

Differential induction of chitinase and β -1,3-Glucanase in rice in response to inoculation with bacterial leaf blight pathogen (*Xanthomonas oryzae* pv. *oryzae*)

Differenzierte Induktion von Chitinase und β -1,3-Glucanase in Reis als Reaktion auf die Inokulation mit dem Erreger der bakteriellen Blattbräune (*Xanthomonas oryzae* pv. *oryzae*)

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Summary

Rice leaves clip-inoculated with *Xanthomonas oryzae* pv. *oryzae* (Xoo) were analyzed for the accumulation of pathogenesis-related (PR) proteins. Inoculation of rice plants with Xoo resulted in a marked increase in activities of chitinase and β -1,3-glucanase. Western blot analysis indicated that protein with a molecular mass of 35 kDa cross-reacting with barley chitinase antibody was induced in rice in response to inoculation with Xoo. The appearance of this chitinase was correlated with increase in (enzyme) activity of that enzyme during the test period.

Key words: Chitinase, β -1,3-glucanase; *Oryza sativa*; PR-proteins; *Xanthomonas oryzae* pv. *oryzae*

Zusammenfassung

Reisblätter wurden nach Inokulation mit *Xanthomonas oryzae* pv. *oryzae* (Xoo) auf die Akkumulation von PR-Proteinen untersucht. Die Inokulation der Reispflanzen mit Xoo hatte einen deutlichen Anstieg der Aktivitäten von Chitinase und β -1,3-Glucanase zur Folge. Western blot Analyse ergab, dass ein Protein mit einer Molekülmasse von 35 kDa, welches mit dem Antikörper der Gerstenchitinase kreuzreagierte, im Reis nach Inokulation mit Xoo induziert wurde. Das Erscheinen dieser Chitinasen war korreliert mit der Erhöhung der Aktivität dieses Enzyms während der Versuchszeit.

Stichwörter: *Xanthomonas oryzae* pv. *oryzae*; Chitinase; β -1,3-Glucanase; *Oryza sativa*; PR-Proteine

1 Introduction

Plants, in general, possess several structural and biochemical defense mechanisms to overcome the infection by pathogens. Some of the chemical compounds are antifungal and are constitutive, existing in healthy plants. For example, phenolics, phenolic glycosides, unsaturated lactones, saponins, cyanogenic glycosides, glucosinolates, 5-alkylated resorcinols and dienes (OSBOURN 1996). In addition to the preformed antimicrobial compounds, several defense mechanisms are induced in response to infection by potentially pathogenic micro-organisms. These inducible defense responses include hypersensitive cell death (LEVINE et al. 1994), production of phytoalexins (KUC and RUSH 1985), accumulation of

Pathogenesis-Related (PR) proteins (VELAZHAHAN et al. 1998, 2000), induction of oxidative burst (BAKER and ORLANDI 1995), lignification (RIDE 1975) and cross-linking of wall glycoprotein (BRADLEY et al. 1992). The speed of expression of the defense genes involved in the production of antimicrobial compounds determines the susceptible or resistant state of the host plant.

In recent years, much research has been focused on PR-proteins (DATTA and MUTHUKRISHNAN 1990). The PR-proteins are a group of extracellular proteins that are induced by different phytopathogens such as fungi, bacteria and viruses, fungal elicitors and certain chemical inducers such as salicylic acid, polyacrylic acid and 2,6-dichloroisonicotinic acid (UKNES et al. 1992). These proteins have been extensively studied in tobacco and are identified in many monocotyledonous and dicotyledonous plants (BOL et al. 1990; LINTHORST 1991). Several studies suggested that the expression of PR-proteins correlated with the development of systemic acquired resistance (SAR) in plants (UKNES et al. 1992; EPPLE et al. 1997; SCHWEIZER et al. 1999; TERRAS et al. 1995). XU et al. (1998) reported that systemic induction of β -1,3-glucanase and chitinase are related with the resistance of bean plants to binucleate *Rhizoctonia* species. Transgenic potato plants expressing a soybean β -1,3-endoglucanase gene exhibited an increased level of resistance to *Phytophthora infestans* (BORKOWSKA et al. 1998). In the present study, we have investigated the induction of PR-proteins in rice in response to infection by *Xanthomonas oryzae* pv. *oryzae* (Xoo), the rice bacterial leaf blight pathogen.

