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Immunocytochemical localization of the pathogenesis-related PR-1 protein in barley leaves after infection by *Bipolaris sorokiniana*

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Abstract

A pathogenesis-related PR-1 protein accumulates in barley (*Hordeum vulgare* L.) leaves upon pathogen attack. The hyphae of *Bipolaris sorokiniana* invades primarily the intercellular space of mesophyll tissue. Light microscopy revealed that infected leaves show PR-1 labeling on mesophyll cell walls and both infected and non-infected leaves show labeling in chloroplasts. Subcellularly, PR-1 was detected on the outer cell wall layer and cytoplasm of primary hyphae, on intercellular electron dense material in junctions between host cells and in host cell wall appositions. The role of PR-1, as a defense and/or senescence related protein, in relation to its subcellular localizations is discussed.

Keywords: Pathogenesis-related protein; PR-1; *Hordeum vulgare*; *Bipolaris sorokiniana*; Immunocytochemistry; Immunogold; Fungal cell wall; Chloroplast; Senescence

1. Introduction

Pathogenesis-related (PR) proteins, a class of host-encoded plant proteins, are by definition induced only in pathological or related situations [1]. PR proteins can be induced both locally and systemically in several plant species, both mono- and dicotyledons, by many different types of pathogens or chemicals [35,52,53]. At present PR proteins are divided into 14 different families [53], classified on the basis of similarities in molecular weight, amino acid sequences and enzymatic and biological activities of the different proteins [51]. The biochemical function of the most abundant PR protein in infected leaves, PR-1, is still not known and the role of PR-1 has been under discussion for several years [54]. Different functions have been suggested for PR-1 proteins; some are thought to be involved in preventing virus diffusion, others to be able to restrict fungal invasion or to protect the plant against environmental stress [2,6,17,18]. PR proteins within the families can possess isoforms according to their isoelectric points, which can be differentially regulated and have separate cellular localization [33,47]. Few acidic isoforms of PR-1 have shown significant antimicrobial activity and they are most commonly targeted to the intercellular space [14,52]. Several basic isoforms of PR-1 have antifungal activity [40]. Some basic PR proteins are known to have a C-terminal vacuolar targeting signal [7,39]. Dual localization, both intra- and intercellular, have been reported for basic PR-1 [40,55]. Bryngelsson et al. [13] isolated two basic PR-1 proteins from barley leaves infected with the powdery mildew fungus. From the derived amino acid sequences, it was concluded that there was no C-terminal vacuolar targeting signal in neither, and they suggested that the basic barley PR-1 proteins are, at least partially, exported to the intercellular space.

We have studied the accumulation and localization of PR-1 in barley leaves, in response to infection by the hemibiotrophic fungus *Bipolaris sorokiniana* [46], using immunohisto- and cytochemical methods. *B. sorokiniana* is a severe pathogen on root and leaf tissue of cereals [32], and can cause spot blotch and root rot on barley [28]. It has a brief initial biotrophic phase where the hyphae penetrate living epidermal cells of the host, followed by intercellular progress of the hyphae in the mesophyll, leading to cell death of the host tissue, the necrotrophic phase [28].

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Our aim in the present paper was to investigate the subcellular localization of a basic PR-1 in barley. It was also of interest to localize possible targets for barley PR-1 that could indicate if PR-1 has a role in arresting fungal invasion of barley leaves. The results obtained in the study support the suggestion that PR-1 has a partial function in the defense mechanisms of plants against fungal pathogens. The results may also support the theory that PR proteins are not only limited to defense mechanisms but may also be expressed during developmental stages of barley.

