Micron 57 (2014) 13–22

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

Localization of hydrogen peroxide accumulation and diamine oxidase activity in pea root nodules under aluminum stress

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ARTICLE INFO

Article history:
Received 2 June 2013
Received in revised form
26 September 2013
Accepted 28 September 2013

Keywords:
Aluminum stress
Apoplast
Cytochemical localization
Diamine oxidase
Hydrogen peroxide
Infection thread
Pea-*Rhizobium* symbiosis

ABSTRACT

Aluminum (Al) is one of the environmental stressors that induces formation of reactive oxygen species (ROS) in plants. Hydrogen peroxide (H_2O_2) and H_2O_2-generated apoplast diamine oxidase (DAO) activity were detected cytochemically via transmission electron microscopy (TEM), in pea (*Pisum sativum* L.) root nodules exposed to high (50 μM $AlCl_3$, for 2 and 24 h) Al stress.

The nodules were shown to respond to Al stress by disturbances in infection thread (IT) growth, bacteria endocytosis, premature degeneration of bacteroidal tissue and generation of H_2O_2 in nodule apoplast. Large amounts of peroxide were found at the same sites as high DAO activity under Al stress, suggesting that DAO is a major source of Al-induced peroxide accumulation in the nodules. Peroxide distribution and DAO activity in the nodules of both control plants and Al-treated ones were typically found in the plant cell walls, intercellular spaces and infection threads. However, 2 h Al treatment increased DAO activity and peroxide accumulation in the nodule apoplast and bacteria within threads. A prolonged Al treatment (24 h) increased the H_2O_2 content and DAO activity in the nodule apoplast, especially in the thread walls, matrix and bacteria within infection threads. In addition to ITs, prematurely degenerated bacteroids, which occurred in response to Al, were associated with intense staining for H_2O_2 and DAO activity.

These results suggest the involvement of DAO in the production of a large amount of H_2O_2 in the nodule apoplast under Al stress. The role of reactive oxygen species in pea-*Rhizobium* symbiosis under Al stress is discussed.

1. Introduction

Aluminum (Al) is one of the most abundant elements in the soil, but it has no known function in biological processes. Aluminum limits the production of many important crops in acidic mineral soils. Consequently, in the past few years extensive studies have been carried out on the mechanism of Al toxicity and tolerance in plants (Matsumoto, 2000; Kochian et al., 2005; Silva, 2012). The common feature of aluminum toxicity symptoms is the enhanced production of reactive oxygen species (ROS), including hydrogen peroxide (Yamamoto et al., 2003; Jones et al., 2006). The studies of Al toxicity in plant roots suggest that ROS production is a determining factor in inhibition of root elongation (Yamamoto et al., 2003; Kobayashi et al., 2004) and cell growth by Al (Yamamoto et al., 2003). High ROS accumulation in response to Al-treatment of roots activates signal transduction pathways that lead to lipid peroxidation, DNA damage (Meriga et al., 2004) and cell death (Pan et al., 2001; Boscolo et al., 2003). In a previous investigation, increased ROS production and high activity of antioxidative enzymes in Al-treated pea root nodules was documented (Sujkowska-Rybkowska, 2012). Increased activity of ROS-scavenging enzymes and elevated levels of antioxidants as a result of aluminum toxicity confirmed the important role of ROS during Al stress (Tamás et al., 2003; Kobayashi et al., 2004).

The recent results suggest that an Al-induced increase in ROS levels in the root apoplast may lead to modifications in cell wall composition and properties (Horst et al., 1999; Sivaguru et al., 2000; Teraoka et al., 2002; Jones et al., 2006). Plant apoplast has received considerable attention for the location of ROS because many enzymes that produce or metabolize ROS are either found in the extracellular matrix or have their activity directed toward it (Bolwell et al., 2002). H_2O_2 produced by several enzymatic systems located in the apoplast and intracellular compartments has a key role in both development and defense (Apel and Hirt, 2004; Mittler et al., 2004). Nagy et al., 2004 have reported that the increase

Abbreviations: Al, aluminum; B, bacteria; Ba, bacteroid; CW, cell wall; DAO, diamine oxidase; H_2O_2, hydrogen peroxide; IS, intercellular space; IT, infection thread; M, mitochondrion; MX, infection thread matrix; N, nucleus; P, plastid; Pa, parenchyma; ROS, reactive oxygen species; S, starch; UC, uninfected cell; W, infection thread wall; V, vacuole.

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http://dx.doi.org/10.1016/j.micron.2013.09.007

in several peroxidase isozymes is the characteristic feature of Al-treated spruce seedlings. Al also activates another H_2O_2-producing enzyme, oxalate oxidase, mainly at lethal Al concentration inducing cell necrosis (Tamás et al., 2004).

