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Original Article

Polygalacturonase-inhibitor proteins in pearl millet: possible involvement in resistance against downy mildew

S. Ashok Prabhu[1], K. Ramachandra Kini[1]*, S. Niranjan Raj[1], Bruno M. Moerschbacher[2], and H. S. Shetty[1]

[1]Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore-570006, Karnataka, India
[2]Institut für Biochemie und Biotechnologie der Pflanzen, Westfälische Wilhelms-Universität Münster, Hindenburgplatz 55, D-48143 Münster, Germany
*Correspondence address. Tel: +91-821-2419882; Fax: +91-821-2414450; E-mail: krk@appbot.uni-mysore.ac.in

Polygalacturonase-inhibitor protein (PGIP) is a defense protein found in plant cell walls. It prevents the degradation of pectin by modulating the endo-polygalacturonase activity. The present study has used heterologous anti-bean PGIP probes to investigate the role of PGIP in pearl millet [*Pennisetum glaucum* (L) R. Br.] resistance against downy mildew caused by oomycete pathogen *Sclerospora graminicola* (Sacc.) Schroet. Northern blot analysis using bean *pgip2* DNA fragment as probe showed an early and marked induction of transcripts (∼1.2 kb) upon pathogen-inoculation in pearl millet cultivar resistant to downy mildew, with the maximum level observed at 24 and 48 h post-inoculation (h.p.i.). Western blot analysis of pearl millet total cell wall proteins using antibodies against bean PGIP showed the presence of a major band of ∼43 kDa, and several minor ones. The protein accumulation was higher in resistant seedlings than in susceptible seedlings with a differential expression observed only in the case of incompatible interaction. Immunocytochemical localization in epidermal peelings of coleoptiles and tissue-printing showed a similar trend in the PGIP accumulation. PGIP was found to localize in the epidermal as well as in the vascular regions of tissues. Higher accumulation was observed in the stomatal guard cells of resistant cultivar inoculated with the pathogen. PGIP activity of pearl millet total protein extracts when assayed against *Aspergillus niger* PG displayed differential PG inhibitory activities between the resistant and suceptible cultivars with resistant sample showing the highest inhibition of 16%, post-pathogen treatment. Thus, PGIP appeared to be an important player in pearl millet–*S. graminicola* interaction leading to host resistance.

Keywords polygalacturonase-inhibitor protein (PGIP); endo-polygalacturonase; pearl millet; downy mildew; transcript accumulation; tissue-printing

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Introduction

Cell walls act as the first line of defense that plant pathogens need to overcome in order to colonize the plant tissue for their nutritional requirements. Endo-polygalacturonases (EPG, E.C. 3.2.1.15) are typically the first enzymes secreted by invading pathogens leading to degradation of polygalacturonic acid component of pectin, a complex polysaccharide found in the middle lamella and primary cell wall of higher plants [1]. Polygalacturonase-inhibitor proteins (PGIPs) were first reported by the observation that plant cell wall proteins of bean cv. Red Kidney inhibited polygalacturonase activity [2]. PGIPs are cell wall glycoproteins involved in plant defense against the invading pathogens by inhibiting/modulating the activity of EPGs [3]. The dicot PGIPs have been shown to be inhibitory against EPGs of microbial origin and not against the plant EPGs [4]. The leucine-rich repeats of PGIPs have the consensus sequence—LxxLxLxxNxLT/SGxIPxxLxxLxx, with a β-strand/β-turn motif which interacts with EPGs [5]. Modulation of EPG activity by the host PGIPs leads to the accumulation of elicitor active oligo-galacturonides and is responsible for an array of host defense responses [6].

Although dicot PGIPs have been studied extensively, scientists were skeptical regarding the presence of EPG inhibitors in monocots due to very low pectin content in their cell walls [7,8]. They were proved otherwise by isolation of PGIPs from monocots such as *Allium cepa* [9] and *Allium porrum* [10], though relatively rich in pectin. However, subsequently PGIPs have been isolated from pectin-poor poaceous monocots such as wheat [11] and recently in oil-palm [12].

Poaceous crops suffer substantial yield and quality reductions due to several diseases as is the case with most agronomic crops. Pearl millet [*Pennisetum glaucum* (L.) R. Br.] is an important crop of semi-arid tropics, which serves as a staple food for the poor parts of Asia and Africa. Downy mildew caused by oomycete pathogen

Sclerospora graminicola (Sacc.) Schroet is a very important disease affecting the production of pearl millet. Under favorable environmental conditions for pathogen, this disease can spread rapidly causing as much as 40% crop loss [13]. In 2002, an EPG sequence, *pipg1*, was reported for the first time from *Phytophthora infestans*, an oomycete. The oomycetes are a unique group of eukaryotic plant pathogens more closely related to brown algae and exhibits fungal-like filamentous growth, but shares little taxonomic affinity to fungi [14]. *Pipg1* was shown to be expressed during both pre-infection and infection stages and was significantly more similar to fungal EPGs than to plant or bacterial ones. This unexpected similarity between *pipg1*and fungal EPGs, the isolation of a PGIP from poaceous monocots, and the lack of any previous literature on the study of PGIPs in millets triggered our interests into investigating a possible role for PGIP in pearl millet–*S. graminicola* interaction. The present study involves identification of PGIPs from pearl millet using DNA and antibody probes generated against bean PGIPs.

Materials and Methods

Plant material

Pearl millet cultivars 7042S (highly susceptible, HS) with >25% downy mildew disease incidence (DMDI) and IP18296 (highly resistant, HR) with 0% DMDI after inoculation with *S. graminicola* under field conditions were used in this study. The seeds were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India.

Pathogen and preparation of inoculum

S. graminicola isolated from pearl millet cv. HB3 and maintained on the same cultivar under greenhouse conditions was used for all inoculation experiments. Leaves of infected plants showing symptoms of downy mildew were collected in evening, washed in running tap water to remove the remnants of previous sporulation, blotted dry, cut to pieces about 4 inches in length, and placed in a moist chamber for sporulation. Fresh sporangia were collected in the next morning and zoospores released by them were used as inoculum [15].

