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FULL RESEARCH PAPER

Chitinase levels in rice cultivars correlate with resistance to the sheath blight pathogen *Rhizoctonia solani*

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Abstract Various rice cultivars were selected and screened for their reaction to sheath blight in the greenhouse. Cluster analysis of percent relative lesion height (% RLH) generated four groups of cultivars with a coefficient of similarity of 3.27. Chitinase activities were detected 24 h after inoculation of moderately resistant cvs Betichikon, Dudruchi, Khatochalani, Padi Pulut Malat, Kakua, IR72, Khakibinni. But in the susceptible cv. IR58, chitinase activity was detected only 36 h after inoculation. Western blot analysis showed that class 1 and class 2 chitinases were induced following *Rhizoctonia solani* infection of these cultivars. The % RLH and the number of infection cushions were negatively correlated with the level of chitinase activity. Moderately resistant rice cultivars had higher levels of chitinase activity and lower disease severity and numbers of infection cushions formed compared to IR58.

Keywords Chitinases · Disease resistance · Rice · Sheath blight

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Introduction

In recent years, much attention has been focused on understanding the complex defence mechanisms of plants in response to pathogenic infection. The rapid accumulation of host-coded proteins, commonly known as pathogenesis-related (PR) proteins, with antifungal activity has been demonstrated in various host–pathogen interactions (van Loon 1997; van Loon et al. 1998, 2006). Among these proteins are hydrolytic enzymes such as chitinases and β-1,3 glucanases which have been purified and characterized from several plants (Legrand et al. 1987; Rasmussen et al. 1992; Buchter et al. 1997; Yeboah et al. 1998). Induction of these enzymes occurs in different plant species in response to fungal infection (Metraux and Boller 1986; Cachinero et al. 1996), wounding (Ignatius et al. 1994), and treatment with ethylene or elicitors (Roby et al. 1988; Mauch et al. 1992; Wubben et al. 1996). Chitinase was detected in up to five leaves above the infected leaf of cucumber plants (Metraux et al. 1988).

Sheath blight of rice caused by *Rhizoctonia solani* has become a major constraint in intensive rice production systems following the introduction of semi-dwarf, high-yielding rice cultivars (Ou 1985; Mew and Rosales 1986; Kalpana et al. 2006). Yield losses have ranged from 8 to 50%, particularly when the infection is well distributed and severe in the field (Savary and Mew 1996; Savary et al. 2000). Many rice cultivars and breeding lines have been screened

for resistance to sheath blight of rice, but cultivars with high levels of resistance have not been identified (Hein 1990). Genetic resistance to *R. solani* has not been recorded in cultivars and wild relatives of rice (Bonman et al. 1992).

Significant progress has been made using molecular techniques to produce disease-resistant plants. Several genes encoding chitinase have been isolated from rice and characterized (Zhu and Lamb 1991; Nishizawa and Hibi 1991; Anuratha et al. 1996; Xu et al. 1996). A large number of pathogenesis-related genes are now reported (Datta et al. 1999). Some of these chitinase genes have been utilized to transform rice in an attempt to increase resistance to sheath blight (Lin et al. 1995; Datta et al. 1999, 2000, 2001, 2002; Nishizawa et al. 1999; Baisakh et al. 2001).

Only limited information is available on the level of chitinase expression in different cultivars of rice (Anuratha et al. 1996). In order to assess the role of chitinase activity in rice in determining disease progression after infection by *R. solani* AG1, 113 rice cultivars were selected and evaluated for the level and timing of chitinase accumulation. The relationship between chitinase activity and relative resistance of selected rice cultivars to sheath blight infection was also determined.

Materials and methods

Inoculation with *R. solani* and disease rating assessment

Two greenhouse experiments were conducted in two seasons using a completely randomized design (CRD) with four replications (one pot/replication/cultivar; Gomez and Gomez 1984) in the International Rice Research Institute (IRRI), Philippines. The rice plants were planted in 20 cm diam plastic pots. *Rhizoctonia solani* was grown on potato dextrose agar at 28°C. The plants were inoculated at 35–40 days after sowing (DAS) with *R. solani* grown on rice hull:rice grain (3:1) mixture (Sharma et al. 1990). Greenhouse temperatures ranged from 28 to 32°C under natural light condition. Disease was assessed using percent relative lesion height (% RLH) where:

$$\%\ \mathrm{RLH} = (\text{lesion height}/\text{plant height}) \times 100$$ (Ahn et al. 1986).