2 Materials and methods

2.1 Plant material

The rice seeds of the susceptible cultivar 'IR 50' were used in all experiments. The seeds were obtained from Paddy Breeding Station, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The plants were grown in 25-cm diameter earthen pots containing rice field soil in a greenhouse according to standard methods.

2.2 Bacterial culture and inoculation

Xanthomonas oryzae pv. *oryzae* (Xoo) was isolated from rice bacterial leaf blight- infected rice plants and maintained on Wakimoto's semi synthetic potato sucrose agar (PSA) medium (WAKIMOTO 1960). After 48 h of incubation at room temperature (28 ± 2 °C), the yellowish bacterial growth on the medium was subcultured in slants containing the above medium and purified by the dilution plate technique (WAKSMAN 1952). Pure culture was maintained on agar slants of PSA medium at -10 °C for further studies. Seeds of rice cultivar 'IR 50' were sown in pots containing field soil, five seeds per pot and grown under greenhouse conditions. Twenty-five plants (in five pots at five plants per pot) were kept as one replication and there were five replications maintained for each treatment. Individual tillers of 21 to 25-day-old-plants were clip-inoculated with the bacterial (Xoo) suspension containing 10^9 colony forming units (cfu)/ml. The plants with mock inoculation with sterile water served as control. Immediately after inoculation, the plants were covered with polyethylene cover to maintain high humidity for infection. At various times after inoculation, plant samples were collected and frozen in liquid nitrogen before protein extraction.

2.3 Assay of chitinase

Rice leaves (1 g) were homogenized in 5 ml 0.1 mol/l sodium citrate buffer (pH 5.0) using a chilled pestle and mortar. The homogenate was then centrifuged for 10 min at $10,000 \times g$ at 4 °C and the supernatant was used in the enzyme assay. Colloidal chitin was prepared from crab shell chitin (Sigma) according to BERGER and REYNOLDS (1958). The commercial lyophilized snail gut enzyme (Helicase, obtained from Sepracor, France) was desalted as described by BOLLER and MAUCH (1988). For the colorimetric assay of chitinase 10μ l of 1 mol/l sodium acetate buffer (pH 4.0), 0.4 ml of 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 $1,000 \times g$ for 3 min. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30μ l of 1 mol/l potassium phosphate buffer (pH 7.1)

and incubated with 20 μ l desalted snail gut enzyme for 1 h. The resulting monomeric N-acetylglucosamine (GlcNAc) was determined according to REISSIG et al. (1955) using internal standards of GlcNAc in the assay mixtures for calculations. Enzyme activity was expressed as nmol GlcNAc equivalents $\text{min}^{-1} \text{g}^{-1}$ fresh weight.

2.4 Assay of β -1,3-glucanase

β -1,3-glucanase activity was assayed by the laminarin-dinitrosalicylate method (PAN et al. 1991). Rice leaves (1 g) were extracted with 5 ml of 0.05 mol/l sodium acetate buffer (pH 5.0) by grinding at 4 °C using a pestle and mortar. The extract was then centrifuged at 10,000 $\times g$ for 15 min at 4 °C and the supernatant was used in the enzyme assay. The reaction mixture consisted of 62.5 μ l of 4 % laminarin and 62.5 μ l of enzyme extract. The reaction was carried out at 40 °C for 10 min. The reaction was then stopped by adding 375 μ l of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting coloured solution was diluted with 4.5 ml of distilled water, vortexed and its absorbance at 500 nm was determined. Enzyme activity was expressed as nmol $\text{min}^{-1} \text{g}^{-1}$ fresh weight.