Inoculation of plant material

The seeds of pearl millet cultivars IP18296 and 7042S were surface sterilized in 0.1% sodium hypochlorite solution for 15 min, washed thoroughly with sterile distilled water, and germinated on moist filter paper under aseptic conditions at $25 \pm 2^{\circ}C$ in dark for 2 days. The 2-day-old seedlings were root-dip inoculated with 4×10^4 zoospores/ml *S. graminicola* [16]. The seedlings were harvested at time intervals of 0, 3, 6, 9, 12, 24, and 48 h post-inoculation (h.p.i.) and coleoptiles were stored at $-20^{\circ}C$ until further use. Suitable un-inoculated water-treated controls were maintained in parallel.

Northern blot analysis

Total RNA was extracted from harvested pearl millet seedlings by the phenol–chloroform method as described previously [17]. RNA (10 μg) was denatured, separated by electrophoresis, transferred to Hybond-N1 membrane (Amersham Pharmacia, Piscataway, USA) in 20× saline-sodium citrate (SSC) (3 M NaCl, 0.3 M sodium citrate), and fixed onto the membrane by baking at 80°C for 90 min. RNA gel blots were pre-hybridized in a solution containing 0.25 M sodium phosphate (pH 7.2), 0.25 M sodium chloride, 7% sodium dodecyl sulfate (SDS, w/v), and 1 mM ethylenediaminetetraacetic acid at 65°C for 3 h. The blots were hybridized with 5 μCi α^{32}P-labeled bean *pgip2* DNA fragment (797 bp, pAD2 clone) as probe (kind gift from Renato D'Ovidio, Universita' della Tuscia, Viterbo, Italy) in the same solution overnight at 55°C. The membranes were washed twice for 20 min each at 55°C in 0.2% SSC and 0.1% SDS (w/v). The hybridized blots were exposed to Phosphorimager plates for 2–3 h and scanned with Multifunctional Image Analysis System (FLA 5000; FujiFilm, Tokyo, Japan).

Pearl millet total protein extraction

Total protein was extracted from the 24-h.p.i. pearl millet seedlings as well as their respective controls of both cultivars using the modified method [18]. All steps were carried out at 4°C. Briefly, 10 g seedlings were homogenized in 2 volumes of cold acetone and centrifuged at 12,000 r.p.m. for 30 min. The pellet was washed twice with cold acetone, air-dried completely, and resuspended in 2 volumes of sodium acetate buffer (20 mM, pH 5 containing 1 M NaCl). It was kept at 4°C for 72 h on a shaker. It was finally centrifuged at 12,000 r.p.m. for 30 min and dialyzed against sodium acetate buffer (20 mM, pH 5), lyophilized, and reconstituted appropriately. The protein extracts were suitably fortified with protease inhibitor cocktail (Sigma, St Louis, USA) during protein extraction. The protein content was estimated by the method described previously [19].

Western blot analysis

Total proteins (50 μg) were separated by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method described previously [20] in a 1 mm-thick, 12% separating polyacrylamide gel under reducing conditions. Immediately after electrophoresis the proteins were electro-transferred onto a polyvinyl difluoride (PVDF) membrane using Multiphor II (LKB, Pharmacia, Uppsala, Sweden) electrophoretic transfer apparatus according to the manufacturer's protocol. The membrane was blocked in

5% (w/v) blotting-grade milk powder in Tris-buffered saline (TBS: 10 mM Tris HCl, pH 8.0, 150 mM NaCl) overnight followed by washing with TBS buffer containing 0.05% Tween-20 (TBST), 3 times for 5 min each. The blot was incubated with the primary antibody (PGIP-11) (1 : 500 dilution in TBST, kind gift from Gabre Kemp, University of Free State, Bloemfontein, South Africa) generated in rabbit against a peptide corresponding to N-terminal residues 10–21 of *Phaseolus vulgaris* PGIP [21] for 2 h at 37°C. After being washed with TBST, 5 times for 5 min each, the blot was treated with secondary antibody, goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugate (1 : 1000 dilution in TBST) for 1 h at 37°C. After washing with TBST, the blot was stained for peroxidase with 3,3′-diaminobenzedine (DAB) and hydrogen peroxide.

Tissue-print and immunoblot analysis

Tissue blot immuno-assay was carried out as described previously [22] with slight modifications. Briefly, 2-day-old pearl millet seedlings (harvested 24 h.p.i.) were cross-sectioned at the coleoptile region with a sharp razor blade. The cut area was lightly dried onto a tissue paper and blotted onto Nitro ME nitrocellulose membranes (Micron Separations Inc., Westboro, USA) with consistent normal thumb pressure for 15 s each. The blotted membranes were air-dried at room temperature and were blocked with 5% (w/v) blotting-grade milk powder in phosphate-buffered saline (PBS) (0.14 M NaCl, 1.0 mM potassium phosphate, 8.0 mM sodium phosphate, and 2.5 mM KCl at pH 7.5) for 1 h. Membranes were washed three times, 5 min each in PBS with 0.5% Tween (PBST), and treated with primary antibody (PGIP-11, 1 : 500) for 1 h. After washing with PBST the membranes were incubated in 1 : 1000 dilution of goat anti-rabbit IgG (Merck Biosciences, Bangalore, India) conjugated to HRP for 1 h. The membranes were developed using DAB and H_2O_2. After air-drying at room temperature they were observed under a stereo binocular microscope (Leica MS5, Wetzlar, Germany) with low magnification. The images were captured with a digital camera (Canon MIC power shot S50) attached to the stereomicroscope.

Immuno-staining of coleoptile-epidermal peelings

Experiments were carried out according to the protocols described previously with modification [23,24]. Briefly, epidermal peelings, both control and inoculated coleoptiles of 2-day-old pearl millet seedlings harvested at 24 h.p.i., were fixed, decolorized in 95% (v/v) ethanol, and submerged in 1% SDS for 24 h at 80°C. Peelings were then subjected to immuno-labeling with PGIP-11 antibody. For this, the peelings were blocked with 3% bovine serum albumin in TBST for 1 h. They were then treated with primary antibody (PGIP-11, 1 : 500) for 1 h and washed three times with TBST. The peelings were later incubated with goat anti-rabbit IgG HRP-conjugate (Merck Biosciences, Bangalore, India), and washed three times in TBST. The color reactions were developed using DAB and H_2O_2. Areas of cell wall localization of PGIP were visualized under a compound microscope (Wild Leitz, Wetzlar, Germany) at a magnification of ×100 and images were captured with a digital camera (Canon power shot S50) attached to the microscope.

