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LOCALIZATION OF REACTIVE OXYGEN SPECIES DURING SYMBIOSIS OF EARLY CLOVER AND *RHIZOBIUM LEGUMINOSARUM* BV. *TRIFOLII*

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In this work, clover was shown to respond to infection with *Rhizobium leguminosarum* bv. *trifolii* by producing reactive oxygen species. Superoxide radical and hydrogen peroxide were detected in infection threads and nodule primordia. The role of reactive oxygen species in clover-*Rhizobium leguminosarum* bv. *trifolii* symbiosis is discussed.

Key words: Cytochemistry, legume-rhizobium symbiosis, reactive oxygen species, *Rhizobium leguminosarum*, *Trifolium pratense* L.

INTRODUCTION

Generation and accumulation of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) is an early plant response to pathogen infection. The role of ROS in plant defense mechanisms is well established. ROS are directly toxic to microorganisms (Mehdy et al., 1996), trigger a localized hypersensitive response (HR) which limits and blocks pathogen penetration and development (Li et al., 2005; Montillet et al., 2005; Zaninotto et al., 2006), and are suggested to mediate the establishment of systemic defenses (systemic acquired resistance, SAR; Durrant and Dong, 2004). H_2O_2 functions as a substrate for crosslinking of proteins in the cell wall, which increases cell wall strength (Iwano et al., 2002), and as a second messenger for activation of defense genes (Orozco-Cardenas et al., 2001; Mellersh et al., 2002; Hu et al., 2003).

Recently, reactive oxygen species have been acknowledged as important regulators in a broad range of physiological processes such as senescence (Bhattachrjee, 2005), programmed cell death (Bethke et al., 2001; Overmyer, 2005; Van Breusegem and Dat, 2006), stomatal movement (Bright et al., 2006) and cell expansion (Foreman et al., 2003; Liszkay et al., 2004; Renew et al., 2005). ROS have been implicated as second messengers in several plant hormone responses (Kwak et al., 2006). H_2O_2 can regulate the plant cell cycle (Suzuki et al., 1999) and is essential for root gravitropism (Joo et al., 2001). In higher plants, ROS are also engaged in such reproductive processes as pollen tube growth (Potocky et al., 2007) and pollen-stigma interaction (McInnis et al., 2006a,b).

There is increasing evidence that ROS also play an important role in legume-rhizobia symbiosis (for review see Hérouart et al., 2002; Pauly et al., 2006). In alfalfa-*Sinorhizobium meliloti* symbiosis, ROS accumulation was observed during the infection process and in mature 6-week-old nodules in the walls of infected cells from the infection zone (zone II), but not in meristematic and fixation zones (Santos et al., 2001; Ramu et al., 2002). High levels of H_2O_2 associated with degrading bacteroids were shown in soybean and alfalfa nodules (Alesandrini et al., 2003; Rubio et al., 2004).

This work investigated the generation and accumulation of reactive oxygen species during the early steps of interaction between clover and *Rhizobium leguminosarum* biovar *trifolii* wild strain TA1.

MATERIALS AND METHODS

PLANT MATERIAL

Trifolium pratense L. cv. Ulka seeds were surface-sterilized with 70% ethanol and 3.5% calcium hypochlorite and pregerminated for 48 h (until root hairs 3–5 mm long appeared) under sterile conditions. After inoculation with *Rhizobium leguminosarum*

