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Hydroxyproline-rich glycoproteins accumulate in pearl millet after seed treatment with elicitors of defense responses against *Sclerospora graminicola*

Neerakkal Sujeeth [a,b,*], Shantharaj Deepak [b,1], Sekhar Shailasree [b,2], Ramachandra K. Kini [b], Shekar H. Shetty [b], Jacques Hille [a]

[a] *Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*
[b] *Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India*

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ABSTRACT

The accumulation of hydroxyproline-rich glycoproteins (HRGPs) was investigated after induction of resistance in pearl millet against downy mildew caused by *Sclerospora graminicola*. Treatment of susceptible pearl millet seeds with various biotic and abiotic elicitors resulted in increased HRGP content in the cell walls of coleoptiles at 9 h after inoculation. Similar results with increased accumulation at 4–6 h after inoculation were obtained in suspension cells of pearl millet. Maximum HRGP accumulation was observed in seedlings raised from susceptible seeds treated with chitosan and *Pseudomonas fluorescens*. Western blot analysis with MAC 265 (a rat monoclonal antibody raised against pea HRGP) identified three proteins of 27, 17 and 14 kDa in resistant cultivars. The absence of the 14 kDa HRGP was observed in susceptible cultivars as reported earlier. The induced accumulation of the 14 kDa HRGP upon elicitor treatments was observed in the present study. Peroxidase and hydrogen peroxide, essential components for HRGP cross-linking, were also increased in samples treated with these elicitors. A tissue specific increase in HRGP at the regions around vascular bundles was observed upon chitosan treatment. The results presented will have a presumed importance in identifying the susceptible pearl millet varieties and improving those using elicitors of defense for field applications.

1. Introduction

Success of a plant defense response depends on the speed by which the plant recognizes the attacking pathogen and the intensity by which appropriate defense responses are activated. The basal resistance response in plants to restrict the colonization of the pathogen can be enhanced by specific biotic and abiotic stimuli in the form of elicitors [1–4]. Protection of pearl millet [*Pennisetum glaucum* (L.) R. Br] against the downy mildew causing oomycete *Sclerospora graminicola* (*Sacc.*) *Schroet* is possible by application of abiotic elicitors such as β-amino butyric acid (BABA) [5], proline [6], chitosan [7], Trichoshield [8] and 2,6-dichloroisonicotinic acid (DCINA) [9]. It has also been shown that microorganisms like *Pseudomonas fluorescens* [10] and plant extracts of *Datura metel* [11,12] have the potential to control *S. graminicola*.

The effect of abiotic and biotic elicitors involves biochemical changes in the host metabolism that may play a role in limiting plant infection by *S. graminicola*. Cell wall reinforcements due to accumulation and cross-linking of hydroxyproline-rich glycoproteins (HRGPs) as a response to *S. graminicola* has been reported [13]. HRGPs are important plant cell wall structural components, which during the course of pathogen invasion are induced in several plant pathogen interactions [13–16]. The involvement of HRGPs in systemic acquired resistance (SAR) has been established recently using transformed tobacco cultivars having the *nahG* gene for salicylate hydroxylase. The transformed plants that were insensitive to salicylic acid signaling showed poor HRGP accumulations [15]. Also a highly co-ordinated localized alteration to plant cell walls with HRGP accumulation was show at the challenge sites of pathogen infection using monoclonal antibodies specific to HRGPs [13,17]. This represents a rapid defense mechanism to strengthen

Abbreviations: HRGPs, hydroxyproline-rich glycoproteins; Hyp, Hydroxyproline; hai, hours after inoculation.

* Corresponding author. Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands. Tel.: +31 50363 2325; fax: +31 50363 8126.
E-mail addresses: s.neerakkal@rug.nl (N. Sujeeth), dshantha@umn.edu (S. Deepak), shailakini@gmail.com (S. Shailasree), krk@appbot.uni-mysore.ac.in (R.K. Kini), hss_uom@hotmail.com (S.H. Shetty), j.hille@rug.nl (J. Hille).
[1] Present address: Department of Plant Biology, 250 Biological Sciences Center, University of Minnesota, 1445 Gortner Avenue, St. Paul, Minnesota 55108.
[2] Present address: Department of Biotechnology, Pooja Bhagavat Memorial Mahajana Post Graduate Center, Metagalli, K.R.S. Road, Mysore 570016, Karnataka, India.

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the cell wall as a barrier to pathogen ingress prior to the development of transcription dependent defenses [18].

The possible mechanism by which HRGP accumulation contributes to disease resistance involves cross-linking between HRGP monomers catalyzed by peroxidase and hydrogen peroxide to form a network, which might provide anchorage for lignifications and creates a barrier impenetrable to fungal hyphae [16,18]. The current study was carried out to investigate the role of HRGPs during the induction of resistance in pearl millet against *S. graminicola* by seed treatment with selected biotic and abiotic elicitors.

2. Materials and methods

2.1. 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 the study. The seeds were obtained from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India. The seeds of each line were sown in the downy mildew disease plot of the Department of Studies in Biotechnology, University of Mysore, Manasagangotri, Mysore 570006, Karnataka, India, for testing their reaction to the disease following the procedure of Williams et al. [19].

