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Accumulation and localization of extensin protein in apoplast of pea root nodule under aluminum stress

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ABSTRACT

Cell wall components such as hydroxyproline-rich glycoproteins (HRGPs, extensins) have been proposed to be involved in aluminum (Al) resistance mechanisms in plants. We have characterized the distribution of extensin in pea (*Pisum sativum* L.) root nodules apoplast under short (for 2 and 24 h) Al stress. Monoclonal antibodie LM1 have been used to locate extensin protein epitope by immunofluorescence and immunogold labeling.

The nodules were shown to respond to Al stress by thickening of plant and infection thread (IT) walls and disturbances in threads growth and bacteria endocytosis. Immunoblot results indicated the presence of a 17-kDa band specific for LM1. Irrespective of the time of Al stress, extensin content increased in root nodules. Further observation utilizing fluorescence and transmission electron microscope showed that LM1 epitope was localized in walls and intercellular spaces of nodule cortex tissues and in the infection threads matrix. Al stress in nodules appears to be associated with higher extensin accumulation in matrix of enlarged thick-walled ITs. In addition to ITs, thickened walls and intercellular spaces of nodule cortex were also associated with intense extensin accumulation.

These data suggest that Al-induced extensin accumulation in plant cell walls and ITs matrix may have influence on the process of IT growth and tissue and cell colonization by *Rhizobium* bacteria.

1. Introduction

One consequence of legume and *Rhizobium* bacteria symbiotic association is the development of root nodules, unique organs for symbiotic nitrogen fixation. Nodules can be classified into two main groups according to their mode of development (Maunoury et al., 2008). Legumes such as *Phaseolus vulgaris* (bean) or *Lotus japonicus* form determinate nodules that have no permanent meristem and adopt a globular shape. The mature nodules contain a homogenous central tissue composed of infected cells fully packed with nitrogen-fixing bacteroids and some uninfected cells. Conversely, legumes such as *Medicago truncatula* or *Pisum sativum* (pea) form indeterminate nodules that possess a permanent meristem and elongate, to become cylindrical. In mature nodule of this type, several histological zones of consecutive developmental states can be distinguished (Vasse et al., 1990). The mature root nodule is made up of a central tissue, containing infected and uninfected cells (bacteroidal tissue), surrounded by a cortex. The nodule has an endodermis which divides the cortex into an outer cortex (nodule cortex) and inner cortex (nodule parenchyma) which contains the vascular bundles, each surrounded by a bundle endodermis (Van de Wiel et al., 1990). The formation of root nodules involves remarkable morphological changes in the apoplast. In plant tissues the apoplast is mainly composed of plant cell walls and intercellular spaces, but in the root nodule there is an additional apoplast zone, which is represented by infection threads (ITs). The IT is a tubular ingrowth, occurring due to invagination of the root hair cell wall, bound by a cylindrical primary wall which comprises esterified and un-esterified pectins, xyloglucans and cellulose fibrils (Rae et al., 1992; Brewin, 2004). Bacteria cells within the lumen of an IT are embedded in a plant cell wall-like matrix containing extensin and other glycoproteins secreted by the plant (Rae et al., 1992) and rhizobial exopolysaccharides (Niehaus et al., 1998). The IT grows as an intrusive tube at its apex within the plant cytoplasm. Extension growth of the IT depends on expansin activity in the IT walls (Sujkowska et al., 2007) and the secretion of matrix glycoproteins into the lumen, and is confined to the tip region of infection threads

Abbreviations: Al, aluminum; B, bacteria; Ba, bacteroid; CW, cell wall; IS, intercellular space; IT, infection thread; M, mitochondrion; MX, infection thread matrix; N, nucleus; P, plastid; Pa, parenchyma; W, infection thread wall; V, vacuole.

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(Gage, 2002). As an IT stops growing, irregular structures, including unwalled droplets of IT matrix material containing rhizobia, are formed (Brewin, 1998).

Extensins are known to be among the key components responsible for cell wall rigidification. They are cell wall structural proteins characterized by a series of $Ser(Hyp)_4$ that become arabinosylated, although their physical properties are highly variable as a result of specific glycosylation, hydroxylation and cross-linking with other cell wall components (Borner et al., 2002). Extensins may be detected in most tissues of the plants analyzed so far. Root nodule extensins (Brewin et al., 2000) are a major component in the infection thread matrix of legume nodules and were first identified using a rat monoclonal antibody MAC265 (VandenBosch et al., 1989; Rae et al., 1991, 1992). These glycoproteins are insolubilized by peroxide-mediated oxidative cross-linking as a rapid plant response to biotic and abiotic stresses. In plants, insolubilization of extensins has been demonstrated to be an inducible phenomenon occurring in response to wounding (Hirsinger et al., 1997; Merkouropoulos et al., 1999), elicitor treatment and pathogen attack (Bradley et al., 1992; Garcia-Muniz et al., 1998), water deficiency (Yoshiba et al., 2001) and aluminum stress (Kenzhebaeva et al., 2001; Kenjebaeva et al., 2001).