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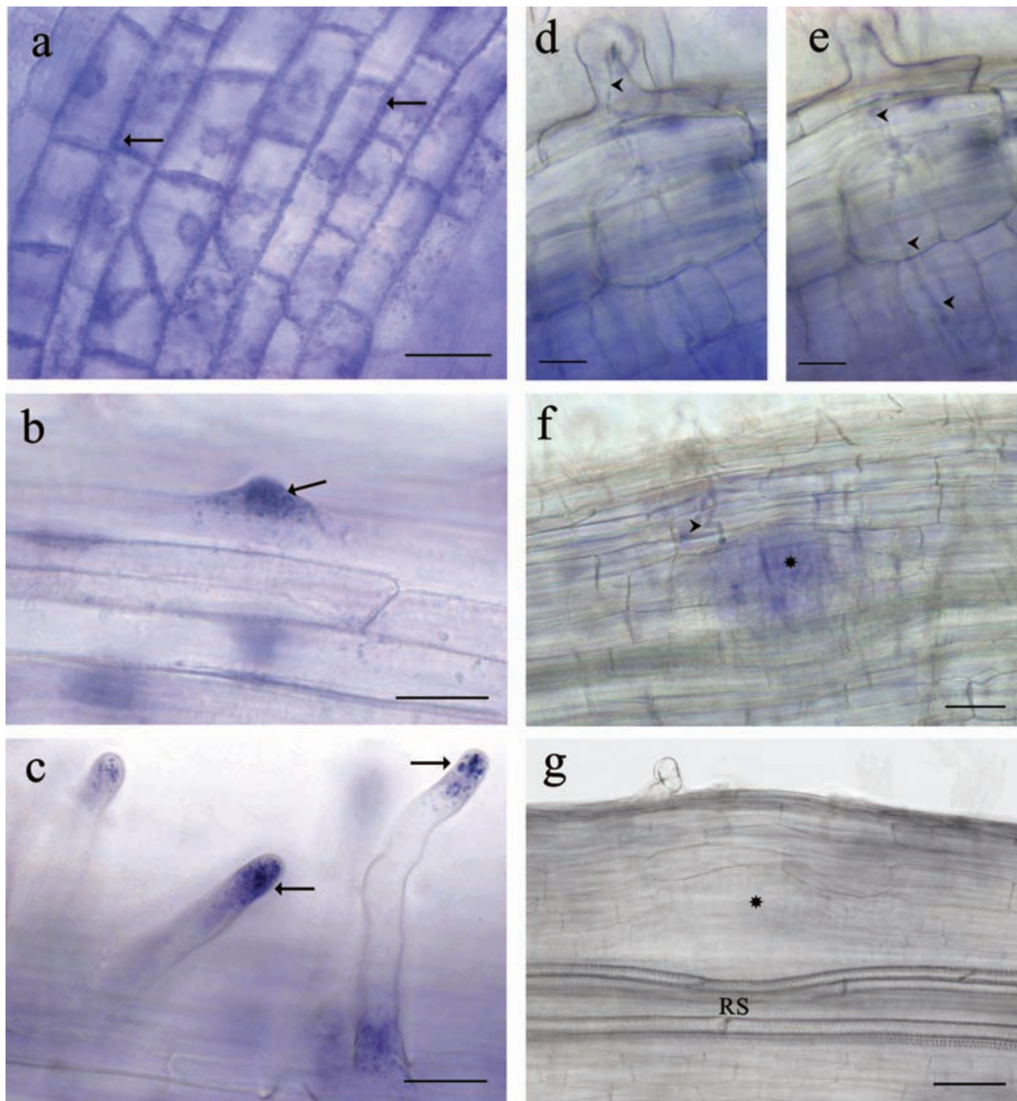

Fig. 1. Histochemical localization of superoxide radical accumulation in clover root inoculated with *R. leguminosarum* bv. *trifolii* wild strain TA1. **(a)** Root elongation zone. Root hair zone with emerging **(b)** and mature **(c)** root hairs. The same infection thread (arrowheads) elongating through root hair **(d)** and root cortex **(e)**. Nodule primordia (rosettes) treated with NBT **(f)** and $MnCl_2$ before NBT staining **(g)**. RS – root stela; arrows – NBT precipitates. Bars = 20 μm in a–c; 80 μm in d–g.

biovar *trifolii* wild strain TA1, they were transferred to modified Fahraeus nitrogen-free medium (Sahlman and Fahraeus, 1963). Plants were cultivated at 20–25°C under light intensity 72 $\mu Es^{-1}m^{-2}$ (PhAR) and a 16 h photoperiod (Łotocka et al., 1997).