2. Materials and methods

2.1. Plants and fungal cultures

Seeds of barley (*Hordeum vulgare* L.) cv. Alva were sown in a peat-based pot-soil with Leca® gravel at a density of 20 seeds per 16-cm-diameter pot. Plants were grown in growth chambers at light/dark temperatures of 18/16 °C (80% RH) with a photoperiod of 16 h and a photon flux density of 400 μmol (s^{-1} m^{-2}). An isolate of *B. sorokiniana* (Sacc. in Sorok.) Schoem. THA1, obtained from J. Hetzler, Institut für Pflanzenpathologie und Pflanzenschutz, Göttingen-Wende, Germany, was used for inoculation. Conidia were produced on V8 juice agar at room temperature for 10–14 days. The conidia were collected by flooding the surface of the fungal colonies with sterile water and hyphal fragments were removed by filtration through a sterile 100 μm mesh nylon net. Hyphal fragments were obtained by inoculating 3% (w/v) Sabouraud dextrose broth (Difco Laboratories, Detroit, USA) with conidia and incubating on a rotary shaker (150 rpm) at room temperature for 48 h. The mycelia were homogenized in a Waring blender and filtered through a sterile 600 μm mesh nylon net. Barley seedlings were sprayed 9 days after sowing with a 10 ml suspension mixture containing conidia (1×10^5 ml^{-1}) and hyphal fragments of *B. sorokiniana* suspended in sterile distilled water with 0.01% (v/v) Tween 20 and 0.1% (w/v) agar (Oxoid No. 1, Oxoid Ltd, Basingstoke, England). Twelve hours before inoculation, light/dark temperatures were changed to 14/12 °C. *RH* was increased to 90% and the pots were covered with plastic tents, to keep a high humidity. Infected plants and control plants were kept in separate chambers under the same set of growth conditions. Control plants were sprayed with sterile distilled water containing 0.01% (v/v) Tween 20 and 0.1% (w/v) agar. Leaf material was collected from control plants and infected plants 5 days post-inoculation and processed further either for protein extraction or for microscopy studies.

2.2. Antibodies

Polyclonal antiserum was raised in rabbits against the basic 15 kDa barley PR-1 (HvPR-1a) protein, purified from intercellular washing fluid of barley leaves infected with the powdery mildew fungus (*Blumera graminis*) [13]. The rabbit non-immune serum used in this study is a pre-immune serum to a barley PR-2c [50], purified as the HvPR-1a antiserum according to Bryngelsson et al. [13]. Before immunomicroscopy, IgG fractions were purified for both antiserum and non-immune by using Mab Trap® G II and PD-10 desalting columns (Amersham Bioscience, Amersham, UK).

2.3. Protein extraction and western analysis

The leaf material from each pot, five replicates per treatment, was frozen in liquid nitrogen and stored at −80 °C. Acid soluble proteins from frozen leaves and homogenized, pure cultured *B. sorokinana* was extracted as described by Bryngelsson et al. [12] modified by Liljeroth et al. [32]. Protein concentration was determined according to Bradford [9]. For western blot analysis, total soluble protein from each replicate was separated by 15% SDS-polyacrylamide gel electrophoresis [13], using a mini Protean™ II apparatus (Bio-Rad, Richmond, CA, USA). After electrophoresis, proteins were electrotransferred to nitrocellulose filters, Hybond-ECL (Amersham Bioscience, Amersham, UK) in a mini Trans-Blot® cell (Bio-Rad, Richmond, CA, USA) as described by the manufacturer. Protein detection was carried out using the BM Chemiluminiscence Western Blotting Kit (Roche Diagnostics Mannheim, Germany) with polyclonal antisera against HvPR-1a.

2.4. Tissue processing for light and electron microscopy

Samples (2×3 mm) were collected from the center and edges of necrotic lesions on the first leaf of infected plants. Control samples were taken from corresponding sites on non-infected plants. Fixation was carried out by placing the tissue samples in freshly made 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, 60 mM sucrose, 5 mM calcium chloride, followed by gentle vacuum pumping for 6×10 min at room temperature. The samples were then thoroughly rinsed with buffer. Before dehydration the samples were preembedded in melted agarose (3% (w/v) (Sigma Chemical Co., St. Louis, USA). After the agarose had solidified on ice it was cut into thin pieces of 4×6 mm, containing one leaf sample each. The agarose embedded samples were dehydrated through a graded ethanol series (10, 30, 51, 70, 90, 95 and 4×100% (v/v)), 15 min each. Infiltration was performed with a mixture of ethanol and LR White medium grade acrylic resin (London Resin Co., Surrey, UK) at the ratios 3:1, 1:1 and 1:3 (v/v) for 90 min each, followed by an overnight incubation in pure resin on a shaker (50 rpm) at room temperature and three subsequent changes, 1 h each. The samples were embedded in resin pretreated with nitrogen gas and positioned in teflon molds (Ted Pella, Inc., Redding, CA, USA). Finally, the molds were covered