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Induction of β-1,3-glucanase in seedlings of pearl millet in response to infection by *Sclerospora graminicola*

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Abstract

Differential resistance of pearl millet cultivars to downy mildew disease was correlated with the levels of β-1,3-glucanase in their seeds. Higher activity of the enzyme in highly resistant cultivars and lower activity in the highly susceptible ones suggested the possible use of β-1,3-glucanase as a biochemical marker for screening pearl millet cultivars for downy mildew disease. Inoculation of seedlings with the downy mildew pathogen *Sclerospora graminicola* resulted in increased enzyme levels in resistant cultivars. Mesocotyl and shoot regions of seedlings recorded higher levels of enzyme than the root. Isoelectric focusing revealed four basic isoforms with pI 9.6, 9.0, 8.9 and 8.2 and two acidic isoforms with pI 4.9 and 6.2 of β-1,3-glucanase in pearl millet. The pI 9.6 isoform was a major isoform of the enzyme in the pearl millet seedlings with a probable developmental function. Isoforms pI 6.2 and pI 8.2 appeared to be involved in resistance and pI 4.9 isoform seemed to be involved in pathogenesis of pearl millet-downy mildew.

Introduction

Sclerospora graminicola (Sacc.) Schroet., an obligate oomycetous fungus causes the downy mildew disease of pearl millet (*Pennisetum glaucum* (L.) R. Br.) and is the major constraint in the production of pearl millet. Even though many cultivars are resistant to the disease, the resistance is not durable and often there is breakdown of resistance in the cultivars (Ball, 1983). The exact reasons for the breakdown of resistance are not known as there is a lack of complete understanding of the biochemical basis of resistance of pearl millet to downy mildew. It is anticipated that a better understanding of the physiological and molecular mechanisms that control resistance can contribute greatly to the improvement of resistance.

Development of defense responses in plants is complex. It involves both structural and biochemical barriers (Paxton and Groth, 1994; Walton, 1994). Though structural barriers are significant, synthesis of new proteins that have direct or indirect action on the course of pathogenesis have the major effect on plant resistance to a given pathogen. These proteins include a heterogeneous group of proteins collectively defined as pathogenesis-related (PR) proteins (Van Loon, 1985). Among these PR proteins, hydrolases such as β-1,3-glucanases and chitinases have been suggested to be involved in plant resistance against fungal pathogens (Boller, 1985; Joosten and De Wit, 1989; Kombrink et al., 1988; Pan et al., 1991; Kim and Hwang, 1994; Lozoyava et al., 1998). These enzymes, developmentally regulated, are induced by pathogens or exogenous chemicals (Simmons, 1994; Cote et al., 1991; Boller, 1985), inhibit fungal growth (Mauch et al., 1988) and release oligosaccharide elicitors inducing the production of phytoalexins (Yoshikawa et al., 1993; Takeuchi et al., 1990). However, in pearl millet–downy mildew interactions

studies on plant defense responses, especially antifungal enzyme activities are limited. This paper reports the changes occurring in the activities of β-1,3-glucanase and expression of its isoforms in susceptible and resistant pearl millet cultivars in response to fungal infection.

Materials and methods

Seed samples

Seeds of 15 different cultivars of pearl millet used in the study were obtained from International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Hyderabad, India and All India Coordinated Pearl Millet Improvement Programme (AICPMIP), Pune, India.

Pathogen

S. graminicola pathotype 1 was maintained on its susceptible host (HB3 cultivar of pearl millet) under greenhouse conditions.