2.5 Western blot analysis

Proteins were extracted from control and Xoo-inoculated leaves by grinding 1 g of tissues with 5 ml of 0.1 mol/l phosphate buffer, pH 6.5, containing 0.5 mmol/l phenylmethylsulphonyl fluoride as described by VELAZHAHAN et al. (1998). Proteins (100 μ g) in aliquots of extracts were separated on 12 % SDS-PAGE (LAEMMLI 1970) and electroblotted onto polyvinylidene difluoride (PVDF) membrane (pore size 0.45 μ m, Bio-Rad) using BioRad semidry transfer cell in accordance with the manufacturer's instructions. Western blotting was carried out as described by WINSTON et al. (1987) with an antiserum raised against barley chitinase. The barley chitinase antiserum was a gift from Dr. S. MUTHUKRISHNAN, Kansas State University, U. S. A. Apparent molecular mass of proteins was determined by comparison with molecular weight standards (Rainbow markers, Amersham Pharmacia, USA). Protein concentrations were determined by Bradford assay (BRADFORD 1976).

2.6 Statistical analysis

All experiments were laid out in a completely randomized design (CRD) with twenty-five plants (in five pots at five plants per pot) kept as one replication and there were five replications maintained for each treatment. The data were statistically analyzed according to Duncan's Multiple Range Test (DMRT) (GOMEZ and GOMEZ 1984).

3 Results

A two-fold increase in chitinase activity was observed in rice leaves after inoculation with Xoo. The maximum enzyme activity was recorded 2 days after inoculation, subsequently the enzyme activity declined but remained significantly at higher levels when analyzed by DMRT (Least significant difference to compare any two means at $P = 0.05$ is 3.09) at all sampling stages when compared with control (Fig. 1). To know whether the increase in chitinase activity in Xoo-inoculated rice leaves is due to *de novo* synthesis or due to post-translational modification of the pre-existing enzyme, the crude proteins from Xoo-inoculated rice leaves at different time intervals were analyzed by Western blot analysis using barley chitinase antibody. Induction of a 35 kDa protein cross-reacting with barley chitinase antibody was detected in rice leaves inoculated with Xoo. A time course study indicated that the 35 kDa chitinase started to increase 12 h after inoculation (Fig. 2) and the level of the protein continued to increase throughout the experimental period of 24 to 48 h after inoculation.

β -1,3-glucanase activity steadily increased in Xoo-inoculated rice leaves from 2 days onwards and reached the maximum 5 days after inoculation (Fig 3). The enzyme activity was significantly at higher levels when analyzed by DMRT (Least significant difference to compare any two means at $P = 0.05$ is 9.43) at all sampling stages when compared with control.

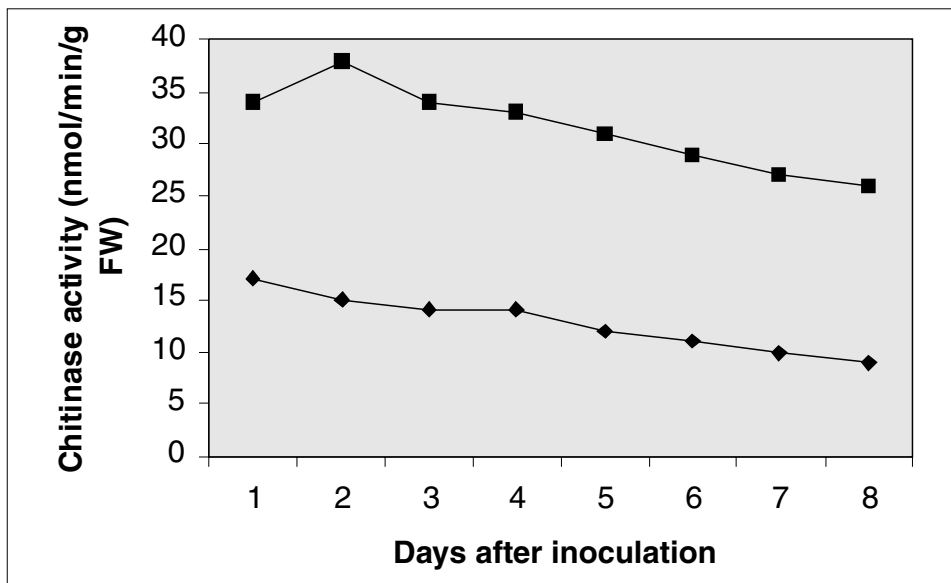


Fig. 1. Changes in chitinase activity in rice in response to inoculation with *Xanthomonas oryzae* pv. *oryzae* (Xoo). Chitinase activity in protein extracts of control (—◆—) and Xoo-inoculated (—■—) rice leaves was measured colorimetrically. Least significant difference to compare any two means at $P = 0.05$ is 3.09.

