Research Note

Oxidative Burst in Alfalfa-*Sinorhizobium meliloti* Symbiotic Interaction

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Reactive oxygen species are produced as an early event in plant defense response against avirulent pathogens. We show here that alfalfa responds to infection with *Sinorhizobium meliloti* by production of superoxide and hydrogen peroxide. This similarity in the early response to infection by pathogenic and symbiotic bacteria addresses the question of which mechanism rhizobia use to counteract the plant defense response.

Additional keywords: hypersensitive response, nitrogen fixation, symbiosis.

The oxidative burst is an early response in plant defense reactions toward pathogens. After a transient and nonspecific weak oxidative burst, a massive production of reactive oxygen species (ROS), namely superoxide (O_2^-) and hydrogen peroxide (H_2O_2), is observed in response to infection with avirulent but not with virulent strains (Baker and Orlandi 1995; Hammond-Kosack and Jones 1996; Van Camp et al. 1998). This constitutes an early event in the protective or hypersensitive response (HR) of plants that have recognized the avirulent pathogen (Lamb and Dixon 1997; Medhy 1994). The HR is characterized by a localized plant cell death at the site of infection that blocks further invasion of bacteria and by the induction of a systemic resistance to virulent pathogens (Alvarez et al. 1998; Dangel et al. 1996; Levine et al. 1994). ROS are believed to act in this process at several levels; for instance, they might participate in the direct killing of the pathogens and plant cells. Although O_2^- and H_2O_2 are moderately reactive, they can be converted to a more reactive species such as the hydroxyl radical and, possibly, peroxynitrite (Bolwell 1999). There is, however, little conclusive evidence available for the active killing of pathogens by ROS. More clear is the role of H_2O_2 in the cross-linking of host cell wall proteins, thereby reinforcing the physical barrier against pathogen ingress (Blumwald et al. 1998; Lamb and Dixon 1997). Perhaps the most important role of ROS, however, is as a signaling intermediate that triggers local defense-related

gene expression, programmed cell death, and systemic acquired resistance (Alvarez et al. 1998; Jabs et al. 1996; Mittler et al. 1996).

What occurs in the symbiotic interaction leading to atmospheric nitrogen fixation? In nitrogen-poor soils, rhizobia respond to a plant signal (flavonoids) by producing nod factors that elicit nodule organogenesis on legume roots (Bladergroen and Spaink 1998; Peters et al. 1986; Schultze and Kondorosi 1996; Truchet et al. 1991). Bacteria enter the developing nodule via an infection thread and, once released into the host cytoplasm, undergo differentiation into nitrogen-fixing bacteroids. In contrast to infection with pathogens, a close association is established with symbiotic bacteria. There are, however, a few experiments suggesting that in the symbiotic interaction with rhizobia, bacteria are initially recognized as intruders but then prevent or overcome plant defense responses (Baron and Zambryski 1995; Djordjevic et al. 1987; Gamas et al. 1998; Niehaus et al. 1993; Parniske et al. 1990; Parniske et al. 1991; Vasse et al. 1993). We therefore investigated whether alfalfa responded to *Sinorhizobium meliloti* infection by producing an oxidative burst.

O_2^- production was specifically detected by the dark precipitate that was formed upon reduction of yellow nitroblue tetrazolium (NBT) by superoxide (Doke 1983). At various times after infection, entire roots were immersed in a solution of 0.1% NBT, 10 mM $NaNO_3$, and 50 μ M NADPH for 15 to 30 min. Staining was found in infection threads and in infected cells in nodules up to 9 days old (Fig. 1A to E). Although the NBT test was less sensitive, in some 16- and 18-day-old nodules faint coloration was seen in the infection zone, suggesting the prolonged production of superoxide (Fig. 1F). NBT staining was completely abolished by a previous 5-min treatment with the superoxide scavenging compound, MnTM-2-PyPCL₅ (25 μ M) (Faulkner et al. 1994), which in contrast to superoxide dismutase, can enter plant cells (Fig. 1D).

