween 20 (v/v) (TBST). Antiserum containing antibodies raised against tobacco chitinase, diluted to 1:1500 in TBS containing 0.05% Tween 20 and 2.5% (w/v) gelatin, was used separately to detect for the induction of chitinase in the rice tissue. The unbound antibody was then removed by four washes with TBST (30 ml) and the blot was incubated for 3 h at room temperature in antirabbit immunoglobulin conjugated with horseradish peroxidase (1:1500 dilution). Unbound secondary antibody was removed by four washes with TBST (30 ml) followed by two 5-min washes with TBS. The protein bands were visualised using 4-chloro-1-naphthol (Bio-Rad).

Molecular weights of the protein bands were determined using pre-stained kaleidoscope protein markers (Bio-Rad, USA).

Results

Induction of resistance by treatment with CGA-245704

Soil-drench application of ASM induced resistance in rice seedlings against BLB (Table 1). All the test concentrations of ASM (1, 10 and 100 µg a.i. ml^{-1}) were effective in inducing resistance to BLB as judged by reduction in lesion length. Of the concentrations of ASM tested, best protection was observed at 100 µg a.i ml^{-1} and the effectiveness persisted up to 3 days after treatment. Furthermore, the shortest lesion lengths were obtained from plants treated with 100 µg a.i. ml^{-1} of ASM when added to the rice plants 3 days prior to inoculation with the pathogen (Table 1).

Induction of defence mechanisms

Induction of PR proteins and accumulation of phenolics in ASM treated or *Xoo*-inoculated rice plants were examined. Significant increases in phenolic content, chitinase or β-1,3-glucanase activity were observed 24 h after inoculation with *Xoo* and increased consistently throughout the experimental period of 72 h (Fig. 1). ASM treatment alone, without inoculation, did not elicit increases in phenolic content, chitinase and β-1,3-glucanase activity. Thus the values obtained were similar to those in uninoculated rice tissues treated with water only. However, when the rice plants were pretreated with ASM followed by pathogen inoculation, phenolic content, chitinase and β-1,3-glucanase activity were significantly increased from 12 h after treatment and reached maximum levels at 72 h after treatment. A two-fold increase in phenolic content, five-fold increase in chitinase activity and four-fold increase in β-1,3-glucanase activity were recorded 72 h after pathogen inoculation in the rice plants pretreated with ASM in comparison with the uninoculated rice tissues treated with water only.

Western blot analysis indicated that a 25 and 35 kDa protein cross-reacting with rice TLP and barley chitinase antiserum respectively were induced due to pathogen inoculation alone and ASM pretreated rice plants followed by *Xoo* inoculation (Fig. 2A, 2B). The induction of TLP and chitinase was greater in plants pretreated with ASM followed by pathogen inoculation than in untreated *Xoo*-inoculated plants. Increase in the induction of TLP and chitinase were observed 3 days after *Xoo* inoculation in the tissues pretreated with ASM when compared with tissues 2 days and 1 day after *Xoo* inoculation in ASM-treated plants. However, pretreatment with ASM without pathogen inoculation and with sterile distilled water (control) did not induce TLP or chitinase in rice plants.

Table 1. *The effect of induction period on the efficacy of ASM as a pretreatment against bacterial leaf blight*

	Bacterial leaf blight lesion length (cm)[a]		
Treatments (µg ml^{-1})	1[b]	2[b]	3[b]
CGA-245704, 1	2.4 ± 0.21	2.1 ± 0.18	1.9 ± 0.15
CGA-245704, 10	1.9 ± 0.16	1.7 ± 0.15	1.4 ± 0.19
CGA-245704, 100	1.7 ± 0.08	1.3 ± 0.09	0.9 ± 0.12
Control	5.8 ± 0.38	5.6 ± 0.31	5.5 ± 0.21

[a]Disease development was recorded 7 days after pathogen inoculation
[b]Days between ASM treatment and inoculation with the pathogen
Data are expressed as means ± SE of three replicates