Protein extraction

Small samples (200 mg) of healthy and inoculated leaves and sheaths were homogenized in 400 μl of 0.2 M sodium acetate buffer (pH 5.0) with a 0.1 mM phenyl methyl sulphonyl fluoride (PMSF). Crude protein extracts were obtained by centrifugation at 13,000×*g* at 4°C for 10 min. The supernatant was decanted and centrifuged again at 13,000×*g* for 10 min at 4°C. The clear supernatant was used for enzyme assay.

Chitinase activity assay

Protein concentrations were determined using the bicinchoninic acid protein assay kit (Pearce, Rockford, IL, USA; Smith et al. 1985; Ignatius et al. 1994). Chitinase activity was determined by a standard colorimetric method using Remazol Brilliant Violet-chitin as the soluble substrate (Wirth and Wolf 1990). A unit of chitinase activity is defined as amount of protein that produces an absorbance change over control of one unit in 1 h using a wavelength of 550 nm.

A total of 113 rice cultivars was selected based on their known reaction to sheath blight infection from previous screening trials conducted at IRRI (T. W. Mew, unpublished). Since no complete resistance was found, only moderately resistant and susceptible cultivars were included in this study. Seeds of selected cultivars were obtained from the Genetic Resource Centre at IRRI.

Leaves and sheaths of rice plants were collected from healthy and inoculated plants of all 113 cultivars 7 days after inoculation to determine the level of chitinase activity. In the case of inoculated plants, the three to four leaf and sheath samples were collected with disease symptoms. All samples were placed in plastic envelopes and stored at −80°C until used. Mean chitinase activity (unit mg^{-1} protein) was calculated.

Time course of chitinase induction

Based on % RLH and chitinase activity, the 113 cultivars were divided into four groups 1, 2, 3 and 4 with increased susceptibility to sheath blight. Three cvs Betichikon, Dudruchi and Khatochalani from group 1, two cvs Padi Pulut Malat and Kakua from group 2, two cvs IR72 and Khakibinni from group 3 and CV. IR58 from group 4 were selected and grown in the greenhouse in a CRD with four replications.

Two experiments were conducted in two seasons. Betichikon, Dudruchi, Khatochalani, Padi Pulut Malat, Kakua, IR72, and Khakibinni were moderately resistant cultivars and only IR58 was a susceptible cultivar. Plants were inoculated 35 to 40 DAS with *R. solani* as described above.

Healthy and inoculated leaves and sheaths were collected separately at 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h after inoculation. All samples were stored at −80°C until analyzed. Crude extracts of leaf and sheath samples were made by homogenizing in 0.2 M sodium acetate buffer at pH 5.0. Aliquots of extracts containing equal amounts of protein (250 μg) were analyzed using 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and by Western blotting using a barley chitinase antiserum (Winston et al. 1987).

Formation of infection cushions

To determine the formation of infection cushions in relation to chitinase activity, the eight cultivars were evaluated as follows. Leaf and sheath pieces 6 cm long were collected 35 to 40 DAS and placed in Petri dishes containing 0.5% water agar medium. A mycelial block from a 3–4 day-old culture of *R. solani* was placed on top of a detached leaf and sheath. Samples for microscopic observation were collected 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, and 72 h after inoculation and stained with lactophenol acid fuchsin. Sections were mounted on microscopic slides in 50% glycerol and the number of infection cushions was counted using a microscope.

Statistical analysis

Data on %RLH, plant height and lesion length were analyzed independently by experiment. The treatment means were compared by Duncan multiple range test (DMRT; Gomez and Gomez 1984). The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute (IRRI), Biometric unit, The Philippines. Means, standard error and least significant difference (LSD) values for the greenhouse experiment to group the cultivars into four groups and constitutive chitinase and induced chitinase activity within the group and between the group were calculated using PROC GLM in SAS for windows, v. 8 (SAS Institute, Cary, NC). The dendrogram was constructed with the Numerical Taxonomy Multivariate Analysis System (NTSYS-pc) version 2.02e software package and clustered the representative data by the UPGMA method. The standard distance of the matrix coefficient was at 3.27.