2.2. Pathogen and preparation of inoculum

S. graminicola was isolated from pearl millet cv. 7042S and maintained on the same cultivar under greenhouse conditions and was used for all inoculation experiments. Leaves of infected plants showing symptoms of downy mildew were collected in the evening, washed in running tap water to remove the remnants of previous sporulation, blotted dried, cut to pieces about 2 inches in length and placed in a moist chamber for sporulation. Fresh sporangia were collected the next morning and zoospores released by them used as inoculum [20].

2.3. Test seedlings used for the study

Seeds of resistant cv. IP18296 and susceptible cv. 7042S cultivars of pearl millet were surface sterilized in 0.1% sodium hypochlorite for 15 min and washed thoroughly with sterile distilled water. Seeds of the susceptible cv. 7042S were treated with the biotic and abiotic elicitors. The concentrations of elicitors used and duration of treatments were chosen based on earlier studies (Table 1). For each elicitor treatment, one hundred seeds were used. Simultaneously, seeds of the resistant and susceptible cultivars were treated with distilled water under similar conditions to serve as a standard control of resistance.

The treated and the untreated/standard control seeds were further germinated on moist filter paper under aseptic conditions at 25 ± 2 °C in darkness for two days. The two-day-old seedlings were inoculated by the root dip technique with a 4×10^4 zoospores ml^{-1} suspension of *S. graminicola* [20]. Seedlings dipped in sterile distilled water served as an uninoculated control. The seedlings were harvested at 8/9 h after inoculation for further experiments.

2.4. Analysis of hydroxyproline-rich glycoproteins (HRGPs)

2.4.1. Hydroxyproline (Hyp) content in cell walls of pearl millet coleoptiles

Test seedlings from resistant, susceptible and elicitor treated susceptible seeds were sampled at 9 hai (hours after inoculation) with *S.graminicola*. Seedlings dipped in sterile distilled water served as an uninoculated control. Cell walls from the coleoptiles regions of the test seedlings were isolated following the procedure of Shailasree et al. [13]. The coleoptiles of the seedlings were homogenized using pestle and mortar at 4 °C in 0.5 M potassium phosphate buffer, pH 7.0. The complete disruption of cells in the paste was examined by light microscopy. The homogenized suspension was centrifuged at 10,000*g* for 10 min. The pellet obtained was repeatedly washed with buffer followed by distilled water for five times. Washed cell walls were suspended by vigorous stirring in 5 volumes of 1:1 (v/v) chloroform–methanol. The organic solvent was carefully removed with out disturbing the cell wall pellet. Cell walls were washed three times with 5 volumes of acetone and then air-dried. The amount of HRGPs was determined by analyzing the Hyp content in the cell wall hydrolysate. Hydrolysis of the cell walls took place with 6 N HCl for 18 h at 110 °C in sealed tubes. Hydrolysates were evaporated to dryness. Hyp was then extracted in the minimum amount of distilled water from the dried hydrolyzed samples and the amount estimated following the spectrophotometric method of Prockop and Udenfriend [21]. Hyp content was expressed as μg Hyp mg^{-1} cell wall (dry weight).

2.4.2. Hydroxyproline in suspension cells of pearl millet

The pearl millet cell culture was raised from the susceptible (7042S) cultivar by following the method of Vasil and Vasil [22]. The well-established suspension cells were regularly sub-cultured onto fresh medium at 1:5 dilution rates at 10-day intervals and after 10 sub-cultures the cells were used for the study. A cell culture (10^8 cells ml^{-1}) at the mid-point of log phase of growth (16 day old) was used for the experiment. The suspension cells were treated with elicitors *P. fluorescens* (UOMSAR 14) at 10^8 cfu/ml or Chitosan

Table 1
List of biological and chemical elicitors selected for HRGP accumulation study. The different concentrations of the elicitors used for the seed treatments in the present study and the treatment time are indicated in the table. The similar treatment gave a field protection, to the susceptible cv. 7042S against *S. graminicola* as reported from the references indicated.

Elicitor	Concentration	Time of seed treatments	Field protection observed	Reference
Chitosan (Sigma)	0.3% in distilled water	9 h	73%	Sharathchandra et al. [7]
2,6 dichloroisonicotinic acid (INA)	0.2 mM in distilled water	6 h	73%	Shivakumar et al. [9]
Pseudomonas fluorescens (UOMSAR– 14)	10^8 cfu/ml^{-1}	6 h	70%	Raj et al. [10]
Trichoshield[a]	5% in distilled water	6 h	67%	Raj et al. [8]
Datura metel	2% leaf extract in distilled water	3 h	67%	Devaiah et al. [11] Shivakumar et al. [12]
Proline	15 mM in distilled water	3 h	67%	Raj et al. [6]

[a] A talc-based formulation containing 100 million spores per gram of *Trichoderma harzianum*, *Gliocladium virens*, and *Bacillus subtilis*, was obtained from Nutri-Tech Solution P/L. Queensland, Australia.