Screening of pearl millet cultivars for downy mildew reaction

Cultivar resistance of pearl millet for downy mildew reaction was screened in the downy mildew sick plot of the Department of Studies in Applied Botany, University of Mysore, Mysore, India. Seeds were sown in downy mildew nurseries containing heavy loads of soil-borne oospores and sporangial inoculation provided from infector rows (Williams et al., 1981). The test entries were sown in a randomized block design with three replicates. Normal agronomic practices were followed to raise the crop. Evaluation of cultivar resistance to downy mildew was carried out by recording disease incidence 30 days after sowing and also at the dough stage (60 days). Plants were rated as diseased when they showed any of the typical symptoms of downy mildew, i.e. stunting, chlorosis, downy growth of asexual spores on the under surface of the leaves and malformed earheads. Percentage disease incidence was rated from the number of systemically-infected plants.

Inoculation of the plant material

The seeds were surface sterilized in 0.1% (v/v) sodium hypochlorite solution for 15 min, washed thoroughly with sterile distilled water and germinated on moist filter paper under aseptic conditions at $25 \pm 1\,^{\circ}$C in dark for 3 days. The three-day-old seedlings were root-dip inoculated with 4×10^4 zoospores ml^{-1} suspension of *S. graminicola* (Safeeulla, 1976). Seedlings dipped in sterile distilled water served as control. The seedlings were harvested at different time intervals (1, 6, 12, 24, 48 and 72 h) after inoculation and used for enzyme extraction. For studying the enzyme activities in different parts of the seedlings, inoculated and control seedlings were harvested 24 h after inoculation and separated into roots, shoots and mesocotyl regions. For enzyme extraction, 0.5 g of tissue was used.

Protein preparation

For enzyme studies, crude protein extracts were prepared in buffer. Seeds (0.5 g) imbibed in distilled water or 1 g seedlings were homogenized in 0.05 M sodium acetate buffer (pH 5.2, 1 ml g^{-1} fresh weight) using acid-washed glass beads and a pre-chilled pestle and mortar at $4\,^{\circ}$C. The homogenate was centrifuged at 10 000 g for 20 min at $4\,^{\circ}$C (Himac Centrifuge, HITACHI) and the supernatant was used as crude extract.

Protein estimation

Protein content in extracts was estimated by the dye binding method (Bradford, 1976) using bovine serum albumin (Sigma) as a standard.

β-1,3-Glucanase assay

β-1,3-Glucanase was assayed with glucose as a standard (Isaac and Gokhale, 1982). Laminarin (Sigma; 0.1%) in 0.05 M sodium acetate buffer (pH 5.2) was used as the substrate. Products released after incubation were estimated for reducing groups at 540 nm using the dinitrosalicylic acid reagent. Enzyme activity was expressed in terms of µmol min^{-1} mg^{-1} protein. Each experiment was repeated three times taking three replicates at a time.

Statistical analysis

Incidence of downy mildew disease of all cultivars in the field and β-1,3-glucanase activities determined in seed were compared by Fischer's least significant difference test.

Isolectric focusing

Total proteins (crude extracts) from whole pearl millet seedlings, inoculated with *S. graminicola* and harvested 24 h after inoculation, were subjected to isoelectric focusing (IEF) on a 1.5 mm 7.5% polyacrylamide gel containing 2% ampholyte (pH 3–10, Sigma) using a Multiphor II (LKB) system according to the manufacturer's protocol. The pI markers (Sigma), ranging from pI 3.6–9.3 were co-electrophoresed to estimate the pI of the proteins. Fifty microgram protein equivalent was loaded at the centre of the horizontal gel maintained at 2 °C. IEF was performed at 2 °C for 3 h increasing the voltage stepwise (200 V for 30 min, 400 V for 30 min, 600 V for 30 min, 800 V for 30 min, and 1000 V for 1 h).

After IEF gels were washed and incubated at 40 °C for 30 min in 0.67% (w/v) laminarin in 0.05 M sodium acetate buffer pH 5.2. β-1,3-Glucanase was detected using the method described by Pan et al. (1989) as modified by Wyatt et al. (1991). The gels were analysed using Bioprofile Image Analysis System (Vilber Lourmat, France). pI of the bands were calculated using the inbuilt software of the Image Analysis System.