PGIP activity assay

The PG activity was determined by measuring reducing end-groups released from polygalacturonic acid (HiMedia). A 500 μl reaction mixture containing 200 μl of 2.5 mg/ml polygalacturonic acid (HiMedia) and 10 ng *Aspergillus niger* PG (Sigma) in 20 mM sodium acetate buffer, pH 4, was incubated at 30°C for 45 min. Reducing end-groups were measured by the method described by Wang *et al*. [25] using D-galacturonic acid (Sigma) as a standard. The PGIP activity was determined by measuring the PG activity in the absence and presence of total protein extracts of susceptible and resistant (control and inoculated) pearl millet seedlings harvested 24 h.p.i. at 1 and 5 μg concentrations, respectively. Boiled as well as trypsin (Sigma)-treated (according to the manufacturer's instruction) pearl millet total protein extracts were also assayed for PG inhibition. The experiment was carried out in triplicates and the PGIP activity was expressed as percent inhibition of PG.

Results

Higher accumulation of *pgip* transcripts in resistant cultivar

Temporal changes in PGIP transcript accumulation in both resistant and susceptible cultivars of pearl millet were analyzed using bean *pgip2* DNA probe. Transcript signals of ~1.2 kb were observed on hybridization. Constitutive expression of *pgip* transcript was clearly evident in resistant cultivar (**Fig. 1**). On pathogen inoculation resistant cultivar showed a differential accumulation of transcripts with maximum signal recorded at 24 and 48 h.p.i. But in resistant control, a minor fluctuation in the transcript level was observed at different time points. The susceptible cultivar in contrast showed low levels of *pgip* transcript without any significant difference on pathogen inoculation.

Differential accumulation of PGIP protein in resistant and susceptible cultivars

Heterologous bean anti-PGIP polyclonal antibody was used in order to assess the nature of protein accumulation in pear millet cultivars. As was the case with the transcripts, a constitutive protein expression was observed in

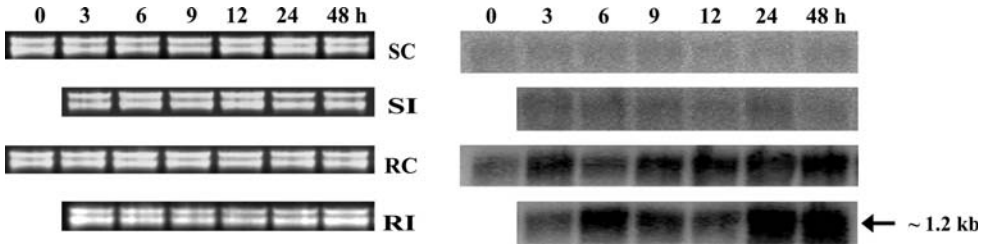

Figure 1 Transcript accumulation analysis by Northern blot hybridization The temporal accumulation pattern of PGIP transcripts in resistant uninoculated control (RC), resistant inoculated (RI), susceptible uninoculated control (SC), and susceptible inoculated (SI) pearl millet seedlings (right side). The corresponding total RNA loading was shown by ethidium bromide staining (left side). The blot shows a transcript signal of ~1.2 kb upon hybridization with 797 bp α^{32}P-labelled PGIP DNA probe from bean *Pv pgip 2*.

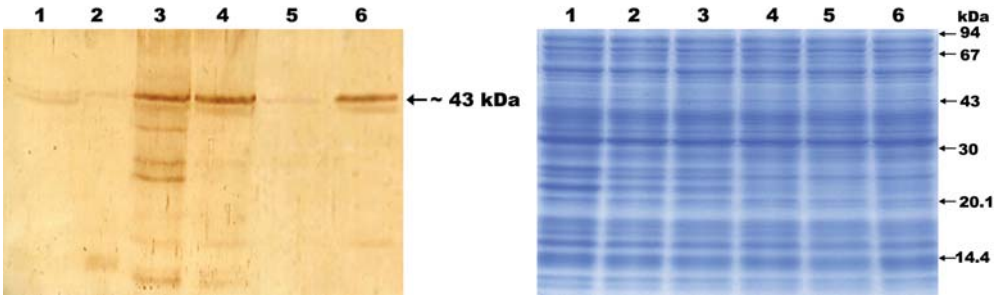

Figure 2 Western blot analysis showing differential accumulation of PGIP Total pearl millet protein (50 μg) from different samples harvested 0 h and 24 h.p.i. were separated by a 12% SDS-PAGE gel and electro-blotted onto a PVDF membrane. Polyclonal antibody (PGIP-11) generated in rabbit against bean PGIP was used for the analysis (left side). The corresponding total protein loading shown by Coomassie blue staining of SDS-PAGE separated proteins (right side). 1, susceptible inoculated (24 h.p.i.); 2, susceptible uninoculated control (24 h); 3, resistant inoculated (24 h.p.i.); 4, resistant uninoculated control (24 h); 5, susceptible uninoculated control (0 h); and 6, resistant uninoculated control (0 h) pearl millet seedlings.

both cultivars with very low level in the case of the susceptible seedlings. The protein accumulation pattern of 0 h susceptible and resistant samples was similar to that of their respective uninoculated control samples harvested at 24 h. Western blot analysis showed that resistant protein samples harvested at 24 h.p.i. resulted a very prominent band of ~43 kDa (**Fig. 2**). Additional bands of lower molecular weights (~41, 37, 29 and 25 kDa) were also observed at much lower intensity. The susceptible sample on the other hand showed a very low accumulation of ~43 kDa band with the appearance of a faint band of ~41 kDa on pathogen inoculation. The treatment of the blot with pre-immune serum and the secondary antibody alone resulted in no bands (data not shown).

