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The arbuscular mycorrhizal fungus *Glomus intraradices* induces intracellular calcium changes in soybean cells

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Abstract — Calcium has been largely hypothesized to be involved in the arbuscular mycorrhizal (AM) signal transduction. A transient elevation in cytosolic Ca^{2+} concentration is considered one of the earliest responses detected in plant cells challenged with different stimuli. In this paper the detection of Ca^{2+} transients was selected as a tool to analyse whether plant cells perceive diffusible molecules released by the AM fungus *Glomus intraradices*. An aequorin-transformed line of soybean cells was used as experimental system. When cells were treated with the medium in which *Gl. intraradices* spores germinated, a rapid and significant increase in cytosolic Ca^{2+} level was induced and dissipated within 30 min. The medium did not induce either intracellular accumulation of reactive oxygen species or cell death. A detectable Ca^{2+} elevation was triggered also by the medium of fungal extraradical mycelium, associated to mycorrhizal hairy roots of carrot. These results indicate that *Gl. intraradices* releases diffusible molecules that are sensed by host plant cells through a Ca^{2+}-mediated signalling during different steps of its life cycle.

Key words: aequorin method, arbuscular mycorrhizal symbiosis, calcium signalling, *Glomus intraradices*, plant cell cultures.

INTRODUCTION

The majority of terrestrial vascular plants are able to establish a mutualistic association, the arbuscular mycorrhizal (AM) endosymbiosis, with soil fungi belonging to Glomeromycota. Both partners benefit from a bidirectional exchange of nutrients: plant mineral nutrition (in particular phosphate) is improved by the fungus, which in turn obtains photosynthates (Harrison 2005). The intimate integration between plant roots and fungal hyphae is achieved through a sequence of steps, and an exchange of molecular signals between the two partners is required.

Advances in understanding AM symbiosis signalling have been made in these last few years (Smith *et al.* 2006). During the pre-symbiotic phase a molecular dialogue between plant and fungus keeps both organisms timely informed about their reciprocal proximity. Plant root exudates containing strigolactones (Akiyama *et al.* 2005) are sensed by the AM fungus, and enhance hyphal growth and branching (branching factor; Buee *et al.* 2000). Plants perceive a diffusible signal generated by the AM fungus, up to now of unknown chemical nature, the myc factor (Kosuta *et al.* 2003), and respond by increasing the development of lateral roots (Oláh *et al.* 2005). In the absence of any physical contact between the two organisms, these diffusible signals induce the activation of specific genes in both plant and fungus (Weidmann *et al.* 2004; Breuninger and Requena 2004). These early responses are part of an "anticipation program", which prepares the symbionts for a successful association (Paszkowski 2006). How plant and fungus perceive and transduce these symbiotic signals is not at the moment fully understood. In analogy with the *Rhizobium*-legume symbiosis (Oldroyd *et al.* 2005), Ca^{2+} ions are envisaged to be involved in the signalling pathway induced in plants by the myc factor (Parniske 2004). Ca^{2+} is considered as the most common intracellular messenger which couples a wide array of extracellular stimuli with specific physiological responses (Hetherington and Brownlee 2004). Transient elevations of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) can be recorded

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in cells undergoing biotic or abiotic stresses, and encode definite information which is subsequently transduced in a cascade of cellular events (SANDERS *et al.* 2002).

In this context, we investigated the possible occurrence of a Ca^{2+} response induced by the culture medium of the AM fungus *Glomus intraradices* in plant cell suspension cultures. As Ca^{2+} reporter we used the aequorin method, which is considered a very reliable, sensitive tool for measuring intracellular Ca^{2+} variations (MITHÖFER and MAZARS 2002). Soybean cells stably expressing the Ca^{2+}-sensitive photoprotein apoaequorin were used as experimental system.

MATERIALS AND METHODS

Plant cell cultures - Cell suspension cultures of soybean (*Glycine max* L., line 6.6.12) stably expressing cytosolic aequorin were grown as described by MITHÖFER and MAZARS (2002).