Results

Varietal differences for sheath blight resistance

The 113 cultivars based on mean %RLH of the two experiments generated four groups of cultivars at a coefficient equivalent to 3.27 (Fig. 1). Coefficient is the fusion level. Group 1, 2 and 3 consisted of 71, 29 and 12 varieties, respectively. Only IR58 was clustered into group 4.

Chitinase activity

The chitinase activities of all 113 cultivars were determined before and after infection with *R. solani*. The chitinase activities of healthy plants were low compared with infected plants (Fig. 2). In group 1, constitutive chitinase activity was significantly different with induced chitinase. But in group 2 and group 3, constitutive chitinase activities were not significantly different with induced chitinase. In group 4 cultivar constitutive chitinase was significantly different with induced chitinase. Similarly, constitutive chitinase of group 1 was significantly different with constitutive chitinase of group 4, but induced chitinase of group 1 was significantly different with induced chitinase of groups 3 and 4, respectively.

The specific chitinase activity of inoculated plants increased after infection, but remained unchanged in healthy plants (Fig. 3). Activity started to increase 24 h after inoculation in all cultivars except IR58, whose chitinase activity started to increase 36 h after inoculation. In all cultivars, there was a rapid increase in activity between 36 and 48 h after inoculation. After 48 h, chitinase levels were nearly steady.

Western blot analysis of healthy and infected plant extracts probed with a barley chitinase antiserum showed that healthy plants had low to undetectable levels of 28 and 35 kDa chitinases (Fig. 4a and b). However, after infection with *R. solani*, the level of 28 and 35 kDa chitinases increased substantially in all