Higher accumulation of PGIP in the epidermal and vascular tissues of resistant cultivar post-inoculation with the pathogen

Tissue-printing showed a low basal level of PGIP localization in both the cultivars [**Fig. 3(A,C)**]. PGIP level was higher in inoculated seedlings than in the uninoculated ones. Localized PGIP level was significantly higher in resistant seedlings compared with the susceptible ones. PGIP staining was intense in epidermal and vascular region of coleoptiles as observed in tissue blots [**Fig. 3(A–D)**]. A very dense localization of PGIP in walls of stomatal guard cells was observed in epidermal tissues of resistant cultivar post-inoculation with the pathogen [**Fig. 4(D)**]. The PGIP localization was higher in the resistant cultivar than in the susceptible cultivar [**Fig. 4(D)**]. Incubation of the epidermal peelings or tissue blots with pre-immune serum and secondary antibody alone did not result in labeling of cellular structures (results not shown).

Higher inhibition of polygalacturonase by the protein from resistant cultivars

The pearl millet cultivars displayed differential PG inhibitory activities. The resistant inoculated sample showed the highest PG inhibition of 7 and 16% at 1 and 5 μg, respectively, whereas resistant uninoculated control showed inhibition of 5 and 11% at the same concentrations (**Fig. 5**). On the other hand, susceptible uninoculated or inoculated protein extracts showed marginal inhibition of 2 and 3% only at 5 μg, but no inhibition recorded at 1 μg. The 0 h susceptible sample showed an inhibition of 2% at 5 μg, whereas the resistant sample showed 5 and 11% at 1 and 5 μg, respectively, which was similar to the trend seen in the case of 24 h uninoculated control samples. A total loss in PG inhibition was observed post-boiling and upon

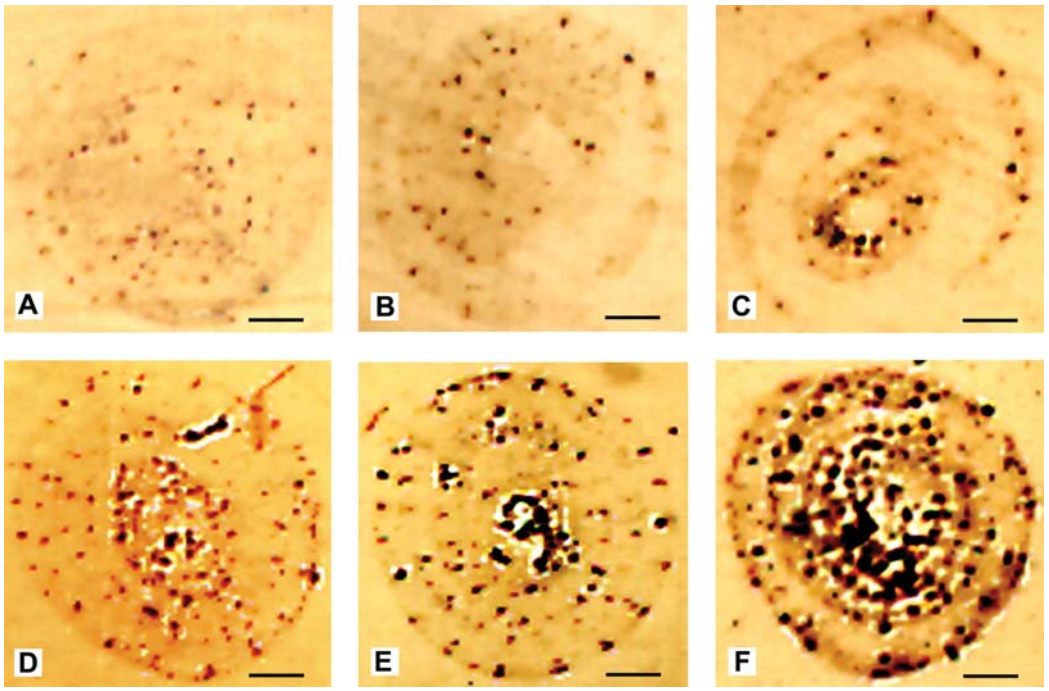

Figure 3 Tissue-print immunoblot localization of PGIPs Tissue blots of the cross-sections of coleoptile regions from resistant and susceptible samples harvested at 0 h and 24 h.p.i. were obtained by printing onto nitrocellulose membrane. Bean polyclonal antibody, PGIP-11, was employed to visualize the occurrence of PGIPs. The samples include susceptible uninoculated control, 0 h (A); susceptible uninoculated control, 24 h (B); susceptible inoculated, 24 h.p.i. (C); resistant uninoculated control, 0 h (D); and resistant uninoculated control, 24 h (E); resistant inoculated, 24 h.p.i. (F) pearl millet seedlings. Bar = 200 μm.