Fungal strains, growth conditions and preparation of culture filtrates - Glomus intraradices DAOM 181602 spores were obtained *in vitro* from mycorrhizal carrot root organ cultures, surface sterilized two times in 3% Chloramine T for 5 minutes, and stored at 4°C. Spore germination was induced by placing the spores in sterile distilled water at 26°C in the dark. After 14 days the majority of spores were germinated and water was collected for cell culture treatments. Mycelial exudates were collected by growing mycorrhizal carrot root cultures in separate Petri dishes: one half of the dish contained the growing mycorrhizas while the other half was filled with sterile water. As roots and extraradical mycelium grew, only hyphae were able to pass over the Petri dish septum and started growing in the water compartment. Fourteen days later water was gently collected with a pipette. Fungal exudates were lyophilized and subsequently resuspended in plant cell culture medium for cell treatments. The final dose applied to cells corresponded to 10-fold fungal medium concentration.

Aequorin-dependent Ca^{2+} measurements - In vivo reconstitution of aequorin was performed by incubating the cells with 5 μM coelenterazine overnight in the dark. After extensive washing with fresh medium, cells were transferred to the luminometer cell chamber. Treatments with the fungal culture medium and calibration of the light signal into $[Ca^{2+}]$ values were carried out as previously described (NAVAZIO *et al.* 2002).

Intracellular ROS detection - Intracellular production of reactive oxygen species was measured according to MAXWELL *et al.* (1999), by loading the cells with 15 μM 2',7'- dichlorodihydrofluorescein diacetate (H_2DCF-DA) for 15 min. After extensive washing with fresh medium, cells were treated with the fungal culture filtrate and observed within 10 min under a Leica DMR light/fluorescence microscope. DCF was excited at 488 nm and emitted fluorescence was detected through a 520 bandpass filter.

Cell viability - Cell viability was determined, after 2 h treatment with the fungal culture filtrate, by the Evans Blue method (BAKER and MOCK 1994).

RESULTS

The culture medium of *Glomus intraradices* spores, collected 14 days after spore germination, was applied to soybean cells stably expressing cytosolic aequorin. A significant transient increase in $[Ca^{2+}]_{cyt}$ was recorded, reaching a maximum peak (about 0.9 μM) after 3.5 min and nearly dissipating within 12 min. A minor flattenened Ca^{2+} elevation (0.34 μM after 16 min) was observed thereafter. No $[Ca^{2+}]_{cyt}$ change was induced by challenging soybean cells with the cell culture medium only (Fig. 1). This result indicates that diffusible molecules released by germinating spores of an AM fungus are perceived by plant cells and trigger a Ca^{2+} response. Interestingly, also the me-

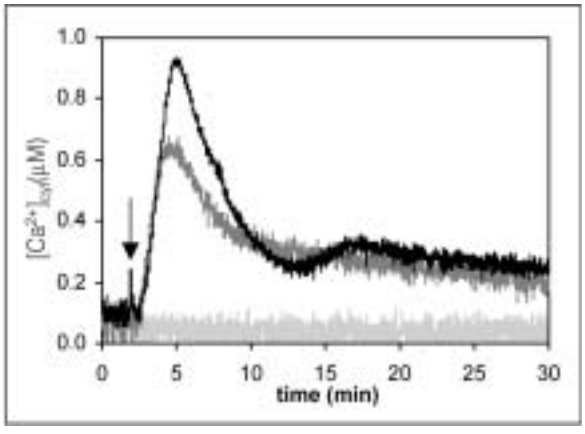

Fig. 1 — Monitoring of $[Ca^{2+}]_{cyt}$ changes triggered in soybean cells by the culture medium of *Gl. intraradices* germinated spores (*black trace*), the culture medium of the fungal extraradical mycelium (*dark grey trace*) and the cell culture medium only (*light grey trace*, control). Addition (100 s) is indicated by the arrow. The traces are representative of two independent experiments with similar results.

dium of the extraradical mycelium, growing in an aqueous compartment kept separate from mycorrhizal carrot root cultures, generated a detectable cytosolic Ca^{2+} transient, although of smaller amplitude (Fig. 1).