In alfalfa nodules induced by *S. meliloti*, steps of bacterial development are correlated with specific histological zones (Vasse et al. 1990). The meristematic zone I does not contain bacteria, which are released from the infection threads in zone II and stop to multiply and start to differentiate into bacteroids. In zone III the bacteroids fix nitrogen, and in zone IV both bacteria and plant cells degenerate. H_2O_2 production was

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detected in ultrathin sections of 6-week-old nodules as an electron-dense deposit stained with cerium chloride (Bestwick et al. 1997). A significant cerium chloride labeling was seen only in the walls of infected cells (zone II) and in some infection threads (Fig. 2A and B). No staining was found in the meristematic zone I and fixation zone III (not shown). The labeling was completely abolished by 25 µg of catalase treatment per milliliter of the sample (Fig. 2C), indicating that it reflects H₂O₂ accumulation. Thus, alfalfa produces a prolonged oxidative burst in response to *S. meliloti* infection.

This oxidative burst in response to symbiotic infection is consistent with rhizobia being initially perceived as invaders by the plant. In normal conditions, however, the plant reaction appears to limit only the infection and does not lead to the rejection of invading symbiont (Vasse et al. 1993). Ramu et al. (1999) recently reported that an oxidative burst at the very early stage of infection is necessary for the induction of Rip1 peroxidase in the root of *Medicago truncatula*. Additionally, this peroxidase induction is dependent on bacterial nod factors. H₂O₂ and peroxidase may be involved in the regulation of infection (Cook et al. 1995; Wisniewski et al. 2000) by cell wall cross linking, limiting the ingress of bacteria in a manner analogous to that of infection with pathogens. Thus, the oxidative burst at the early stage of infection appears to be involved in the control of infection and nodulation. Why does an oxidative burst during symbiosis not trigger plant defense reaction? Presumably, rhizobia

inhibit signaling pathways, leading to the deleterious defense cascade. There are suggestions that alfalfa can react with a HR-like response as long as the symbiont is not recognized as useful. Autofluorescence that indicates the presence of phenolic substances and phytoalexins that accumulate in cells undergoing HR (Graham and Graham 1991) has been observed in abortive infections as a result of nodule number feedback control (Vasse et al. 1993). A general plant defense response is induced in nodules elicited by exopolysaccharide (EPS I)-deficient mutants, and it has been proposed that EPS I or a related compound may act as a suppressor of the alfalfa defense system (González et al. 1996; Niehaus et al. 1993). Thus, the prolonged maintenance of the oxidative burst in the infection zone might keep the plant's options open, allowing it, in the absence of the appropriate bacterial signal, to switch to a defense response against what could ultimately prove to be a useless parasite.

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Fig. 1. Histochemical localization of O₂⁻ generation in infection threads and nodules of alfalfa by nitroblue tetrazolium (NBT) staining. NBT forms a dark formazan precipitate upon reduction by superoxide. **A**, Infection threads. **B**, Root hair and nodule primordia. **C**, Nodule 5 days after infection. **D**, Nodule treated with MnTM-2-PyPCl₅ before NBT staining 6 days after infection. **E and F**, Nodules, 9 and 18 days after infection, respectively. Ten to nineteen entire, treated roots were examined in each experiment; nodules showed a similar pattern. Arrows indicate formazan precipitate in infection threads. Scale bars: **A**, 50 µm; **B to F**, 100 µm.

Fig. 2. Detection of H_2O_2 production in 6-week-old nodules as electron-dense precipitate formed in the presence of cerium chloride. **A**, Staining in the cell walls (arrows) of infected cells from the infection zone. **B**, Staining in the infection thread matrix and cell wall (arrows). **C**, No staining in catalase pretreated samples. A similar pattern was observed in 20 nodules stained with cerium chloride. Nodules were fixed with 2.5% glutaraldehyde in 50 mM cacodylate buffer (pH 7.4) for 2 h at room temperature, postfixed with 1% osmium tetroxide for 45 min, dehydrated in a graded series of alcohol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and Reynold's lead citrate and examined in a CM12 electron microscope (Philips Electronic Instruments, Mahwah, NJ, U.S.A.) operating at 60 kV. b = bacteroids; cw = cell wall; ic = infected cell; it = infection threads; m = mitochondria; nic = uninfected cell; pbm = peribacteroid membrane; v = vacuole; vs = vesicles. Scale bars: **A and C**, 1 μm ; **B**, 0.2 μm .

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