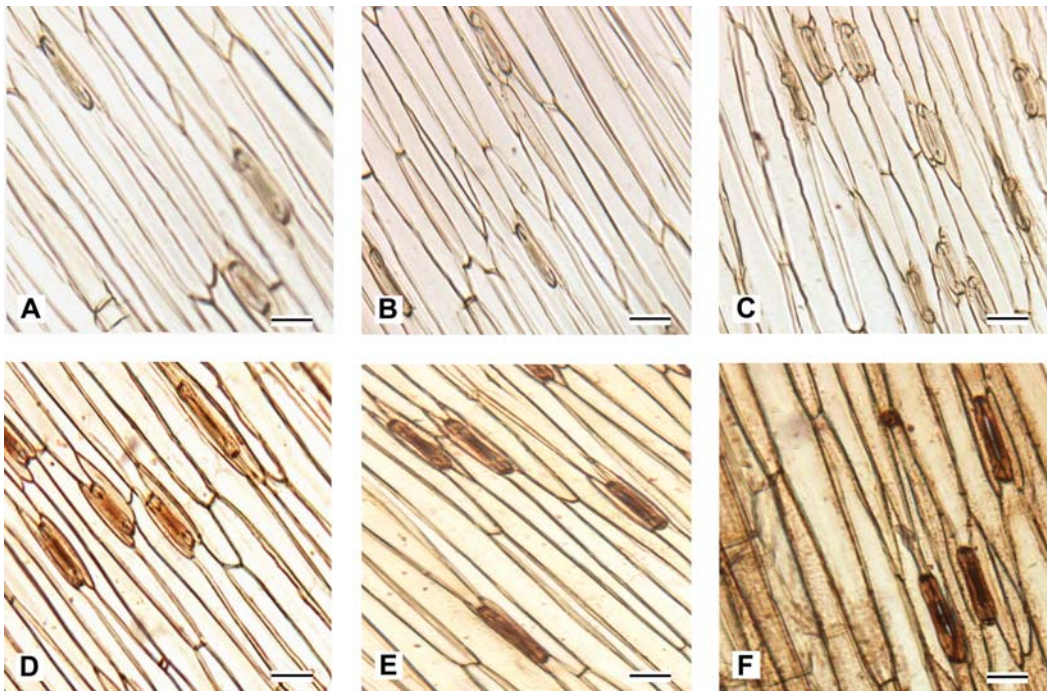

Figure 4 Immuno-histochemical localization of PGIPs in epidermal peelings of coleoptile Epidermal peelings from the coleoptile region of 2-day-old pearl millet seedlings harvested at 0 h and 24 h.p.i. with *S. graminicola* was used. Polyclonal antibody (PGIP-11) generated in rabbit against bean PGIP was employed in the localization study. The samples include susceptible uninoculated control, 0 h (A); susceptible uninoculated control, 24 h (B); susceptible inoculated, 24 h.p.i. (C); resistant uninoculated control, 0 h (D); resistant uninoculated control, 24 h (E); and resistant inoculated, 24 h.p.i. (F) pearl millet seedlings. Bar = 20 μm.

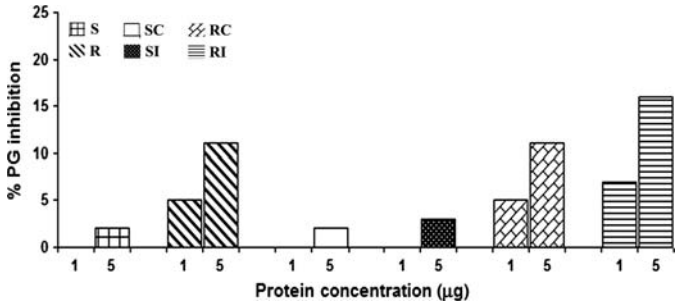

Figure 5 PGIP activity assay The PGIP activity was determined against *A. niger* PG (10 ng) by measuring the PG activity in the absence and presence of total protein extracts of different pearl millet seedlings harvested 24 h.p.i. at 1 and 5 µg. The samples include susceptible uninoculated control, 0 h (S); resistant uninoculated control, 0 h (R); susceptible uninoculated control, 24 h (SC); susceptible inoculated, 24 h.p.i. (SI); resistant uninoculated control, 24 h (RC); and resistant inoculated, 24 h.p.i. (RI) pearl millet seedlings. The experiment was carried out in triplicates and the PGIP activity was expressed as percent inhibition of PG.

protease (trypsin) treatment of the pearl millet total protein extracts (data not shown).

Discussion

Abundant literature is available on the presence, expression, and inhibitory action of dicotyledonous PGIPs against various EPGs [18,26–31]. However, very little information exists on their presence and role in monocotyledonous plants. Recently though, biochemical and immunological evidences have confirmed the presence of PGIP in monocotyledonous species, both in non-graminaceous plant, *A. porrum* [10] and in the graminaceous member, wheat [11]. Since the isolation of wheat PGIP, characterization of *pgip* genes from rice and wheat [32,33] and more recently in oil-palm [12] added information on economically important graminaceous monocot PGIPs. The present study was undertaken in order to investigate the presence and role of PGIPs in the defense response of pearl millet against downy mildew pathogen *S. graminicola*.

Pathogen-induced accumulation of *pgip* transcripts is an indication of the involvement of PGIP in active plant defense [34]. Hence, in order to assess the role of PGIP in pearl millet–*S. graminicola* interaction, a kinetic transcript accumulation study was carried out by Northern hybridization analysis. A bean *Pv pgip2* DNA fragment was used as probe against total RNA extracted at different time intervals from both pearl millet cultivars. A constitutive level of *pgip* transcripts (~1.2 kb) was observed in the resistant cultivar, and it was necessary to counter EPGs released during initial pathogen entry. However, only the resistant cultivar showed a differential expression post-pathogen challenge with maximum accumulation at 24 and 48 h.p.i. Similar minor fluctuations in the transcript signals of resistant controls has been reported earlier for various defense genes in pearl millet [35,36]. In bean–*Colletotrichum lindemuthianum* interaction, it has been reported that there is a differential *pgip* transcript accumulation between the susceptible and resistant genotypes with significantly higher signal in incompatible interaction, similar to what was observed in the present study [37–39]. Similarly in pear, *pgip* gene was induced significantly only in the resistant cultivars Kinchaku and Flemish Beauty upon inoculation with the pathogen, *Venturia nashicola* race 1 [40]. On the other hand, the susceptible cultivar showed a delayed, weak induction at 72 h.p.i. The soybean *pgip* genes, *Gmpgip1* and *Gmpgip3*, were up-regulated within 8 h after inoculation with *Sclerotinia sclerotiorum* [41]. Whereas soybean plants inoculated with *Phytophthora sojae* zoospores showed a delayed accumulation of the transcripts at 48 h.p.i. in comparison with plants inoculated with *P. sojae* mycelium, which was explained by the time needed for zoospore germination and plant penetration [34]. Since the downy mildew of pearl millet is also established by the infection of *S. graminicola* zoospores, the delayed PGIP accumulation observed in the present study is in agreement with the above-mentioned report. However, in the soyabean–*P. sojae* interaction it has been reported that plants infected with compatible *P. sojae* race 20 showed higher *pgip* expression levels in comparison with plants infected with the incompatible race 1. In this interaction, *pgip* behaves differently from what has been reported in this and other papers [34]. Such an observation has been speculated to be due to the different manner in which the soyabean gene is regulated and/or to the differential interaction with the pathogen.