In pea, as in most temperate legumes, rhizobia enter host tissues through a specialized structure, the infection thread (IT). The infection thread, filled with dividing and growing bacteria, is a tubular ingrowth of the plant cell wall. Within the thread, rhizobia are normally surrounded by a matrix containing plant glycoproteins and rhizobial exopolysaccharides (Gage, 2004; Monahan-Giovanelli et al., 2006). A major component of infection thread matrix and intercellular spaces are glycosylated extensins (Rathbun et al., 2002). These glycoproteins are insolubilized by peroxide-mediated oxidative cross-linking as a rapid plant response to pathogen attack (Otte and Barz, 1996), wounding (Bradley et al., 1992) and abiotic stresses, including aluminum (Kenzhebaeva et al., 2001). Extensin insolubilization causes wall stiffening and regulated polarized growth of the infection threads and tissue invasion by *Rhizobium* (Rae et al., 1991; Peretto et al., 1994; Wisniewski et al., 2000). Reduction of hydrogen peroxide levels in the threads after infection with the *Sinorhizobium meliloti* catalase-overexpressing mutant resulted in irregular, enlarged infection threads (Jamet et al., 2007). One possible source of hydrogen peroxide formation during infection and nodule organogenesis is diamine oxidase (DAO) (Wisniewski et al., 2000). Diamine oxidase from microbes, animals and plants oxidase the diamines putrescine (Put) and cadaverine (Cad) (Cona et al., 2006). Enzymes have been mostly found in the apoplast of mature tissues and it has been proposed that these enzymes have a special role in the localized production of hydrogen peroxide from polyamine substrates (Angelini et al., 1990; Padiglia et al., 1991). Increasing evidence suggested that peroxide generated by diamine oxidase participates in plant development and defense responses (Angelini et al., 1990; Wisniewski et al., 2000; Cona et al., 2006). The activity of diamine oxidase is modulated by various stress factors (Maccarrone et al., 1997; Cona et al., 2006), and a parallel increase in DAO activity was demonstrated, together with ligno-suberised depositions in wounded chick-pea (Angelini et al., 1990) and maize stems (Angelini et al., 2008). However, diamine oxidase activity under aluminum stress and its subcellular colocalization with hydrogen peroxide has not been demonstrated.

The mechanisms by which legumes respond and tolerate aluminum stress are poorly understood. The establishment and activity of the legume-*Rhizobium* symbiosis have both been found to be extremely sensitive to Al stress (Bordeleau and Provost, 1994). Legumes treated with Al show decreased nodulation and nitrogenase activity (Igual et al., 1997; Balestrasse et al., 2006). Aluminum 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).

This work focuses on hydrogen peroxide and diamine oxidase activity detection in pea root nodules under short-term aluminum stress using cerium chloride for electron microscopy. The role of reactive oxygen species in pea-*Rhizobium leguminosarum* symbiosis under Al stress is discussed.

2. Materials and methods

2.1. Plant material

Pea plants (*Pisum sativum* L. cv. Sześciotygodniowy) were inoculated with *R. leguminosarum* bv. *viciae* wild-type strain 248. Bacterial strains, plant inoculation and growth conditions have previously been described (Borucki and Sujkowska, 2008). The plants were watered with a nitrogen-free medium according to Fahraeus (1957) that had been adjusted to pH 4.5 with HCl. Two-week-old plants were treated with 50 μM $AlCl_3$ for 2 and 24 h. After treatment, the root nodules were collected for investigation.

2.2. Cytochemical localization of the hydrogen peroxide (H_2O_2) accumulation

Hydrogen peroxide was detected in pea root nodules treated with 0 (control material) and 50 μM $AlCl_3$ for 2 and 24 h. Samples were fixed, embedded, sectioned, and stained for conventional electron microscopy, as described by Bestwick et al. (1997). The histochemical method developed for localization of H_2O_2, based on the generation of cerium perhydroxides, was as follows: Fresh nodules were quickly cut lengthwise into small pieces (<1 mm) and were immediately perfused in 5 mM $CeCl_3$ in a 50 mM (w/v) 3-morpholinopropanesulfonic acid (buffer MOPS) (pH 7.2) for 2 h, prior to fixation in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2–7.4) for 2 h at room temperature. As a negative control, pea tissues were incubated without cerium chloride. Afterwards, nodules with and without cerium chloride (negative control) were post-fixed for 45 min at 4 °C in 1% osmium tetroxide in 0.1 M cacodylate buffer, were dehydrated in an ethanol series at room temperature, followed by three rinses in propylene oxide, embedded in epoxy resin Epon 812 (Fluka) and polymerized for 24 h at 60 °C. Ultrathin sections were stained with lead citrate for 30 s, followed by 1.2% aqueous uranyl acetate for 3 min. H_2O_2 was localized as electron-dense precipitates of cerium perhydroxides. Observations were made using a Morgagni 268C (FEI) transmission electron microscope. Photographic documentation was prepared with a Morada (SIS) digital camera and an iTEM (SIS) computer program.

2.3. Cytochemical localization of the diamine oxidase (DAO) activity

The diamine oxidase activity in nodule tissues was localized directly by utilizing the $CeCl_3$ method to visualize the distribution of the H_2O_2 product of amine oxidation, according to Slocum and Furey (1991). This method involves the trapping of enzyme-generated H_2O_2 by $CeCl_3$ to form an insoluble, electron-dense cerium perhydroxide product. Material for the investigation was collected as above. Sections of nodules were immediately perfused in 5 mM $CeCl_3$ in a 0.1 M Hepes buffer (pH 7.5) containing 50 mM 3-amino-1,2,4-triazole (AT, an irreversible inhibitor of catalase and some peroxidases) for 1 h at 25 °C. The tissues were then transferred to a fresh change of reaction buffer containing 10 mM putrescine (the amine substrate) and incubated for 4 h. All samples were provided with a fresh change of reaction buffer after an initial 2 h incubation. After incubation, all samples were washed in two 15-min changes of 0.1 M Na-cacodylate buffer (pH 6.0). Afterwards, nodules were fixed in 2% glutaraldehyde buffered with 0.1 M cacodylate (pH 7.0) overnight at 4 °C, rinsed in buffer, and post-fixed with 1% osmium tetroxide in the same buffer for 1 h, at 4 °C. Finally, the material was dehydrated, embedded, sectioned and observed under a Morgagni transmission electron microscope, as mentioned above.

3. Results

3.1. Localization of hydrogen peroxide using transmission electron microscopy

In control nodules cerium perhydroxide precipitates were found surrounding bacteria within the infection thread and in the thread wall, but not in thread matrix (Fig. 1A–D). The unwalled infection thread tip, where bacteria were released by endocytosis into