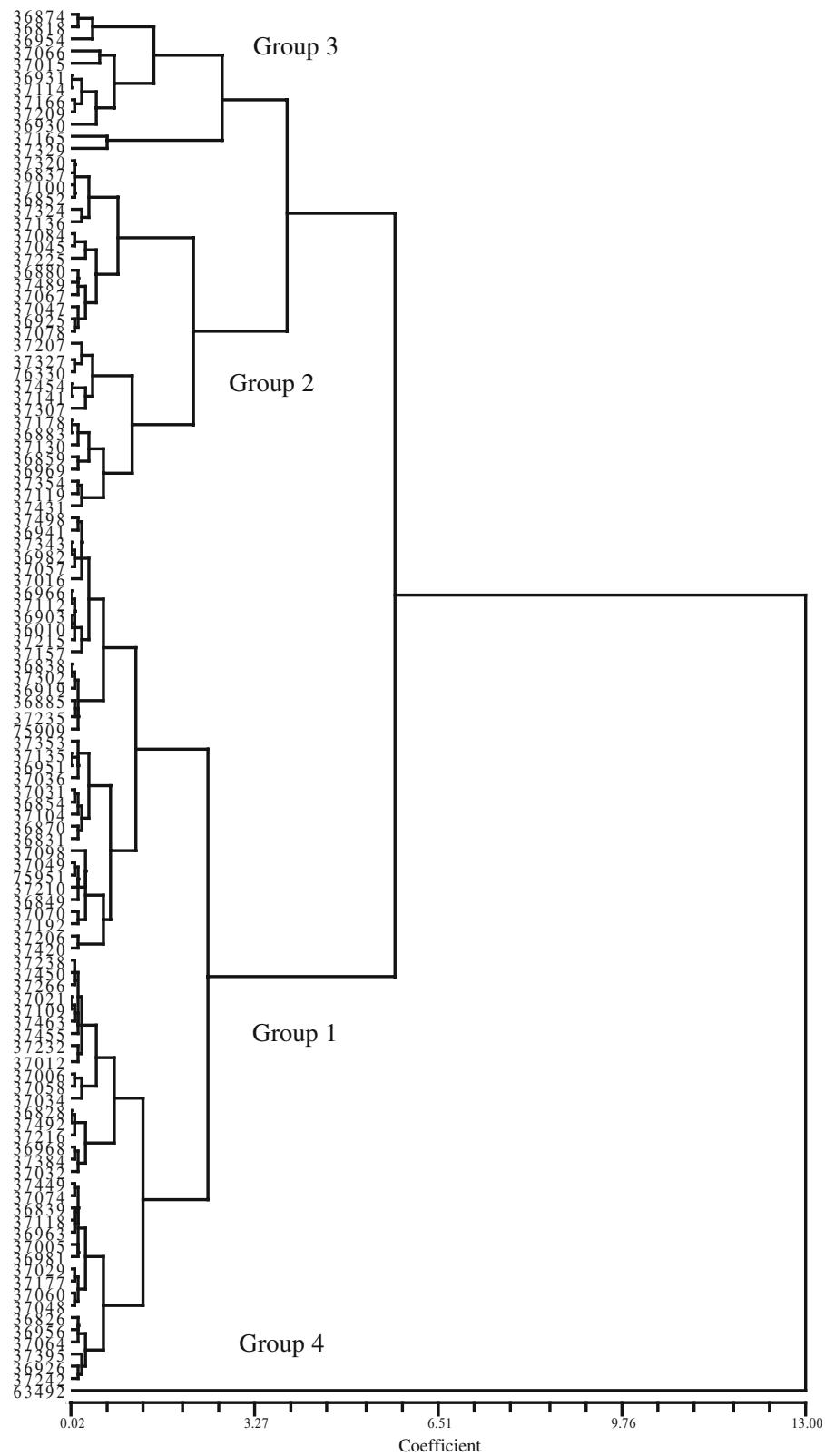

Fig. 1 Cluster analysis of 113 rice cultivars based on % relative lesion height (RLH). This analysis clustered 113 cultivars into four groups. *Group 1* consisted of 71 cultivars, *Group 2* consisted of 29, *Group 3* consisted of 12 and *Group 4* consisted of one cultivar. Mean of two experiments

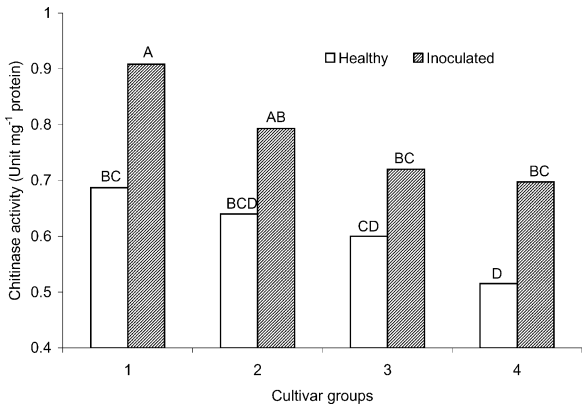

Fig. 2 Chitinase activities of healthy and infected rice cultivars inoculated with *R. solani* (mean of two experiments). The chitinase activities with same capital letters are not significantly different ($p<0.05$)

cultivars tested indicating that these chitinases were induced after infection (Fig. 4a and b). In Khatochalani (Fig. 4a, lane 4), these protein bands were present even in the uninoculated plants. The susceptible cv. IR58, had very low levels of chitinase even after infection (Fig. 4a, lanes 7 and 8).

Formation of infection cushions

The numbers of infection cushions on the leaves and sheaths were significantly different among the eight cultivars. The number of infection cushions on the leaf was nearly the same as those on the sheath. The numbers of infection cushions formed were significantly lower in group 1 cvs, Betichikon, Dudruchi and Khatochalani (Table 1).