To extract the wall-bound proteins, a buffer containing 1 M NaCl was used, as high salt is known to disrupt strong ionic interactions through which some cell wall proteins are bound to the cell wall matrix. The resistant sample at 24 h.p.i. was chosen for protein extraction as maximum accumulation of its transcripts was observed at that time interval. The crude pearl millet cell wall extract, as expected, showed numerous bands of varying molecular weights when subjected to SDS-PAGE. The western blot analysis of the pearl millet proteins using the bean polyclonal antibody, PGIP-11, showed the presence of a prominent ~43 kDa band in case of the resistant samples. In contrast, susceptible samples showed bands of very low intensity, which was in agreement with transcript studies. Altough there was no significant change in the accumulation level of the ~43 kDa protein band between the resistant control and inoculated samples, the appearance of additional lower molecular protein bands at 24 h.p.i. only in the resistant inoculated lane accounted for the significantly higher transcript signal observed in the resistant

inoculated sample. In bean, the amounts of preformed cell wall-bound protein as well as the soluble PGIP were always higher in the resistant line, but following inoculation with the pathogen, *C. lindemuthianum*, small variations were documented [42]. This corroborates the results of the present study. The resistant inoculated sample did show the presence of additional bands of lower molecular weight, which could possibly be the result of pathogen-triggered expression of additional *pgip* genes and/or isoforms and/or glycoforms of PGIP, as this protein has been known to exist as a small multigene family [41]. In addition to the pathogen trigger, the additional bands could also be attributed to the differential genetic background of the pearl millet cultivars. These results were further supported by the reports of existence of multiple isoforms of PGIP within a species, each of which exists as a series of glycoforms [43,44]. Such instances of pathogen-induced expression of additional PGIPs have been reported in soybean in which *Gmpgip1*, *Gmpgip3*, *Gmpgip4* are expressed in 7-day-old seedlings, whereas *Gmpgip2* is expressed only upon *S. sclerotiorum* infection [41]. PGIP-11 antibody was used in the present study, as it has been successfully used in analyzing PGIPs from cotton [45] and wheat [11]. The PGIP isolated from bean had a molecular weight of 45 kDa [28] and that in wheat was of 40.3 kDa [11]. A number of other PGIPs have also been found to fall in the range of 34–45 kDa [29,46,47]. However PGIP with sizes of 15 kDa in peach and 91 kDa in pear have been isolated [26,48]. Thus, the identification of a putative PGIP of ~43 kDa along with several smaller bands by western blot analysis in pearl millet is consistent with earlier reports.

To visualize the accumulation pattern of PGIP in cell walls of pearl millet coleoptiles, immuno-localization study including both epidermal peelings staining and tissue-printing was carried out after inoculation with the pathogen. The samples harvested at 24 h.p.i. were chosen for the study as the maximum accumulation of its transcripts was observed at that time interval. Previous such studies in our laboratory have successfully demonstrated localization of hydroxyl-proline-rich glycoproteins (HRGPs) in the pearl millet cell wall using similar techniques [49]. Consistent with the transcript accumulation studies, the protein also showed a differential expression with significantly higher accumulation observed in incompatible interaction. These studies have further corroborated PGIP involvement in pearl millet defense. Similar localization of PGIP in cell wall of wheat leaves was demonstrated using PGIP-11 antibody with a very low level of non-specificity [11]. The basal levels of PGIP observed in cell walls in the current study are in agreement with earlier reports [21,50]. PGIP mRNA localization by *in situ* hybridization experiments in *P. vulgaris* have shown an intense accumulation in hypocotyls in incompatible interaction with *C. lindemuthianum* [38]. Since the downy mildew pathogen is known to enter the host through stomatal openings, a strong accumulation of the protein observed in guard cells of resistant cultivar could indicate an important role for PGIPs in prevention of pathogen entry. A strong presence of the protein along the epidermal cell walls in the resistant coleoptiles is important as well, since the pathogen is also known to penetrate into host cells by wall degradation.

Earlier studies on pearl millet cell wall proteins like HRGPs [49] have shown a differential expression only in incompatible interactions with an early induction, as is the case with PGIPs. This is consistent with the fact that, cell wall is the first impediment that any pathogen encounters and that an early fortification of cell wall defense proteins is observed in incompatible interactions leading to inhibition of pathogen ingress.

Since a differential expression of PGIP both at the RNA and protein level has been recorded between the pearl millet cultivars, PGIP activity assay was conducted in order to verify if the results further translated into differential inhibition of fungal PGs as well. It is practically not possible to obtain good amounts of endo-polygalacturonase from the native pathogen, as its axenic culture is not possible. Hence, the commercially available PG from *A. niger* was used in the present study for PGIP activity assay. PGIP activity assay result, as expected, proved the existence of differential PG inhibitory activities between the pearl millet cultivars. Increase in total protein concentration of pearl millet samples led to increased percent PG inhibition, irrespective of the cultivar. Another trend observed across pearl millet cultivars was the partial inhibition of *A. niger* PG. This could be explained by the fact that, PGIPs from different sources are known to inhibit various fungal PGs to different extents, depending on recognition specificity [44]. Such low inhibitions of *A. niger* and *Fusarium moniliforme* PGs by wheat PGIP have been reported earlier [11]. The protein samples of the susceptible cultivar, both unioculated and inoculated, showed very low inhibition of the *A. niger* PG only at 5 μg, which could be explained by the weak PGIP bands observed in western blot. A marginal difference in inhibition of 1% between the control and inoculated samples could be due to the appearance of ~41 kDa in the susceptible inoculated lane. A strong immuno-reaction of the ~43 kDa PGIP band observed in both resistant uninoculated control and inoculated lanes clearly explained the significantly higher PG inhibition observed in the PGIP activity assay. A slight difference in the PGIP activity between the resistant samples might be due to the expression of additional PGIP proteins and its isoforms and/or glycoforms induced post-pathogen inoculation. The pattern of inhibition in the case of the 0 h samples confirmed basal expression of proteins in both

cultivars and, in addition, the differential inhibition between the cultivars was consistent with the trend observed in both western blot and immuo-histochemical analysis. The proteinaceous nature of the inhibitor was demonstrated by the complete loss in PG inhibition observed, post-boiling and upon protease (trypsin) treatment of all the pearl millet total protein extracts. Similar PGIP activity assays, reported in pectin-poor grass species such as wheat, have proved the importance of PGIP in its defense against the PG of *Cochliobolus sativus*, native fungal pathogen [11]. Additional corroborative evidence in the grass species is provided by the inhibition of various fungal PGs from *S. sclerotiorum*, *Fusarium graminearum*, *A. niger*, and *Botryitis cinerea* by rice PGIP, OsPGIP1 [33].