HISTOCHEMICAL LOCALIZATION OF SUPEROXIDE ($O_2{\cdot}^-$)

Production of $O_2{\cdot}^-$ was detected with nitro blue tetrazolium (NBT) forming dark blue insoluble precipitates in the presence of $O_2{\cdot}^-$. Roots excised 3 and 7 days after inoculation were incubated in 25 mM K-HEPES [4-(2-hydroxyethyl)-1-piperazineethane-sulphonic acid] buffer (pH 7.6) containing 0.1 mg mL^{-1} NBT for 1 h in the dark (Hernandez et al., 2001). The roots were than cleared in lactic acid and examined with a Provis AX light microscope (Olympus). The specificity of NBT staining for $O_2{\cdot}^-$ was demonstrated by incubation of roots in the infiltration buffer with 10 mM $MnCl_2$ as the superoxide radical scavenger.

HISTOCHEMICAL LOCALIZATION OF HYDROGEN PEROXIDE (H_2O_2)

H_2O_2 localization was determined by light microscopy after the method of Thordal-Christensen et al. (1997) using 1 mg mL^{-1} 3, 3'-diaminobenzidine (DAB)-HCl

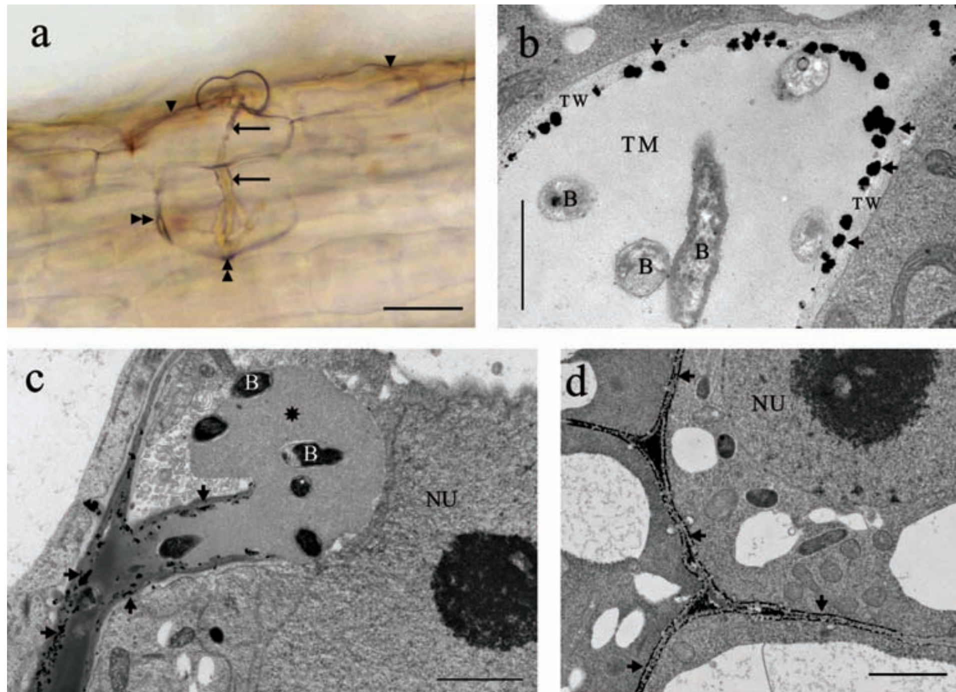

Fig. 2. (a) Detection of hydrogen peroxide accumulation. Infection thread (arrows), rhizodermis (arrowheads) and cortex cell walls (double arrowheads) show positive DAB staining. Bar = 40 μm. Electron-dense precipitates formed in the presence of cerium chloride (short arrows) are visible in the cell walls of the infection thread **(b, c)** and nodule primordium cells **(d).** Note the absence of precipitates in the infection thread tip (rosette; **c**). B – bacteria in infection thread; NU – cell nucleus; TM – infection thread matrix; TW – infection thread wall. Bars = 1 μm in (b); 2 μm in (c, d).