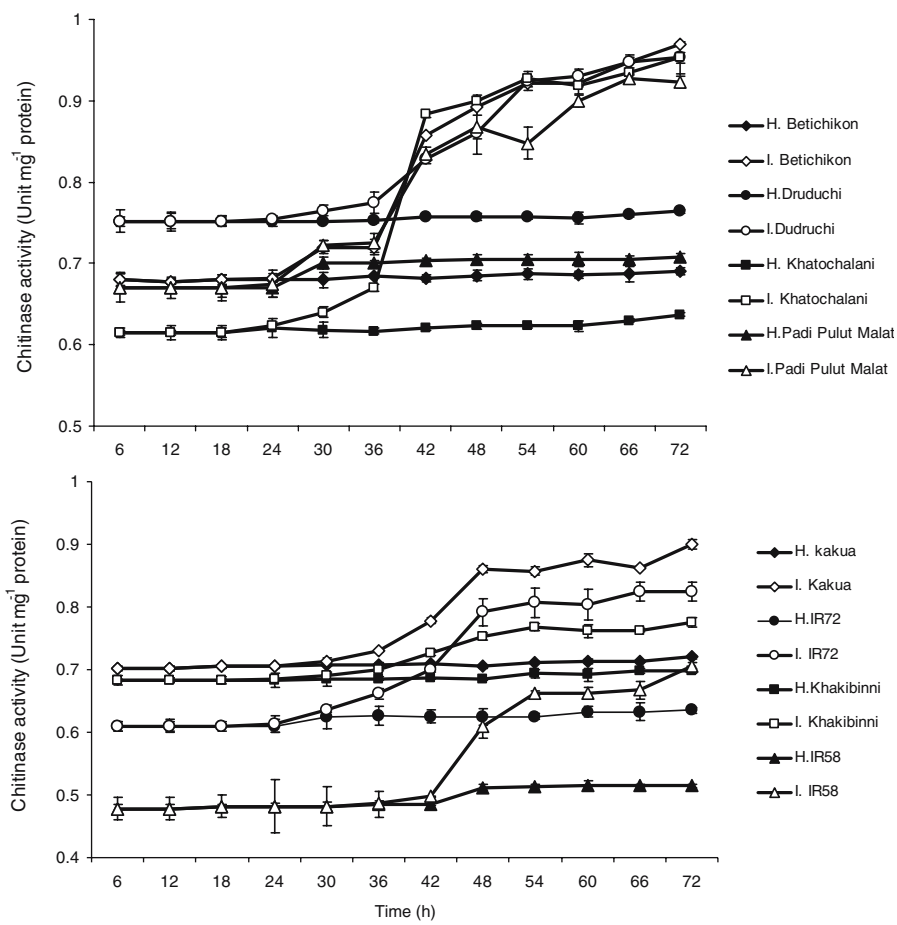

Fig. 3 Induction of chitinase activity at 6-h intervals in healthy (*H*) and infected (*I*) rice cultivars inoculated with *R. solani*. The *closed symbols* represent chitinase activities of healthy plants and *open symbols* represent chitinase activities of inoculated plants with *R. solani* (mean of two experiments). *Vertical bars* represent standard error of the mean

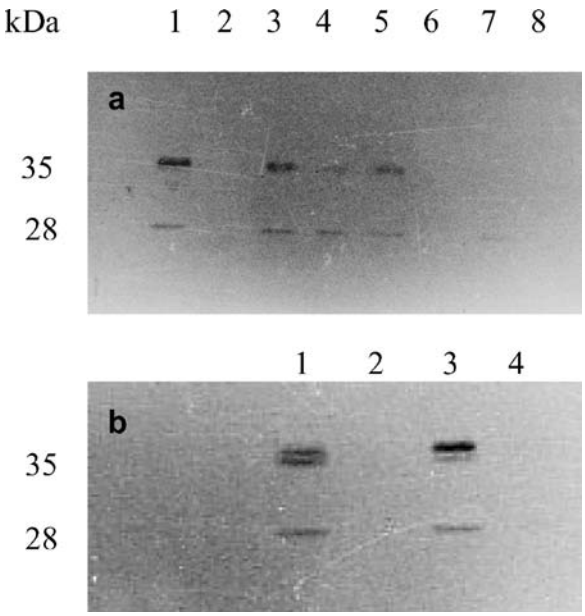

Fig. 4 Western blot analysis of chitinase induction in healthy and infected rice cultivars 72 h after inoculation with *R. solani*: **a** *Lanes 1*, and *2*, Dudruchi infected and healthy; *lanes 3*, and *4*, Khatochalani infected and healthy; *lanes 5* and *6*, IR72 infected and healthy; *lanes 7*, and *8*, IR58 infected and healthy; **b** *Lanes 1* and *2*, Betichikon infected and healthy, *lanes 3* and *4* Kakua infected and healthy. The sizes of the marker are on the *left*