In conclusion, the present study has given an evidence for the presence of PGIPs in pearl millet. The investigation has also indicated a strong role for them in incompatible interactions with the oomycete pathogen, *S. graminicola*. Further studies on the characterization of PGIPs and demonstration of their interaction with *S. graminicola* EPGs as well as isolation and characterization of their genes are being carried out to prove conclusively, a role for PGIPs in pearl millet–*S. graminicola* interactions.

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References

1 Esquerre-Tugaye MT, Boudard G and Dumas B. Cell wall degrading enzymes, inhibitory proteins, and oligosaccharides participate in the molecular dialogue between plants and pathogens. Plant Physiol Biochem 2000, 38: 157–163.

2 Albersheim P and Anderson AJ. Proteins from plant cell walls inhibit polygalacturonases secreted by plant pathogens. Proc Natl Acad Sci USA 1971, 68: 1815–1819.

3 Ridley BL, O'Neill MA and Mohnen D. Pectins: Structure, biosynthesis, and oligogalacturonide-related signaling. Phytochemistry 2001, 57: 929–967.

4 Gomathi V and Gnanamanickam SS. Polygalacturonase-inhibiting proteins in plant defense. Curr Sci India 2004, 87: 1211–1217.

5 De Lorenzo G, Cervone F, Bellincampi D, Caprari C, Clark AJ, Desiderio A and Devoto A, *et al*. Polygalacturonase, PGIP and oligogalacturonides in cell–cell communication. Biochem Soc Trans 1994, 22: 396–399.

6 D'Ovidio R, Mattei B, Robertia S and Bellincampi D. Polygalacturonases, polygalacturonase-inhibiting proteins and pectic oligomers in plant–pathogen interactions. Biochim Biophys Acta 2004, 1696: 237–244.

7 Albersheim P and Anderson-Prouty AJ. Carbohydrates, proteins, cell surfaces, and the biochemistry of pathogenesis. Annu Rev Plant Physiol 1975, 26: 31–52.

8 De Lorenzo G, Castoria R, Bellincampi D and Cervone F. Fungal invasion enzymes and their inhibition. In: Carrol G and Tudzynski P eds. The mycota. V. Plant Relationships, Part B. Berlin: Springer, 1997, 61–83.

9 Favaron F, Castiglioni C and Di Lenna P. Inhibition of some rot fungi polygalacturonases by *Allium cepa* L. and *Allium porrum* L. extracts. J Phytopathol 1993, 139: 201–206.

10 Favaron F, Castiglioni C, D'Ovidio R and Alghisi P. Polygalacturonase-inhibiting proteins from *Allium porrum* L. and their role in plant tissue against fungal endo-polygalacturonases. Mol Plant Microbe Interact 1997, 50: 403–417.

11 Kemp G, Bergmann CW, Clay R, Van der Westhuizen AJ and Pretorius ZA. Isolation of polygalacturonase-inhibiting protein (PGIP) from wheat. Mol Plant Microbe Interact 2003, 16: 955–961.

12 Al-Obaidi JR, Mohd-Yusuf Y, Chin-Chong T, Mhd-Noh N and Othman RY. Identification of a partial oil palm polygalacturonase-inhibiting protein (*EgPGIP*) gene and its expression during basal stem rot infection caused by *Ganoderma boninense*. Afr J Biotechnol 2010, 9: 7788–7797.

13 Shetty SA, Shetty HS and Mathur SB. Downy Mildew of Pearl Millet. Technical Bulletin. Mysore, India: Downy Mildew Research Laboratory, University of Mysore, 1995.

14 Torto TA, Rauser L and Kamoun S. The *pipg1* gene of the oomycete *Phytophthora* encodes a fungal-like endo-polygalacturonase. Curr Genet 2002, 40: 385–390.

15 Singh SD and Gopinath R. A seedling inoculation technique for detecting downy mildew resistance in pearl millet. Plant Dis 1985, 72: 425–428.

16 Safeeulla KM. Biology and Control of the Downy Mildew of Pearl Millet, Sorghum and Finger Millet. Mysore: Wesley Press, 1976.

17 DeVries SC, Springer J and Wesseles JGH. Diversity of abundant mRNA sequences and patterns of protein synthesis in etiolated and greened pea seedlings. Planta 1982, 156: 129–135.

18 Favaron F, D'Ovidio R, Porceddu E and Alghisi P. Purification and molecular characterization of a soybean polygalacturonase-inhibiting protein. Planta 1994, 195: 80–87.

19 Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951, 193: 265–275.

20 Laemmli UK. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature 1970, 227: 680–685.

21 Bergmann CW, Ito Y, Singer D, Albersheim P, Darvill AG, Benhamou N and Nuss L, *et al*. Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L.in response to wounding, elicitors and fungal infection. Plant J 1994, 5: 625–634.

22 Whitefield AE, Campbell LR, Sherwood JL and Ullman DE. Tissue blot immunoassay for detection of tomato spotted wilt tospovirus in *Rananculus asiaticus* and other ornamentals. Plant Dis 2003, 387: 618–622.

23 Thordal-Christensen H, Zhang Z, Wei Y and Collinge DB. Sub-cellular localization of H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J 1997, 11: 1187–1194.

24 Mellersh DG, Foulds IV, Higgins V and Heath MC. H_2O_2 plays different roles in determining penetration failure in three diverse plant-fungal interactions. Plant J 2002, 29: 257–268.

25 Wang G, Michailides TJ and Bostock RM. Improved detection of polygalacturonase activity due to *Mucor piriformis* with a modified dinitrosalicylic acid reagent. Phytopathology 1997, 87: 161–163.