The possible induction of defence responses in soybean cells by *Gl. intraradices* culture medium was evaluated by measuring intracellular accumulation of reactive oxygen species (ROS) and cell viability. Both parameters were found not to be affected by the fungal diffusible molecules (Fig. 2A and B).

DISCUSSION

The present findings demonstrate that Ca^{2+} ions are involved in the transduction by plant cells of an AM fungal diffusible signal. These data support previous hypotheses based on a parallelism with the *Rhizobium*-legume symbiosis, in which the role of Ca^{2+} signalling has been already ascertained (Oldroyd and Downie 2004). Furthermore, they indicate that a signal exchange is already in place very early in the rhizosphere, before any direct contact between the plant and the fungus, favouring the following encounter of the two symbionts. The observed transient Ca^{2+} elevation may be considered as an initial event in a progression of coordinated, sequential cellular responses leading in plants to the complete functional integration of the two symbionts.

Previous results have demonstrated that even non-germinated spores of AM fungi can produce the signalling molecules (Navazio *et al.* 2006). The release of a signal into the culture medium by the extraradical mycelium, which is primarily associated with phosphate and inorganic nitrogen acquisition from the rhizosphere (Harrison 2005), indicates that the AM factor is secreted also by the fungal hyphae even when the symbiosis is in place, possibly in view of their ability to colonize other neighbouring roots.

The diffusible signalling molecules were not found to alert defence responses, such as intracellular ROS production and cell death, suggesting that the AM fungus is perceived as a compatible host since the very beginning of its possible association with the plant.

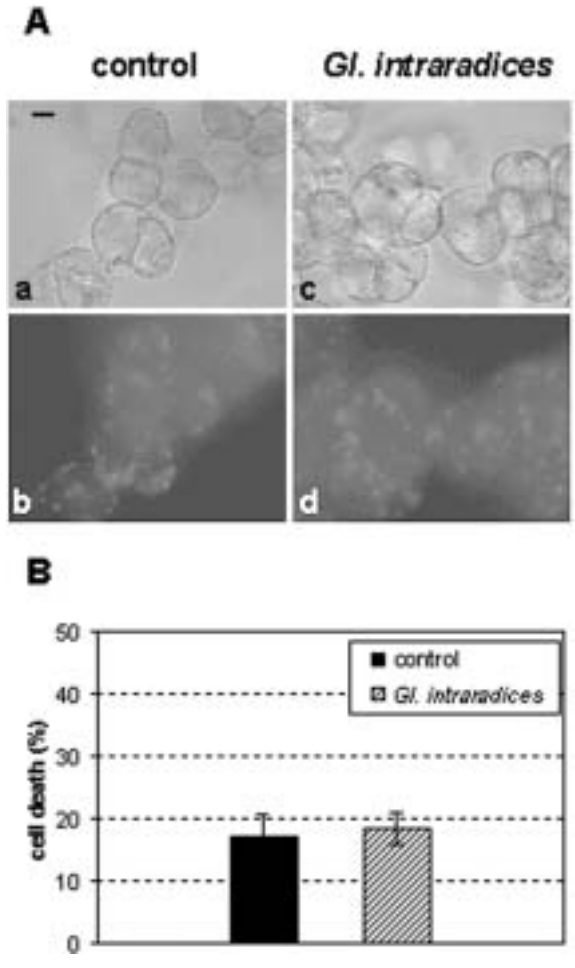

Fig. 2 — **A.** Detection of intracellular accumulation of ROS in control cells (a, b) and cells treated with *Gl. intraradices* medium (c, d). Light (a, c) and fluorescence (b, d) microscope images of the same field are reported. Bar: 10 µm. **B.** Cell viability in control cells and cells treated with *Gl. intraradices* medium. Results are the means ± SD of three experiments.

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