(pH 3.8). DAB polymerizes in contact with H_2O_2 in the presence of peroxidase and is visualized as brown coloration. Entire roots (3 or 7 days after inoculation) were incubated in DAB for 1.5 h under -0.04 MPa air pressure. The material was cleared in lactic acid and examined with a Provis AX microscope (Olympus).

Histochemistry based on generation of cerium perhydroxide precipitate was used for detection of H_2O_2 accumulation by transmission electron microscopy (Bestwick et al., 1997). Samples of inoculated roots were incubated in freshly prepared 5 mM $CeCl_3$ in 50 mM MOPS [3-(N-morpholino)propanesulfonic acid] at pH 7.2 for 1 h. The control for H_2O_2 localization was run in the presence of 10 mM ascorbic acid as H_2O_2 scavenger.

TREATMENT WITH INHIBITORS

Samples of inoculated roots were preincubated for 30 min in 50 mM MOPS containing either 3 mM sodium azide to inhibit peroxidase or 20 μM ATZ (amino-1,2,4-triazole) to inhibit catalase. Next they were transferred to $CeCl_3$ solution supplemented with inhibitors at the same concentrations and incubated for 1 h (Bestwick et al., 1997).

The material was processed for TEM as described by Łotocka et al. (1997). Ultrathin sections were contrasted with uranyl acetate for 3 min and lead citrate for 0.5 min and analyzed with a Morgagni 268 (FEI Company) transmission electron microscope operating at 80 kV.

RESULTS

The insoluble blue formazan precipitates indicating $O_2{\cdot}^-$ generation and accumulation were found in uninfected and infected roots in the root elongation zone, mostly in the epidermal cell walls (Fig. 1a). In the root part proximal to the elongation zone, staining was restricted to the emerging and the newly formed root hairs (Fig. 1b). In the root elongation zone, root hairs were strongly stained at the growing tip (Fig. 1c).

In infected roots, superoxide radical accumulation was observed also in infection threads, which elongated through root hairs, rhizodermis cells, root cortex cells (Fig. 1d,e) and in nodule primordia (Fig. 1f). $O_2{\cdot}^-$ was abolished by treating roots with 10 mM $MnCl_2$ (Fig. 1g).

In infected roots, colored products of the DAB reaction, which show sites of H_2O_2 accumulation, were located by LM in the infection threads and in the cell walls of the rhizodermis and root cortex cells (Fig. 2a). Samples incubated with $CeCl_3$ and ana-

lyzed in TEM gave more precise H_2O_2 localization. Cerium perhydroxide precipitates formed in intercellular and intracellular infection threads, principally between the thread wall and thread matrix (Fig. 2b). Close to the unwalled infection thread tip, H_2O_2 accumulation decreased and no H_2O_2 could be detected at the thread tip, where bacteria were released by endocytosis into the plant host cell (Fig. 2c). Nor was H_2O_2 observed around the bacteria in infection threads and in symbiosomes. In the host plant cells, H_2O_2 was located in the cell walls of nodule primordium cells (Fig. 2d). The labeling was completely abolished by ascorbic acid (data not shown), indicating that it reflects H_2O_2 accumulation.

Treatment of the samples with catalase and peroxidase inhibitors did not substantially alter H_2O_2 accumulation (data not shown).

DISCUSSION

In clover, as in most temperate legumes, rhizobia infect host root via root hairs and enter host tissues through a specialized structure, the infection thread. Bacteria are released from the infection threads into host cells by endocytosis. Within the infection threads, rhizobia are surrounded by a matrix containing material of plant and rhizobial origin (Krishnan, 2002; Gage, 2004). According to Wisniewski et al. (2000) and Rathbun et al. (2002), matrix extensin-like glycoprotein, a major component of the infection thread matrix, is present in the thread in two forms: soluble and thus extractable (only at the tip region) and insoluble (elsewhere). Glycoprotein insolubilization probably is the result of H_2O_2-dependent protein crosslinking, and serves in the regulation of polarized growth of the infection thread. Reduction of the H_2O_2 level in threads after infection with the *Sinorhizobium meliloti* catalase-overexpressing mutant resulted in irregular, enlarged infection threads (Jamet et al., 2007).

