Journal of Plant Physiology 167 (2010) 625–632

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.de/jplph

# Boron deficiency results in induction of pathogenesis-related proteins from the PR-10 family during the legume–rhizobia interaction

María Reguera, Ildefonso Bonilla, Luis Bolaños *

*Departamento de Biología, Facultad de Ciencias, Universidad Autónoma de Madrid, Darwin 2, 28049 Madrid, Spain*

ARTICLE INFO

*Article history:*
Received 1 September 2009
Received in revised form
31 October 2009
Accepted 20 November 2009

*Keywords:*
ABR17
Boron
Legume–rhizobia interaction
Pathogenesis-related proteins
Symbiosis

ABSTRACT

Boron (B) deficiency has a strong effect on molecular and cellular plant–bacteria interactions during the development of the legume–rhizobia symbiosis, leading to reduced infection and early necrosis of nodules, resembling a pathogenic-like rather than a symbiotic interaction. Therefore, induction of pathogenesis-related (PRs) proteins was investigated here in legume root nodules. Following two-dimensional electrophoresis and MALDI-TOF spectrometry analysis of proteins extracted from *Pisum sativum* B-sufficient (+B) or B-deficient (–B) root nodules, two proteins from the family PR10, ABR17 and PR10.1, were identified as highly induced in –B nodules. Analysis of gene expression and the use of anti-ABR17 confirmed that induction occurred in B-deficient young nodules and increased during nodule development. ABR17 was also induced in –B nodules of *Phaseolus vulgaris*. Boron deficiency did not significantly increase the expression of these PR10 in uninfected plant tissues. Moreover, independent of B, induction was detected in senescent tissues, although at a level weaker than in −B nodules. The immunochemical study of ABR17 antigen distribution showed that it was localized in all tissues of poorly invaded B-deficient nodules and accumulated around bacteria, which showed advanced degradation. These results suggest that, under B deficiency, the rhizobia–legume dialogue fails and the bacterium is recognized as a pathogen by the plant, which reacts to prevent infection by inducing at least these two identified PR10 proteins.



## Introduction

Plant roots are continuously interacting with soil microorganisms, establishing either pathogenic relationships, which trigger both constitutive and inducible mechanisms to prevent attack of pathogens, including the expression of defense proteins (Van Loon, Rep, 2006), or symbioses, like those established between legumes and rhizobia, that result in a $N_2$-fixing nodule, without eliciting defense mechanisms (Parniske, 2000).

Some features of rhizobial infections resemble pathogenic interactions (for a recent review, Soto et al., 2009), and after allowing development of a number of nodules, the legume may initiate a defense reaction, characterized by early abortion of infection threads in necrotic cells, as a plant mechanism to control the extent of infection (Vasse et al., 1993). Successful nodulation strongly depends on the suppression of the host defense response (Mithöfer, 2002). At each stage of nodule development, the rhizobia must survive in the endophytic environment without eliciting or overcoming defense mechanisms. Failure to adapt to the changing ecological niches offered by host legume would lead the plant to treat the bacterium more like a pathogen than like a symbiont. Therefore, host cell colonization would be prevented.

The legume–rhizobia symbiotic interaction is largely dependent on boron (B), a micronutrient required for plants and other organisms in micromolar concentrations. Boron deficiency leads to aberrant nodule organogenesis (Reguera et al., 2009), with an important reduction of infection (Bolaños et al., 1996; Redondo-Nieto et al., 2001), and a block of maturation of endophytic bacteria to $N_2$-fixing bacteroids (Bolaños et al., 2001; Redondo-Nieto et al., 2007), which could be at least partially due to a recognition of the bacterium as a potential pathogen. Therefore, it is likely that failure of plant–rhizobia interaction triggers some typical plant defense reactions to prevent infections under B-deficient conditions.