Results

Resistance of pearl millet cultivars to downy mildew disease

Differential downy mildew disease incidence in pearl millet cultivars, recorded in the field screening experiment under artificial epiphytotic conditions with infector rows of the pathogen, confirmed the occurrence of resistance variation among cultivars. The cultivars IP18292, IP18293, IP18294, IP18295, IP18296, IP18297 did not exhibit any downy mildew symptom expression despite exposure to continuous supply of inoculum for over 60 days. Maximum disease of over 90% was observed in cultivars HB3, NHB3 and 7042(S)-1 while other cultivars showed intermediate disease reactions. Based on the reaction in the field, pearl millet cultivars were categorised as highly resistant (HR) with 0% disease incidence (IP18296, IP18294, IP18297, IP18293, IP18292, IP18295), resistant (R) with 1–10% incidence (7042R, P310-17), susceptible (S) with 11–25% incidence (P7-4R) and highly susceptible (HS) with > 25% disease incidence (7042S, 5141B, 23D_2B, 7042(S)-1, NHB3, HB3).

β-1,3-Glucanase activities in seeds

Among the seeds tested, those of pearl millet cultivars with resistance to downy mildew showed higher activities of β-1,3-glucanase, while lower activities were recorded in susceptible cultivars (Table 1). Highest activity was recorded in the seeds of highly resistant cultivar IP18296 and lowest activity in that of highly susceptible cultivar HB3.

β-1,3-Glucanase in seedlings of pearl millet

Activities of β-1,3-glucanase in the seedlings of IP18296 (highly resistant) and HB3 (highly susceptible) cultivars of pearl millet studied at different time intervals are shown in Figure 1. At the time of inoculation, β-1,3-glucanase activity in IP18296 seedlings was about seven times higher than the activity in HB3 seedlings. After inoculation, the activity in the former increased by about 27% compared to uninoculated control and reached a peak at 24 h. In HB3, β-1,3-glucanase activity was lower in the inoculated seedlings than in uninoculated controls. After 24 h, enzyme activity started to decline gradually and remained stable at a higher level in IP18296, while in the cultivar HB3, there was a further decrease. The activity trends of β-1,3-glucanase in other resistant and susceptible cultivars were similar (data not shown).

When different parts of the seedlings were analyzed for β-1,3-glucanase, mesocotyl and shoot regions of the IP18296 seedlings showed higher activity while root had the least activity (Figure 2). In cultivar HB3, the enzyme was not detected in root while mesocotyl and shoot recorded only low activities of the enzyme.

β-1,3-Glucanase isoforms

Isoelectric focusing of extracts from resistant (IP18296) and susceptible (HB3) seedlings of pearl millet showed the presence of six isoforms of β-1,3-glucanase (Figure 3). Of these, four were basic and two were acidic isoforms. The basic isoform (pI 9.6) was the major isoform present in inoculated and control samples of both resistant and susceptible cultivars. Isoforms of pI 9.0 and 8.9 were found only in susceptible control samples and not in susceptible inoculated or resistant samples. pI 8.2 isoform was observed in resistant samples induced after inoculation, while

Table 1. β-1,3-Glucanase activity in seeds of different cultivars of pearl millet

Sl. no.	Cultivar	Mean downy mildew incidence in field	Grouping based on field screening	β-1,3-Glucanase activity* (μmol min^{-1} mg^{-1} protein) $\pm$ SD values
1	HB3	94[a]	Highly susceptible (HS)	0.355 ± 0.006^{m}
2	NHB3	94[a]	HS	0.412 ± 0.012^{l}
3	7042(S)-1	94[a]	HS	0.422 ± 0.012^{k}
4	23D2B	90[a]	HS	0.422 ± 0.012^{k}
5	5141B	70[b]	HS	0.467 ± 0.020^{j}
6	7042S	70[b]	HS	0.488 ± 0.022^{i}
7	P74R	25[c]	Susceptible (S)	0.554 ± 0.026^{h}
8	P310-17	10[d]	Resistant (R)	0.600 ± 0.020^{g}
9	7042R	5[e]	R	0.621 ± 0.028^{f}
10	IP18294	0[f]	Highly resistant (HR)	0.689 ± 0.028^{e}
11	IP18297	0[f]	HR	0.710 ± 0.030^{d}
12	IP18293	0[f]	HR	0.710 ± 0.032^{d}
13	IP18292	0[f]	HR	0.777 ± 0.042^{c}
14	IP18295	0[f]	HR	0.800 ± 0.040^{b}
15	IP18296	0[f]	HR	0.887 ± 0.032^{a}