The establishment and activity of the legume-*Rhizobium* symbiosis have both been found to be extremely sensitive to aluminum (Al) stress (Bordeleau and Provost, 1994; Igual et al., 1997; Balestrasse et al., 2006). Al has been shown to adversely affect the nodulation process through inhibition of lateral root extension (Silva et al., 2001) and nodule initiation (Flis et al., 1993). In a previous work, we observed that Al-stress induced thickening of the infection thread walls of *M. truncatula* (Sujkowska-Rybkowska et al., 2012) and pea (Sujkowska-Rybkowska and Borucki, 2014), which may have an important influence on the process of tissue and cell colonization by *Rhizobium* bacteria. The increased evidence supports the view that the apoplast plays the major role in Al perception (Horst, 1995; Rengel, 1996). The rapid inhibition of root growth has been related to arrested cell elongation (Horst, 1995; Kataoka et al., 2003), cell wall thickening (Budíková et al., 1997), enhanced lignin and callose deposition (Zhang et al., 1994; Kochian, 1995), decreases in the mobility of the apoplast protein (Kataoka et al., 2003) and changes in the polysaccharide content of the cell wall (Tabuchi and Matsumoto, 2001), which causes cell wall thickening. Changes in the mechanical properties of plant cell walls exposed to Al-stress have been documented, but information on the Al-induced modifications in cell wall composition is very limited. To the best of our knowledge, findings on the occurrence of extensin in nodule apoplast during Al stress have not been reported so far, although this molecule is essential structural component of cell walls and infection threads.

In this work, the possible role of extensin in the plant-mediated control of nodule and infection thread growth during short Al stress was investigated. Structural changes in the nodule apoplast were analyzed using LM, rat anti-extensin monoclonal antibody.

2. Materials and methods

2.1. Biological material and electron microscopy investigations

Pea (*P. sativum* L. cv. Sześciotygodniowy) seeds were surface sterilized with 1% sodium hypochloride and germinated on 0.8% (w/v) water agar. Young seedlings with straight primary roots, 0.5 cm long, were selected and inoculated with *Rhizobium leguminosarum* bv. *viciae* wild-type strain 248 and grown as previously described (Sujkowska et al., 2007). The plants were watered three times a week with nitrogen-free Fahraeus medium and all solutions for plants were adjusted to pH 4.5 with HCl. Since long-term (measured days after the addition of Al) responses are not directly caused by Al, but might rather be a consequence of numerous other Al-related biochemical and physiological processes, they might be more misleading than short-term studies in determining the primary toxic effect of Al. Therefore, two-week-old plants were treated with 50 μM $AlCl_3$ for 2 and 24 h. Al concentration was selected based on previous work (Sujkowska-Rybkowska and Borucki, 2014). After treatment, all the plants were washed with distilled water and root nodules (ten per plant, five plants per pot) were collected for the investigation.

For electron microscopy investigations, hand sections of the 18-day-old nodules were fixed and embedded in glycid ether 100 epoxy resin (SERVA) according to Borucki and Sujkowska (2008).

2.2. Protein isolation and Western-blotting

Cell walls from the pea root nodules were obtained by modifying the procedure of York et al. (1986). Total cell wall proteins were extracted following the procedure described by Shailasree et al. (2004). Pea root nodules were macerated and repeatedly washed with 0.5 M phosphate buffer, pH 7.0, and water. The resultant pellet was suspended in 1.25 N HCl: absolute ethanol (1:1, v/v) at 4 °C overnight. Proteins were precipitated by 3 v of cold acetone. Acetone was decanted and the dried proteins were subjected to 12% SDS-PAGE with a protein concentration of 40 μg per lane. Protein concentration was determined using bovine serum albumin (BSA, Sigma) as standard. The separated proteins were blotted onto PVDF membrane using Hoefer mini Ve (Amersham Pharmacia Biotech) electrophoretic transfer apparatus according to the manufacturer's protocol. The blots were blocked in 2% fat-free milk in Tris-buffered saline (TBS: 10 mM Tris–HCl, pH 8.0, containing 150 mM NaCl) and probed with LM1, rat anti-extensin monoclonal antibody (1:50 dilution) (Smallwood et al., 1995; Rathbun et al., 2002). Subsequently, the blots were incubated with a goat anti-rat IgG (whole molecule) antibody conjugated with alkaline phosphatase (Sigma) diluted 1:1000 for 1 h, washed with TBS three times, and then stained with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma).

2.3. Immunolocalization

For immunolocalization experiments the tissue samples where embedded in butyl-methylmethacrylate resin (BMM) for fluorescence microscopy (FM) or LR White resin, an utrathin sectioned material for transmission electron microscopy (TEM). Rat monoclonal antibodie LM1 was used in nodule tissue sections (Dahiya and Brewin, 2000; Rathbun et al., 2002). The primary antibodie LM1 was a kind gift from K. Wieczorek (University of Natural Resources and Applied Life Sciences, Vienna).

For immunofluorescence labeling pea nodules (18-d-old) were fixed in a 4% paraformaldehyde MSB buffer for 2 h at room temperature. The material was dehydrated in a graded ethanol series, embedded in BMM resin and sectioned at 2 μm on microtome (Jung RMM 2065). After resin removal with acetone, sections were incubated in a blocking solution containing 5% skimmed milk powder in PBS (phosphate-buffered saline) for 1 h before application of the primary monoclonal antibody. Sections were washed in PBS and incubated in a primary antibody solution (1:20) with 1% skimmed milk powder for 90 min at room temperature before washing in PBS and applying a secondary (1:2000) goat anti-rat antibody conjugated to Alexa Fluor 488 (Molecular Probes) for 1 h. Control sections were incubated without the primary antibody in an incubation medium. Then sections were washed thoroughly as described above, and stained with 0.1% toluidine blue for 10 min, to minimize tissue autofluorescence. Longitudinal hand sections through the nodules were observed under Olympus-Provis