The suggestion that glycoprotein insolubilization is due to H_2O_2-dependent protein crosslinking is supported by the our observations of ROS localization during clover root infection. In this work, H_2O_2 accumulation was observed within the infection thread, where matrix glycoprotein insolubilization is supposed to occur. Unlike in mature parts of the infection thread, no H_2O_2 was detected at the thread tip, where, as reported by Wisniewski et al. (2000) and Rathbun et al. (2002), the thread matrix is in soluble form. Two processes are related to the thread tip: continuous extension of the thread, and bacteria endocytosis. They result in host cell colonization and the formation of symbiosomes. Both of them depend on matrix fluidity, and both are easier when secretion of the (initially fluid) matrix glycoprotein is faster than crosslinking.

In this work H_2O_2 was detected neither in bacteria within threads nor in symbiosomes in infected young host cells. Santos et al. (2001) reported similar results in alfalfa-*Sinorhizobium meliloti* symbiosis. This suggests that bacteria and bacteroids have an efficient antioxidant system. The superoxide dismutases (Santos et al., 2000; Rubio et al., 2004) and three catalases differentially expressed during nodule formation (Jamet et al., 2003) are the best-characterized elements of the *Sinorhizobium meliloti* antioxidant machinery, but other enzymatic and non-enzymatic systems might add to the rhizobia antioxidant defense (Barloy et al., 2004; Dombrecht et al., 2005).

ROS generation in response to symbiotic infection is consistent with the idea that rhizobia are initially perceived as invaders by the plant, which reacts with a defensive response as long as the symbiont is not recognized as such (Vasse et al., 1993). In the absence of an appropriate bacterial signal, superoxide and subsequent hydrogen peroxide accumulation in clover nodule primordia can be interpreted as a prolonged oxidative burst which triggers the plant's defensive response when it has recognized an incompatible organism (Santos et al., 2001).

Which enzymes are responsible for ROS formation during infection and nodule organogenesis? The lack of significant changes in $CeCl_3$ "staining" of the infection thread and cell walls after preincubation in sodium azide (peroxidase but not NADPH oxidase inhibitor) suggests that the superoxide radicals detected in early symbiosis of clover and *Rhizobium leguminosarum* bv. *trifolii* (this work) were generated by membrane-bound NADPH oxidase. Ramu et al. (2002) and Rubio et al. (2004) observed inhibition of the oxidative burst with DPI (a specific inhibitor of NADPH oxidase) in early stages of alfalfa-*Sinorhizobium meliloti* symbiosis. Other sources for H_2O_2 such as cell wall peroxidases, germin-like oxalate oxidases and diamine oxidases are also possible (Wojtaszek, 1997; Wisniewski et al., 2000).

The failure of ATZ (catalase inhibitor) to induce H_2O_2 accumulation demonstrates that reduced catalase activity does not necessarily lead to a corresponding increase in H_2O_2 concentration. I can suggest that the potential increase in detectable H_2O_2 must be prevented by its utilization in oxidative-reductive processes or by scavenging by other antioxidant systems.

Superoxide radical generation detected in the growing part of the hair cell and in the elongation zone of both uninfected and infected clover roots is related to physiological processes of cell elongation. It was shown in *Zea mays* (Liszkay et al., 2004) and *Arabidopsis thaliana* roots (Foreman et al., 2003) that superoxide radicals can be converted to H_2O_2 and then to hydroxyl radicals (·OH), which by the polysaccharide-cleaving reaction affect wall-loosening and as a result cell elongation growth. Thus ·OH

controls root elongation and root hair cell polar growth.

In this work, ROS accumulation was observed during infection and the early stages of clover-*Rhizobium leguminosarum* bv. *trifolii* symbiosis. It is important to note, however, that H_2O_2 production is inhibited in the first hours of infection (Shaw and Long, 2003). Moreover, bacterial strains with impaired or enhanced H_2O_2 detoxification capacity show defective symbiosis (Jamet et al., 2003). It clearly is a complex process, and the relationship between ROS and legume-rhizobia symbiosis is a subtle one.

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