*Average of three independent experiments each with three replicates. The values in the column followed by same letter(s) are not significantly different from each other according to Fischer's least significant difference test ($P = 0.05$).

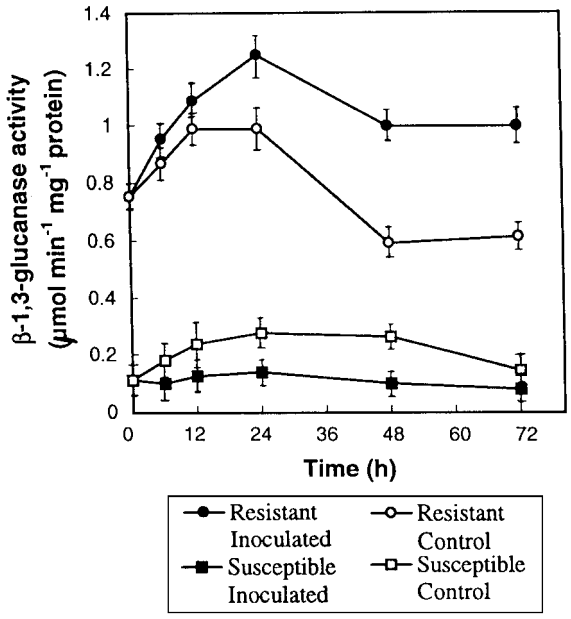

Figure 1. Changes in β-1,3-glucanase activity in seedlings of pearl millet cultivars resistant (IP18296) and susceptible (HB3) to downy mildew as a response to inoculation with *Sclerospora graminicola*. The data are means of three independent experiments. Bars indicate $\pm$ SE.

the acidic isoform of pI 4.9 was highly induced in susceptible cultivar after inoculation. Another acidic isoform (pI 6.2) which was unique to resistant cultivar was highly induced after inoculation.

Discussion

Constitutive hydrolases and peroxidases have been associated with resistance reaction in various plants (Reuveni and Karchi, 1987; Reuveni et al., 1991; Ahl Goy et al., 1992; Mozzetti et al., 1995). The results of the present study substantiate this in pearl millet downy mildew interactions. The variation in cultivar resistance studied by field screening correlated with the activities of β-1,3-glucanase in the seeds, with high activity in highly resistant cultivars and low in highly susceptible cultivars.

The higher activity of β-1,3-glucanase detected in seeds of resistant cultivars was also found in seedlings. Increase in enzyme activity in the resistant cultivars after inoculation and the activity reaching a peak at 24 h of inoculation suggested a role for this enzyme in the expression of effective resistance by pearl millet seedlings to *S. graminicola* invasion since in downy mildew disease, maximum infection of the host by