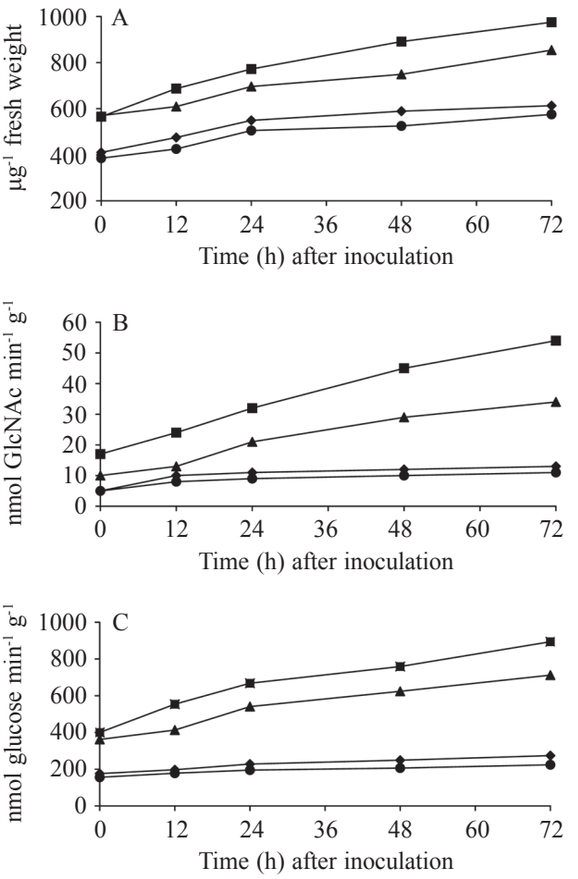

Fig. 1. Changes in phenolic content (A) and chitinase (B) and β-1,3-glucanase (C) activities in rice leaf tissues of ASM-treated susceptible rice cv. IR 50 after inoculation with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). Data are means of three independent samples taken up to 3 days after *Xoo* inoculation. 100 µg a.i. ml^{-1} ASM-treated plants (■); untreated; *Xoo* inoculated (▲); ASM treated, uninoculated (◆); control (sterile water) (●).

Discussion

The phenomenon of SAR has attracted much attention as a strategy for controlling plant diseases. Although the principle of induced resistance has long been known (Hammerschmidt & Kuc, 1995), the first commercially available resistance inducer was not developed until recently. Many reports have demonstrated the efficiency of BTH in controlling plant diseases caused by pathogenic fungi and bacteria (Friedrich *et al.,* 1996; Gorlach *et al.,* 1996; Lawton *et al.,* 1996; Benhamou & Bélanger, 1998; Cole, 1999; Godard *et al.,* 1999; Buonaurio *et al*., 2002). However, little is known about the ability of BTH to trigger SAR in rice against *Xoo*. Although earlier reports have highlighted the potential of BTH in activating SAR against bacterial diseases (Gorlach *et al.,* 1996; Lawton *et al.,* 1996; Benhamou & Bélanger, 1998; Cole, 1999; Godard *et al.,* 1999), our data provide the first evidence that BTH triggers SAR and protects rice plants against *Xoo* infection.

It is well known that plants are endowed with various defence mechanisms against pathogens. However, in susceptible plants the defence mechanisms are not induced. For the induction of defence mechanisms, signals are needed. The defence mechanisms can be triggered even in susceptible cultivars by manipulating the signal transduction system (Vidhyasekaran, 1997; Lucas, 1999). Many defence mechanisms are triggered in plants in response to infection by pathogens (M'piga *et al.,* 1997), including phenolics (Vidhyasekaran *et al.,* 2001), phytoalexins, callose, PR proteins and hydroxyproline-rich glycoproteins (HRGPs) (Vidhyasekeran, 1997). The genes encoding PR proteins are expressed not only in the infected leaves, but also in the non-infected leaves of the same plant (Uknes *et al.,* 1992). This phenomenon, SAR, plays a major role in disease resistance of plants (Schweizer *et al.,* 1999). Naturally induced SAR is not predictable in timing and level of expression and therefore would not be useful for agricultural practice. Thus it is quite obvious that from a practical point of view only a chemical means of plant activation would have definite advantages in plant disease control. Novel synthetic signal molecules that are able to induce SAR offer great potential for disease control in economically important crops. Many such chemical compounds are also known to induce SAR in plants (Cohen *et al.,* 1999; Hong *et al.,* 1999; Narusaka *et al.,* 1999; Toal & Jones, 1999). ASM and its metabolites are known to have no direct antimicrobial activity (Kunz *et al.,* 1997; Raum, 1997; Ruess *et al.,* 1995). The results of the present