One important feature of the plant defense response to pathogens is the induction and accumulation of various pathogenesis-related (PR) proteins (Van Loon, Rep, 2006). Even in the homologous symbiotic interaction, accumulation of PRs has been described as part of the mechanism by which the legume controls infection and regulates nodulation (Vasse et al., 1993). Many PRs

*Abbreviations:* 2DE, two-dimensional gel electrophoresis; BSA, bovine serum albumin; DTT, dithiothreitol; IEF, isoelectric focusing; MALDI-TOF, Matrix-assisted laser desorption/ionization time-of-flight; pI, isoelectric point; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TBS, tris-buffer saline; TCA, trichloroacetic acid

* Corresponding author: Tel.: +34 91 497 82 72; fax: +34 91 497 83 44.
*E-mail address:* luis.bolarios@uam.es (L. Bolaños).

0176-1617/$ - see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2009.11.017

are induced not only by pathogens, but also by abiotic stresses, developmentally regulated or constitutively expressed in some tissues (Van Loon, Rep, 2006), including uninfected roots of pea plants (Mylona et al., 1994). Some genes homologous to PRs are even expressed in the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti* (Gamas et al., 1998). However, expression of root-constitutive proteins from the PR-10 family, structurally related to ribonucleases (Bufe et al., 1996), was reduced in young and mature lupin nodules (Sikorski et al., 1999). These reports suggest that expression of some PR-10, and perhaps other PR proteins, could be a part of a constitutive defense mechanism that must be suppressed by the symbiotic interaction. Therefore, one main goal of this work was to examine whether B deficiency led to induction of pathogenesis-related proteins in legumes, which could partially explain the failure of its interaction with *Rhizobium*.

## Materials and methods

### *Plant growth and inoculation*

Pea (*Pisum sativum* cv. Lincoln) or bean (*Phaseolus vulgaris* cv. Delinel) seeds were surface-sterilized with 70% (v/v) ethanol for 1 min and 10% (v/v) sodium hypochlorite for 20 min, soaked for 4 h in sterile distilled water and then germinated on wet filter paper at 25 °C. After 4 d the seedlings were transferred to plastic growth pots and cultivated on perlite with FP medium for legumes (Fahraeus, 1957).

To induce B deficiency, B was removed from the micronutrient solution. For cultures with the normal B content, the micronutrient (as $H_3BO_3$) was added to a final concentration of 0.1 mg B $L^{-1}$. All solutions were prepared and stored in polyethylene containers previously tested not to release boron, even under sterilizing conditions. Boron was determined using Azomethine H at pH 5.1 (Wolf, 1974) and a Technicon Automatic Analytical System (Tarry Town, New York, NY, USA) in the solutions and media prior to use, and it was found to be below the detection limit (0.02 mg $mL^{-1}$). Before inoculation, the pea seedlings were grown without added B over 15 d (including germination time) to drain the B stored in the seeds.

Plants were inoculated with 1 mL per seedling of about $10^8$ cells $mL^{-1}$ of *Rhizobium leguminosarum* bv. *Viciae* (strain 3841 for pea or strain B625 for bean), from an exponential culture in tryptone–yeast extract (TY) medium (Beringer, 1974). Inoculated plants were maintained in a growth cabinet at 22 °C day/18 °C night temperatures with a 16/8 h photoperiod and an irradiance of 190 μmol $m^{-2}$ $s^{-1}$. Relative humidity was kept between 60% and 70%.