26 Abu-Goukh AA, Greve LC and Labavitch JM. Purification and partial characterization of 'Bartlett' pear fruit, polygalacturonase inhibitors. Physiol Plant Pathol 1983, 23: 111–122.

27 Barmore C and Nguyen TK. Polygalacturonase inhibition in rind of Valencia orange infected with *Diplodia natalensis*. Phytopathology 1985, 75: 446–449.

28 Cervone F, De Lorenzo G, Degra L, Salvi G and Bergami M. Purification and characterization of polygalacturonase-inhibiting protein from *Phaseolus vulgaris L*. Plant Physiol 1987, 85: 631–637.

29 Mahalingam R, Wang G and Knap KH. Polygalacturonase and polygalacturonase inhibitor protein: gene isolation and transcription in *Glycine max–Heterodera glycines* interactions. Mol Plant Microbe Interact 1999, 12: 490–498.

30 Berger DK, Oelofse D, Arendse MS, Du Plessis E and Dubery IA. Bean polygalacturonase-inhibiting protein-1 (PGIPs-1) inhibits polygalacturonases from *Stenocarpella maydis*. Physiol Mol Plant Pathol 2000, 57: 5–14.

31 Deo A and Shastri NV. Purification and characterization of Polygalacturonase-inhibitory proteins from *Psidium guajava* Linn. (guava) fruit. Plant Sci 2003, 164: 147–156.

32 Jang S, Lee B, Kim C, Yim J, Han JJ, Lee S and Kim SR, *et al*. The OsFOR1 gene encodes a polygalacturonase-inhibiting protein (PGIP) that regulates floral organ number in rice. Plant Mol Biol 2003, 53: 357–369.

33 Janni M, Di Giovanni M, Roberti S, Capodicasa C and D'Ovidio R. Characterization of expressed *Pgip* genes in rice and wheat reveals similar extent of sequence variation to dicot PGIPs and identifies an active PGIP lacking an entire LRR repeat. Theor Appl Genet 2006, 113: 1233–1245.

34 Favaron F, Destro T and D'Ovidio R. Transcript accumulation of polygalacturonase-inhibiting protein (PGIP) following pathogen infections in soybean. J Plant Pathol 2000, 82: 103–109.

35 Raj SN, Sarosh BR and Shetty HS. Induction and accumulation of polyphenol oxidase activities as implicated in development of resistance against pearl millet downy mildew disease. Funct Plant Biol 2006, 33: 563–571.

36 Sarosh BR, Sivaramakrishnan S and Shetty HS. Elicitation of defense related enzymes and resistance by L-methionine in pearl millet against downy mildew disease caused by *Sclerospora graminicola*. Plant Physiol Biochem 2005, 43: 808–815.

37 Nuss L, Mahe A, Clark AJ, Grisvard J, Dron M, Cervone F and De Lorenzo G. Differential inhibition of polygalacturonase-inhibiting protein (PGIP) in two near-isogenic lines of *Phaseolus vulgaris* L. upon infection with *Colletotrichum lindemuthianum*. Physiol Mol Plant Pathol 1996, 48: 83–89.

38 Devoto AJ, Nuss CL, Cervone F and De Lorenzo G. Developmental and pathogen-induced accumulation of transcripts of polygalacturonase-inhibiting protein in *Phaseolus vulgaris* L. Planta 1997, 202: 284–292.

39 Kalunke RM, Janni M, Sella L, David P, Geoffrey V, Favaron F and D'Ovidio R. Transcript analysis of the bean polygalacturonase inhibiting protein gene family reveals that pvpgip2 is expressed in the whole plant and is strongly induced by pathogen infection. J Plant Pathol 2011, 93: 141–148.

40 Faize M, Sugiyama T, Faize L and Ishii H. Polygalacturonase-inhibiting protein (PGIP) from Japanese pear: possible involvement in resistance against scab. Physiol Mol Plant Pathol 2003, 63: 19–327.

41 D'Ovidio R, Roberti S, Di Giovanni M, Capodicasa C, Melaragni M, Sella L and Tosi P, *et al*. The characterization of the soybean polygalacturonase-inhibiting proteins (*Pgip*) gene family reveals that a single member is responsible for the activity detected in soybean tissues. Planta 2006, 224: 633–645.

42 Lafitte C, Barthe JB, Montillet JL and Touze A. Glycoprotein inhibitors of *Colletotrichum lindemuthianum* endo-polygalacturonase in near isogenic lines of *Phaseolus vulgaris* resistant and susceptible to anthracnose. Physiol Plant Pathol 1984, 25: 39–53.

43 Desiderio A, Aracri B, Leckie F, Mattei B, Salvi G, Tigelaar H and Van Roekel JS, *et al*. Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol Plant Microbe Interact 1997, 10: 852–860.

44 Favaron F. Gel detection of *Allium porrum* polygalacturonase-inhibiting protein reveals a high number of isoforms. Physiol Mol Plant Pathol 2001, 58: 239–245.

45 James JT and Dubery IA. Inhibition of polygalacturonase from *Verticillium dahliae* by a polygalacturonase-inhibiting protein from cotton. Phytochemistry 2001, 57: 149–156.

46 Stotz HU, Contos JJ, Powell AL, Bennett AB and Labavitch JM. Structure and expression of an inhibitor of fungal polygalacturonases from tomato. Plant Mol Biol 1994, 25: 607–617.

47 Conway WS, Yao C, Ross GS and Sams CE. Apple polygalacturonase-inhibiting protein cDNA cloning and transcription. Proceedings of 7th International Congress of Plant Pathology, Edinburgh, Scotland, 16 August, 1998.

48 Fielding AH. Natural inhibitors of fungal polygalacturonases in infected fruit tissues. J Gen Microbiol 1981, 123: 377–381.

49 Deepak S, Shailasree S, Kini RK, Hause B, Shetty HS and Mithöfer A. Role of hydroxyproline-rich glycoproteins in resistance of pearl millet against downy mildew pathogen *Sclerospora graminicola*. Planta 2007, 226: 323–333.

50 Cervone F, De Lorenzo G, Pressey R, Darvill AG and Albersheim P. Host-pathogen interactions. XXXIII. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol 1989, 90: 542–545.