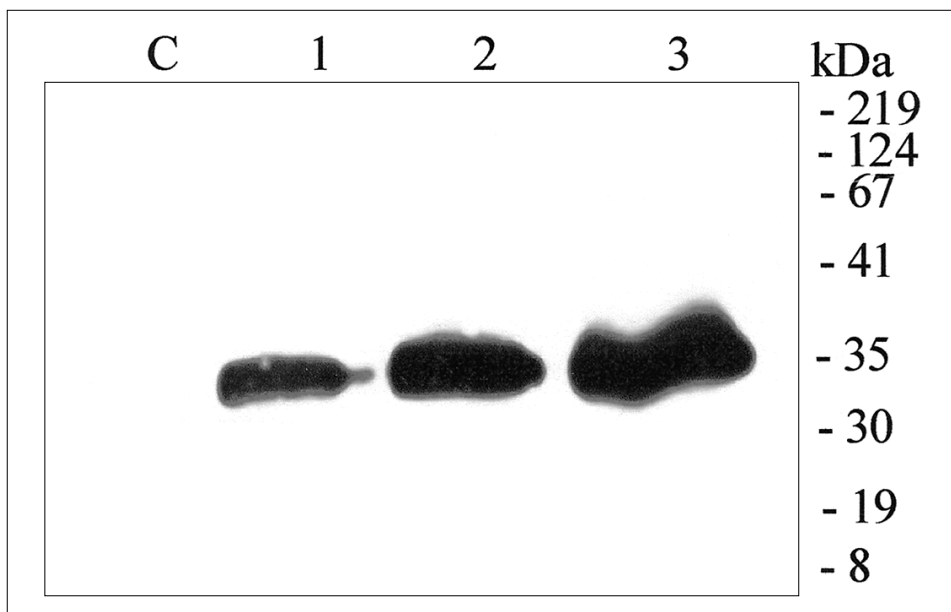


Fig. 2. Induction of chitinase in rice in response to inoculation with *Xanthomonas oryzae* pv. *oryzae* (Xoo). Aliquots (100 μ g) of proteins extracted from control (lane C) and Xoo-inoculated rice leaves (lanes 1–3) were analyzed by Western blotting after SDS-PAGE, using a barley chitinase antiserum. Lane 1: 12 h after inoculation; Lane 2: 24 h after inoculation; Lane 3: 48 h after inoculation. Sizes of marker proteins are indicated on the right.