### *Protein extraction and 2-DE analyses*

Nodules at a comparable stage of development were harvested and homogenized to a fine powder in liquid nitrogen. Proteins from 0.25 g of nodule homogenate were extracted with 10% w/v trichloroacetic acid (TCA) for 2 h. The samples were centrifuged at 13,000*g* for 20 min and the pellets were resuspended in 80% acetone containing 0.07% dithiothreitol (DTT) and then incubated at −20 °C for 1 h. The samples were centrifuged and the pellet washed again in ice-cold acetone-DTT and incubated at −20 °C for an additional hour. The wash step was repeated two more times and the final pellet was dried at room temperature for 30 min. Protein extracts were analyzed by high resolution 2-DE (O'Farrell, 1975), with isoelectric focusing (IEF) using immobilized pH gradient strips in the pH 3–10 range to separate proteins in the first- and SDS (sodium dodecyl sulphate)-PAGE (polyacrylamide gel electrophoresis) in the second dimensions. IEF was carried out using a Bio-Rad IEF system. Strips (Inmobiline Drystrip pH 4–7, 7 cm) were rehydrated overnight at room temperature with a total of 125 μL solution containing the extracted protein at a concentration of 1 μg $\mu L^{-1}$. Rehydrated strips were positioned in the focusing tray and covered with mineral oil and focused in the IEF unit. Initially, a low voltage of 300 V $cm^{-1}$ was applied for 30 min to remove salt ions and charged contaminants, followed by a linear voltage ramping step to reach 8000 V in 3 h, after which focusing took place at a constant 8000 V for 4 h without exceeding the 50 μA per strip. At the end of focusing, the strips were removed from the IEF unit and equilibrated in 10 mL of 50 mM Tris–HCl, pH 8.8, containing 6 M urea, 20% glycerol, 2% w/v SDS and 2% w/v DTT for 10 min at room temperature, followed by another 10 min in 10 mL of 50 mM Tris–HCl buffer, pH 8.8, containing 6 M urea, 20% glycerol, 2% w/v SDS and 2.5% w/v iodoacetamide to reduce and alkylate the proteins. Separation in the second dimension was performed by SDS-PAGE using a vertical slab of 12% acrylamide at 30 mA. Protein spots were visualized by silver staining.

### *Mass spectrometry MALDI-TOF*

Analysis of protein spots from 2-DE gels was performed at the Proteomic Unit of Parque Científico de Madrid (http://www.fpcm.es/protIHP.htm). Spots of interest were excised from the gels using a sterile scalpel, placed in numbered wells of a polypropylene plate, and submerged with 40 μL of sterile water. The gel pieces were de-stained, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with 6 ng $\mu L^{-1}$ trypsinin 50 mM ammonium bicarbonate for 5 h at 37 °C. After enzymatic digestion, the tryptic peptides were extracted and then subjected to mass spectrometry MALDI-TOF. Mass spectral data were acquired from detected peptides with a charge state selection from 2 to 5, and protein identification was performed using the database MASCOT (Matrix Science, UK) (http://www.matrixscience.com).

### *Immunoblotting*

Samples for gel separation were extracted by heating in SDS buffer for 10 min at 100 °C. After centrifugation to remove insoluble debris, the extracts (10 μg protein loaded per lane) were subjected to conventional unidimensional electrophoresis using 12% acrylamide minigels (Laemmli, 1970). Bi- or unidimensional gels were transferred electrophoretically to membranes of nitrocellulose (Bittner et al., 1980). Blots were blocked with 5% bovine serum albumin (BSA) in Tris-buffered saline (50 mM Tris–HCl, pH 7.4; and 200 mM NaCl) buffer (TBS) and then incubated with BSA–TBS containing anti-ABR17 antiserum (Barratt et al., 1989). Immunostaining was visualized using a goat anti-rabbit IgG peroxidase-conjugated secondary antibody (Bradley et al., 1988). Duplicate gels were silver stained to show protein profiles and to reveal the specificity of anti-ABR antiserum.

### *RNA extraction and RT-PCR*

Total RNA was extracted from control and boron-deficient nodules frozen in liquid nitrogen. The tissues (ca. 250 mg) were ground with a mortar and pestle and RNA was prepared with an RNA extraction kit (ARN SV Total RNA Isolation System Promega, Madison, USA). RNA concentration and quality was determined by absorption at 260, 280 and 230 nm with a Biophotometer Eppendor (Hamburg, Germany) and integrity was estimated by