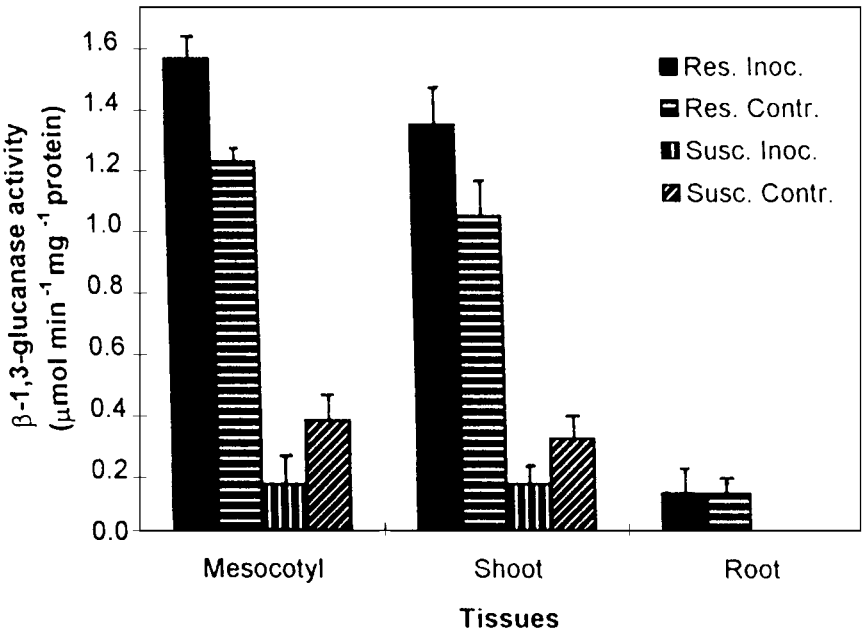

Figure 2. β-1,3-Glucanase activity in different tissues of seedlings of pearl millet cultivars resistant (IP18296) and susceptible (HB3) to downy mildew as a response to inoculation with *Sclerospora graminicola* (24 h after inoculation). The data are means of three independent experiments. Bars indicate ± SE.

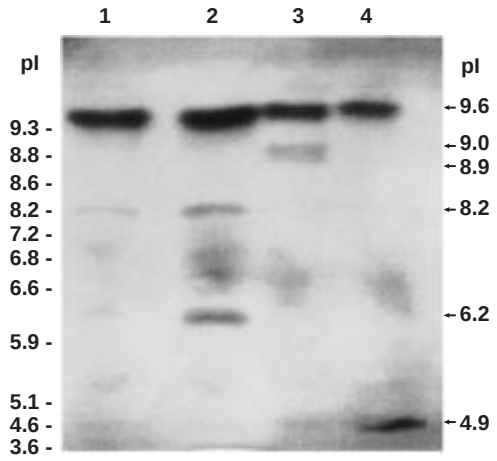

Figure 3. Induction of β-1,3-glucanase isoforms in pearl millet seedlings upon *S. graminicola* infection. Total proteins (50 μg) from resistant (IP18296) and susceptible (HB3) seedlings inoculated and harvested 24 h after inoculation were electrophoresed on IEF gel and stained for β-1,3-glucanase activity. Lane 1: resistant control; Lane 2: resistant inoculated; Lane 3: susceptible control; Lane 4: susceptible inoculated.

the fungus is achieved within 24 h of inoculation (Subramanya et al., 1983). Similar increases in activity and accumulation of β-1,3-glucanase and chitinase enzymes in incompatible interactions of maize, pepper, barley and wheat with pathogenic fungi have been reported (Cordero et al., 1994; Kim and Hwang, 1994; Roulin et al., 1997; Caruso et al., 1999), suggesting a role for these enzymes in determining resistance against fungal pathogens. Reduction in the activity of β-1,3-glucanase after inoculation of the host by the pathogen in susceptible cultivars probably indicates pathogen inactivation of the enzyme and/or reduced host protein synthesis due to pathogen colonization as evidenced by differential induction of the enzyme isoforms. Earlier studies on lipoxygenase and phenylalanine ammonia lyase enzymes in pearl millet downy mildew system have recorded decreased enzyme activity in susceptible cultivars and increased activity in resistant cultivars after fungal inoculation (Nagarathna et al., 1992; 1993)