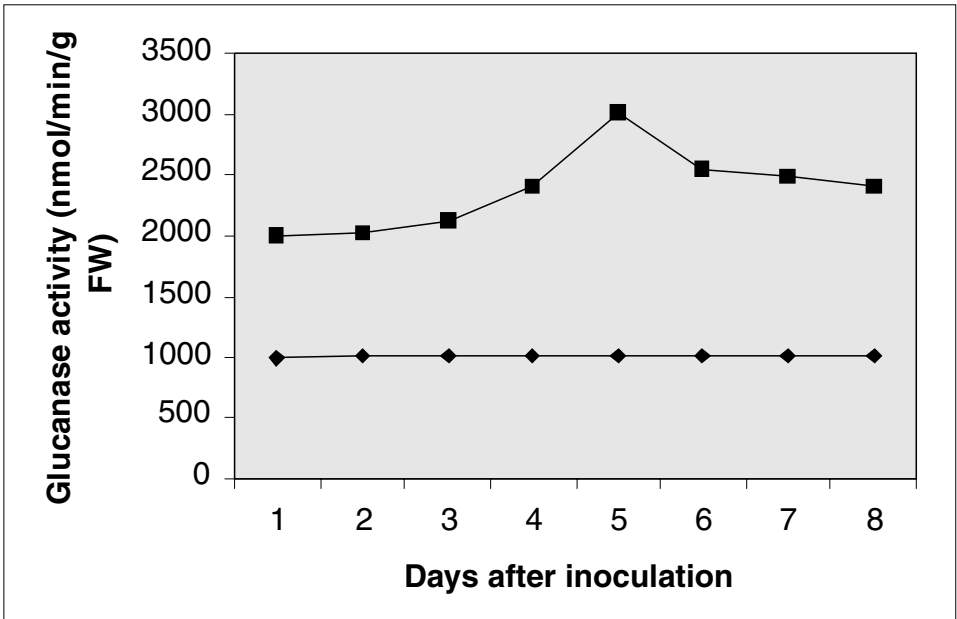


Fig. 3. Changes in β -1,3-glucanase activity in rice in response to inoculation with *Xanthomonas oryzae* pv. *oryzae* (Xoo). Glucanase activity in protein extracts of control (—◆—) and Xoo-inoculated (—■—) rice leaves was measured colorimetrically. Least significant difference to compare any two means at $P = 0.05$ is 9.43.

4 Discussion

Chitinases and β -1,3-glucanases have been suggested to be involved in resistance of plants against fungal and bacterial pathogens (KIM and HUANG 1994; XU et al. 1998; SHEWRY and LUCAS 1997; LIN et al. 1995; MOHAN BABU et al. 2002). Chitinases and β -1,3-glucanases have the potential to hydrolyze chitin and β -1,3-glucan, respectively, which are major components of fungal cell walls, leading to direct inhibition of growth of several fungi (SCHLUMBAUM et al. 1986; LEAH et al. 1991). It has been demonstrated that both chitinases and β -1,3-glucanases from pea tissues acted synergistically in partial degradation of isolated fungal cell walls (MAUCH et al. 1988). In addition, chitinases and glucanases play an indirect role in stimulating plant defense by releasing oligosaccharides from the fungal cell walls by their enzymatic action which act as “elicitors” or inducers of several defense genes (RYAN 1987; VIDHYASEKARAN and VELAZZHAN 1996). High constitutive levels of β -1,3-glucanase activity was found to be associated with leaf rust resistance in wheat (ANGUELOVA et al. 1999). Similarly, higher levels of β -1,3-glucanase activity were recorded in resistant muskmelon plants than in susceptible plants (WARD et al. 1991).

Early increase in chitinase and glucanase activities has been correlated with resistance to plants against pathogens. In a resistant *Brassica napus* cultivar, the level of chitinase mRNA was three-fold higher than in the susceptible cultivar one day after inoculation (RASMUSSEN et al. 1992). The induction of systemic acquired resistance (SAR) in response to inducers is correlated with the systemic accumulation of β -1,3-glucanase and chitinase (VIDHYASEKARAN and VELAZZHAN 1996). In the present study, the time courses of chitinase and glucanase activities in response to infection by *Xanthomonas oryzae* pv. *oryzae* were examined. Inoculation of rice leaves with *Xanthomonas oryzae* pv. *oryzae* resulted in a marked increase in activities of both chitinase and glucanase. Several studies have shown that PR-proteins are also induced in plants upon treatment with *Pseudomonas fluorescens* (MAURHOFER et al. 1994; M'PIGA et al. 1997). Accumulation of PR-proteins is associated with systemic acquired resistance (SAR) in plants (RYALS et al. 1996). SHEWRY and LUCAS (1997) reported the role of

defense-related enzymes in disease resistance. Studies have also shown that *Pseudomonas fluorescens* induces resistance in radish (HOFFLAND et al. 1995) and in *Arabidopsis* (PIETERSE et al. 1996).

However, PUNJA and ZHANG (1993) reported higher levels of chitinase activity in infected susceptible plants than in resistant plants. They attributed this to stress levels induced by higher fungal biomass in susceptible plants. The results of the present study indicated that the increased activities and accumulation of chitinase and β -1,3-glucanase in rice in response to clip-inoculation with Xoo have contributed to increased resistance against bacterial leaf blight. Since these enzymes were more pronounced in rice-Xoo interaction it is suggested that these hydrolytic enzymes may play a role in resistance of rice to bacterial leaf blight. The defense related genes encoding chitinase and β -1,3-glucanase are already been characterized, cloned and expressed in other crops (VIDHYASEKARAN and VELAZHAHAN 1996). The utility of these genes in developing transgenic rice gains importance.

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