Table 1 Number of infection cushions on detached leaves and sheaths of rice plants 72 h after inoculation with *R. solani*

Cultivars	No. of infection cushions cm^{-2} (72 h after inoculation)		
	Leaf[a]	Sheath[b]	Average
Betichikon	139.9 a[c]	139.0 a	139.1 a
Dudruchi	150.9 a	165.2 b	158.1 b
Khatochalani	170.7 b	179.1 b	171.9 c
Padi PulutMalat	219.3 c	225.1 c	222.2 d
Kakua	335.9 d	332.4 d	331.0 e
IR 72	376.1 e	369.6 e	372.9 f
Khakibinni	372.1 e	375.9 e	374.0 f
R 58	578.4 f	494.5 f	536.3 g
Mean	292.9	285.0	

[a] No. of infection cushions cm^{-2} leaf 72 h after inoculation

[b] No. of infection cushions cm^{-2} sheath 72 h after inoculation

[c] Means followed by common letters are not significantly different at 5% level by DMRT

Relationship between chitinase activity, infection cushion formation and % RLH

Percent RLH and chitinase activity were negatively correlated (R^2=0.931; Fig. 5a). Chitinase activity and the number of infection cushions formed were also negatively correlated (R^2=0.922) indicating that the causal relationship between sheath blight disease resistance was correlated with an increase in chitinase activity (Fig. 5b).

Discussion

Although there were differences in the levels and time-course of chitinase induction among the different rice cultivars evaluated, chitinase activity was significantly elevated in most infected plants as compared to their healthy controls. The moderately resistant cultivars showed an increase in activity within 24 h after inoculation whereas the susceptible cv. IR58 exhibited an increase in chitinase activity only 36 h following inoculation. Similar observations have been reported previously in other plants demonstrating that chitinase activity in resistant cultivars was higher and accumulated earlier than in susceptible cultivars (Rasmussen et al. 1992; Ignatius et al. 1994; Neha et al. 1994). In rice, Anuratha et al. (1996) detected more isozymes and higher concentrations of chitinases in moderately resistant cultivars compared to a susceptible cultivar

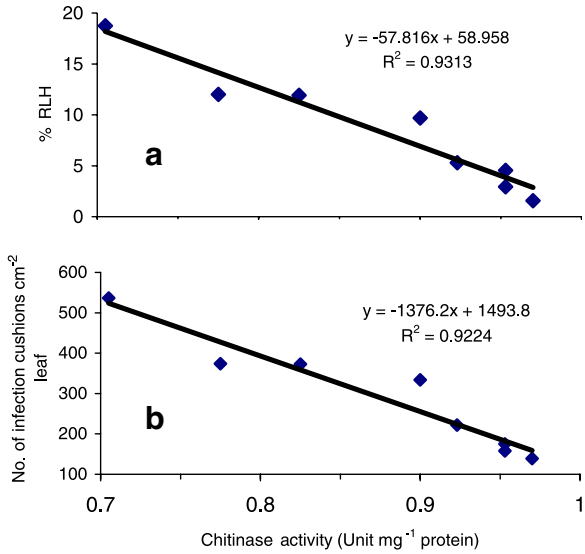

Fig. 5 Relationship between chitinase activity and **a** % RLH, and **b** number of infection cushions formed, in eight rice cultivars representing the four different groups (Mean of two experiments)

24 h after infection with *R. solani*. Two size classes of chitinases (28 and 35 kDa) were induced in rice (Anuratha et al. 1996; Nandakumar et al. 2001; Radiacommare et al. 2004), and these two classes of chitinases were also found in this study. In most rice cultivars tested in our study these size bands were not visible in healthy plant extracts, but were present in inoculated leaves. But in Khatochalani, we found a faint chitinase band in uninoculated plants. Even in these cultivars the chitinase levels increased further after *R. solani* infection. These observations suggest that chitinase activity in rice was induced after *R. solani* infection and that chitinase levels may contribute to the resistance of these cultivars to sheath blight. Similar kinds of results were observed when plants were inoculated with *R. solani* by Radiacommare et al. (2004). This is similar to the results observed in melon plants, where chitinase contributed to increased resistance of the plants against infection by *Colletotrichum lagenarium* (Roby et al. 1988). In the case of IR58, even though the induced chitinase activity was more in susceptible plants as compared to healthy plants, the expression of the 35 kDa chitinase in the Western blot was low. From this we can conclude that the expression of 35 kDa chitinase might be the main contributing factor to disease resistance.