Tissue-specific expression of β-1,3-glucanase is also known (Cordero et al., 1994; Cabello et al., 1994; Yi and Hwang 1996; Cachinero et al., 1996) and the present study confirms this in pearl millet-downy mildew host–pathogen interaction. In downy mildew disease of pearl millet, though the root and

coleoptile are preferred sites for infection by zoospores of the pathogen (Subramanya et al., 1983), most of the pathogen is localised in the mesocotyl and shoot regions of the susceptible seedlings while the resistant seedlings did not show any colonization by the pathogen (Sharada et al., 1995). Hence, higher activity of β-1,3-glucanase in the shoot and mesocotyl regions of the resistant seedlings recorded in the present study further suggests their role in defense against the invading pathogen. It is likely that β-1,3-glucanases offer protection against pathogens through their capacity to hydrolyse the branched ($1\rightarrow3$, $1\rightarrow6$) β-glucans that are commonly found in fungal cell walls. This suggestion is supported by *in vitro* experiments in which erosion of fungal walls caused by β-1,3-glucanases led to lysis of the hyphal tips especially when β-1,3-glucanases acted in concert with chitinases (Mauch et al., 1988; Ji and Kuć, 1996; Kim and Hwang, 1997). It is proposed that the resistance reaction is also due to the release of β-1,3-glucan and chitin oligomers by β-1,3-glucanases and chitinases that act as elicitors responsible for invoking general resistance in plants (Takeuchi et al., 1990; Yoshikawa et al., 1993; Ebel and Mithofer, 1998).

Isoelectric focusing studies revealed differential expression of β-1,3-glucanase isoforms during pathogenesis of *S. graminicola*. Not all the isoforms of β-1,3-glucanases identified in the study were associated with resistance of pearl millet to *S. graminicola* infection. The basic isoform of pI 9.6 appeared to have a developmental role since it was present in both resistant and susceptible cultivars of pearl millet before and after inoculation. The constitutive or developmental-regulated expression of β-1,3-glucanases are reported in many plants (Cote et al., 1991; Caruso et al., 1999) and are thought to be involved in seed germination process. The pI 8.2 isoform was probably involved in the expression of resistance since, unlike the other isoforms, the concentration of this isoform increased substantially in the resistant seedlings after inoculation with the pathogen and was not observed in susceptible seedlings. Presence of an acidic isoform of the enzyme (pI 6.2) only in the resistant cultivars was identified in the study. Probably, this isozyme is also involved in determining resistance of pearl millet against downy mildew pathogen *S. graminicola* since in the incompatible reaction this isoenzyme was induced many fold. The higher level of isoform pI 9.6 and the presence of pI 8.2 and pI 6.2 isoforms might be responsible for the higher basal level of β-1,3-glucanase activity in the resistant seedlings than in the susceptible ones. Higher induced expression of pI 8.2 and pI 6.2 isoforms due to inoculation with the pathogen may account for the increased β-1,3-glucanase activity in resistant seedlings after inoculation. The appearance of an acidic isoform of β-1,3-glucanase (pI 4.9) in pathogenesis was apparent in this study as it was induced only in the compatible interaction. This isoform seemed to be selectively induced during pathogenesis at the expense of the two basic isoforms of pI 8.9 and 9.0 that were present in the uninoculated susceptible seedlings. Similar reports on the induction of acidic and basic isoforms during infection are also available in a number of plant pathogen interactions (Linthorst, 1991; Simmons, 1994; Yi and Hwang, 1996; Caruso et al., 1999).

These studies on β-1,3-glucanases in pearl millet-downy mildew host–pathogen interactions suggest involvement of β-1,3-glucanase in resistance of pearl millet to the downy mildew pathogen. Differential expression of the enzyme in resistant and susceptible pearl millet cultivars and induction of different isoforms of the enzyme during infection indicate the possible use of β-1,3-glucanase enzyme activity as a biochemical marker for screening pearl millet cultivars against downy mildew.

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