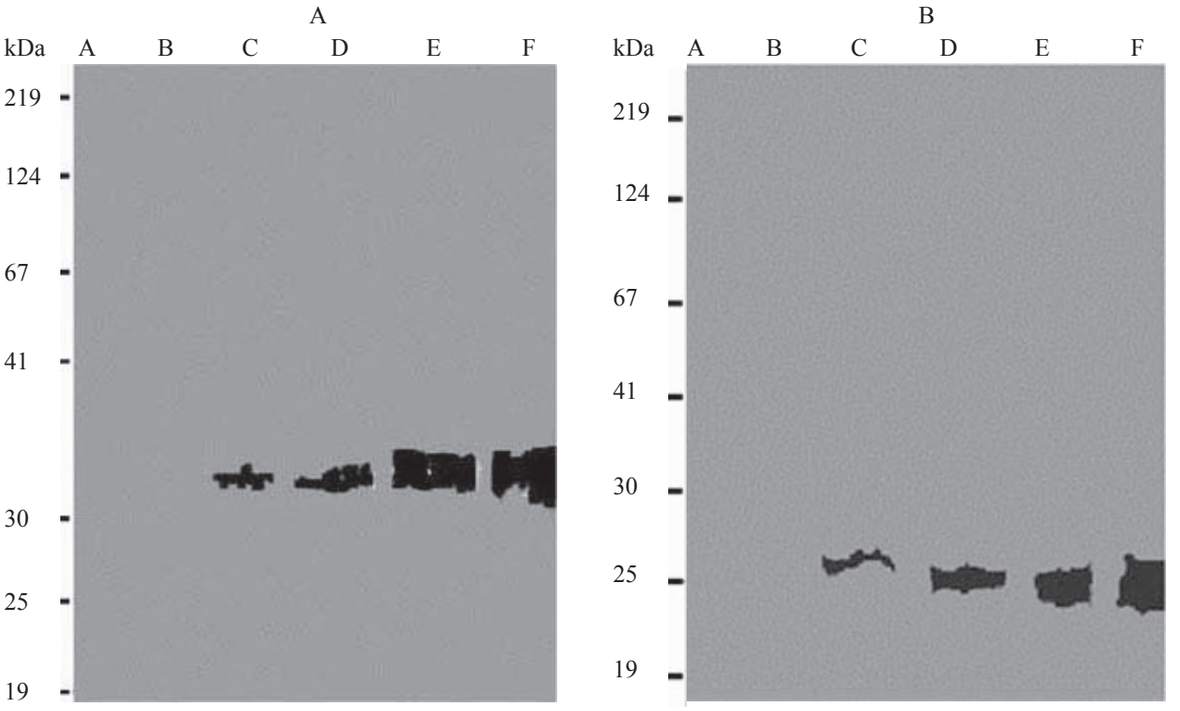

Fig. 2. Induction of (A) 35 kDa chitinase and (B) 25 kDa thaumatin-like protein (TLP) in rice in response to ASM treatment and *Xanthomonas oryzae* pv. *oryzae* inoculation. A, Control; B, ASM treated, uninoculated; C, untreated, *Xoo*-inoculated; D, 1 day after *Xoo* inoculation in 100 µg a.i. ml^{-1} ASM-treated plants ; E, 2 days after *Xoo* inoculation in 100 µg a.i. ml^{-1} ASM-treated plants; F, 3 days after *Xoo* inoculation in 100 µg a.i. ml^{-1} ASM-treated plants. Aliquots (100 µg) of proteins were analysed by western blotting after SDS-PAGE, using rice TLP antiserum and tobacco chitinase antiserum. Sizes of the marker protein are indicated on the left.

study indicate clearly that plants treated with ASM were effective in inducing systemic resistance in rice plants against *Xoo*. When ASM was applied to the rice plants, it was found that host defence was activated. When the pathogen was clip-inoculated, the untreated plants died completely within 3 days, whereas in the ASM-treated plants the pathogen could not colonise from the site of inoculation. Similar induction of SAR has been reported in cucumber against *Colletotrichum orbiculare*, *Pseudomonas lachrymans* and Tobacco necrosis virus A (Oostendorp *et al.,* 1996) and in cereals against *Septoria* spp. and *Puccinia* spp. (Ruess *et al.,* 1995).

The accumulation of PR proteins such as chitinase, β-1,3-glucanase and TLP, and phenolics was also induced in rice plants due to inoculation with *Xoo* in ASM-treated rice plants. Accumulation of PR proteins is associated with SAR in plants (Ryals *et al.,* 1996). Many PR proteins exhibit antifungal activity (Mauch *et al.,* 1998; Velazhahan *et al.,* 2000). PR proteins such as chitinases (PR-3) and β-1,3-glucanases (PR-2) have the potential to hydrolyse chitin and β-1,3 glucan respectively, the major components of fungal cell walls (Ham *et al.,* 1991; Ren & West, 1992). Several studies have demonstrated that over-expression of chitinases, β-1,3-glucanases and TLPs in transgenic rice is associated with enhanced resistance to various fungal pathogens (Lin *et al.,* 1995; Chen *et al.,* 1999; Datta *et al.,* 1999, 2001). Recently, Narusaka *et al*. (1999) reported that both resistance against *Cladosporium cucumerinum* causing scab disease and accumulation of PR protein were rapidly induced in cucumber plants after treatment with ASM. They observed induction of chitinase in the first and second leaves of cucumber when the third leaves were treated ASM. In the present study, an increase in the levels of β-1,3-glucanase and chitinase was observed in response to pretreatment with ASM followed by *Xoo* inoculation. Increase in phenolic content in both dicotyledonous and monocotyledonous plants has been correlated with resistance to pathogens (Vidhyasekeran, 1997). It is well known that resistant plants accumulate phenols or produce polyphenols more rapidly than susceptible ones (Lyon & McGill, 1988). The increased phenolic content and accumulation of PR proteins due to inoculation with *Xoo* in ASM-treated rice plants may be involved in the resistance of rice against *Xoo*. Western blot analysis revealed the rapid induction and up regulation of 25 and 35 kDa proteins cross-reacting with rice TLP and tobacco chitinase antiserum, respectively, when the plants were pretreated with ASM followed by pathogen inoculation. It can be concluded that these proteins induced in rice plants may be TLP and chitinase respectively.

The data in the present studies have demonstrated an additional method for protecting rice plants against BLB by a synthetic chemical inducer. Further studies in this area should allow for the better development of induced resistance strategies by activating defence responses.

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