Chitinase is constitutively present at low concentrations in non-inoculated plants, as observed in this experiment, in which faint bands of 28 and 35 kDa were detected in rice cv. Khatochalani. It is likely that chitinase is present in the other healthy rice cultivars but in such small amounts so as to be undetectable. It is likely that plants are subjected to low levels of stress under most types of growth conditions and the response of different cultivars to low pathogen stress levels varies. Low levels of chitinase expression in most healthy plants indicates the potential of these rice cultivars as possible materials for genetic transformation for enhancing the level of expression of both constitutive and induced chitinases. With the rapid advancement in genetic transformation, any resistance of these cultivars to sheath blight infection might be improved by regulation of chitinase gene expression and other genes controlling host resistance (Broglie et al. 1991; Vierheilig et al. 1993; Datta et al. 1999, 2000, 2001; Kumar et al. 2003; Tabai et al. 1998). Latest studies showed constitutive expression of the chitinase alone might not have contributed to increased sheath blight disease resistance. In addition to chitinase, several other defence genes are also triggered (break-down products of chitin released from fungal cell walls as a result of the action of chitinase) and might have also contributed to sheath blight resistance (Sareena et al. 2006).

Other reports have shown that chitinases and β-1,3-glucanases show a parallel increase after pathogen attack and that they act synergistically in enhancing resistance (Roberts and Selitrennikoff 1988; Mauch et al. 1992; Vierheilig et al. 1993). These enzymes target different structures in the cell walls of the fungal pathogen and greater protection was observed in hybrid transgenic tobacco plants expressing both basic chitinase and acidic glucanase genes than in the lines expressing either transgene alone (Benhamou et al. 1990; Zhu et al. 1994). Assays for β-1,3-glucanase were not done in this study and it is uncertain whether the induced chitinase alone is sufficient to account for the reduction in *R. solani* infection of inoculated rice plants. However, cytochemical investigations of chitin distribution using wheat germ agglutinin/ovomucoid-gold labelling following chitinase treatment revealed that chitinase alone is sufficient to cause lysis of *R. solani* hyphae and that the chitin at the hyphal tips of this fungus is readily accessible to chitinase attack (Benhamou et al. 1993).

The number of infection cushions formed 72 h after inoculation with *R. solani* was significantly different among the four groups of rice cultivars tested. Fewer infection cushions were formed in all three groups of moderately resistant rice cultivars than in the susceptible cv. IR58. The eight cultivars belonging to the four groups showed a negative correlation between chitinase activity and number of infection cushions as well as with sheath blight infection (%RLH). Testing different variables such as %RLH, constitutive chitinase level (chitinase of healthy plants), and induced chitinase level (chitinase of inoculated plants) using correspondence analysis, it was found that the medium to high levels of expression of induced chitinase was associated with the cultivars exhibiting medium and low disease. High levels of expression of induced chitinase was always associated with a low disease index. It is evident, therefore, that both constitutive and induced chitinase activity were associated with low to medium disease (C. L. Shrestha, unpublished data). Results presented here demonstrate that chitinase activity is associated with moderate resistance of rice cultivars against sheath blight.

In summary, by analyzing a large number of rice cultivars differing in sheath blight resistance, we were able to show that there are cultivar-specific differences between moderately resistant and susceptible rice cultivars in the timing and level of chitinase activity after *R solani* infection. The low level of constitutive expression of chitinases in rice cultivars indicates their potential as materials for genetic transformation by regulating the expression of hydrolase genes in these cultivars for enhanced resistance to sheath blight disease where no high level of resistance is known. Further, the induced expression of chitinase after *R. solani* infection would indicate a defence role of this enzyme in limiting the spread of sheath blight infection.

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