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Arbuscular mycorrhizal fungi induce differential activation of the plasma membrane and vacuolar H$^+$ pumps in maize roots

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Abstract Roots undergo multiple changes as a consequence of arbuscular mycorrhizal (AM) interactions. One of the major alterations expected is the induction of membrane transport systems, including proton pumps. In this work, we investigated the changes in the activities of vacuolar and plasma membrane (PM) H$^+$ pumps from maize roots (*Zea mays* L.) in response to colonization by two species of AM fungi, *Gigaspora margarita* and *Glomus clarum*. Both the vacuolar and PM H$^+$-ATPase activities were inhibited, while a concomitant strong stimulation of the vacuolar H$^+$-PPase was found in the early stages of root colonization by *G. clarum* (30 days after inoculation), localized in the younger root regions. In contrast, roots colonized by *G. margarita* exhibited only stimulation of these enzymatic activities, suggesting a species-specific phenomenon. However, when the root surface H$^+$ effluxes were recorded using a noninvasive vibrating probe technique, a striking activation of the PM H$^+$-ATPases was revealed specifically in the elongation zone of roots colonized with *G. clarum*. The data provide evidences for a coordinated regulation of the H$^+$ pumps, which depicts a mechanism underlying an activation of the root H$^+$-PPase activity as an adaptative response to the energetic changes faced by the host root during the early stages of the AM interaction.

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Introduction

The bidirectional exchange of nutrients is the basis of the arbuscular mycorrhizal symbiosis; in this way, the fungus interacts with host plant roots to increase their absorption of water, phosphate, and other nutrients from the soil. In turn, the plant provides photosynthesized sugars to the fungus, a phenomenon that provokes many cellular, physiological, and energetic changes in the host roots (Harrison 2005; Ramos et al. 2008a). Variations have been observed in the

host growth responses depending on the particular plant genotype and fungal species involved. In fact, it has been observed in many instances that during the early stages of mycorrhizal colonization, the host plant exhibits no growth increase or even growth depression. These depressions will occur when fungal demands for photoassimilates outweigh the benefits obtained to the host. Nevertheless, it is now becoming clear that the cost/benefit relationship is more complex than was previously thought (Li et al. 2008).

The development of AM interaction starts when hyphal growth and branching are followed by the formation of appressorium leading to the hyphal penetration in the root system (Giovannetti et al. 1993, 1996). Inside the root cortex, fungal hyphae form highly branched arbuscules invaginated in the plasma membrane of several cortical cells. A specific genetic program is triggered to change the expression profile of the plant cell membrane proteins, resulting in the periarbuscular membrane (Gianinazzi-Pearson 1996; Rausch et al. 2001; Bestel-Corre et al. 2002; Gianinazzi-Pearson and Brechenmacher 2004; Hohnjec et al. 2005; Valot et al. 2005; Lambais 2006). Several lines of evidence suggest that a H^+-ATPase transport protons across the periarbuscular membrane (Marx et al. 1982; Gianinazzi-Pearson et al. 1991) and is responsible for generating an acidic intercellular compartment (Guttenberger 2000). This proton gradient seems to be related to the exchange of phosphate (Poulsen et al. 2005), sugars (Harrison 1996; Schußler et al. 2006) and amino acids (Cruz et al. 2007; Cappellazzo et al. 2008) at the symbiotic interface.

Both plant and fungi cells use P-type H^+-ATPases to energize their secondary systems of nutrient transport (Portillo 2000; Palmgren 2001; Buch-Pedersen et al. 2006). Two other H^+ pumps are located in the vacuolar membrane, namely the H^+-ATPase (V-ATPase) and H^+-pyrophosphatase (H^+-PPases). It has been postulated that a coordinated control of the plasmalemma and vacuolar H^+ pumps is a key process for root development and physiology (Gaxiola et al. 2007; Zandonadi et al. 2007). It was previously reported that the host P-type H^+-ATPases are up-regulated during the AM fungal colonization (Murphy et al. 1996; Gianinazzi-Pearson et al. 2000; Ferrol et al. 2002; Krajinski et al. 2002). However, several studies have failed to demonstrate a respective enzymatic activation of these pumps. In these reports, specific ATPase activities from microsomal membrane vesicles were found to be not affected (Bago et al. 1997; Benabdellah et al. 1999) or at most marginally influenced (McArthur and Knowles 1993). On the other hand, Bago et al. (1997) have shown that plasmalemmal, mitochondrial, and vacuolar ATPase activities could be increased in mycorrhizal roots at later stages of colonization. Furthermore, Fieschi et al. (1992) reported on cell membrane potential hyperpolariza-tion in arbuscular mycorrhizae of *Allium porrum*, but only indirect correlations with a H^+-ATPase activation were provided.

The H^+-PPases have been found in higher plants, protozoa, eubacteria, and archeabacteria (Drozdowicz and Rea 2001). However, there is limited information on H^+-PPases in fungi cells (Lichko and Okorokov 1991; Okorokov et al. 2001). In previous work, we have reported the possible involvement of a microsomal pyrophosphatase activity in AM interactions (Ramos et al. 2005). In this paper, we report a coordinate activation of the host root H^+ pumps during the AM symbiosis by isolating the plasma membrane and tonoplast-enriched fractions from two regions of maize roots colonized with *Gigaspora margarita* or *Glomus clarum*. This pattern varies with the fungal species, the stage of colonization, and region of the colonized root.

Materials and methods

Plant material, inoculation, and growth conditions

Experiments were conducted in the greenhouses of the Universidade Estadual do Norte Fluminense Darcy Ribeiro (Campos dos Goytacazes) located in the Northern region of Rio de Janeiro State, Brazil (latitude=21°45′ S, longitude=41°18′ W, altitude=11 m). The three experiments were performed in a completely randomized design consisting of three treatments (control, inoculated with *G. margarita* or *G. clarum*), four periods (20, 30, 40, and 60 days), and ten replicates. In the 60-day experiment, 20 replicates were done to overcome the difficulty of extracting proteins of plant roots in advanced growth stages. Seeds of maize (*Zea mays* L., var. UENF 506-6) were surface-sterilized with 10% NaClO solution for 3 min, rinsed four to five changes of sterile distilled water, and then sown in pots (3 L) with sterilized substrate (Cambisoil/sand, 1:1) containing the arbuscular mycorrhizal inoculum. The inoculum consisted of a mixture of hyphae, spores, and mycorrhizal root fragments of *G. margarita* Becker & Hall and *G. clarum*. Control pots received the same inoculum, but after prior sterilization. Plants were maintained in a greenhouse during the experiment and watered daily and fertilized twice a week with 200 ml of a diluted (1\4 F) Clark's solution (Clark 1975).

Plant harvest for root membrane preparations

The plants were harvested and divided into shoots and roots, their fresh weight and shoot height were determined, and a small amount of roots for mycorrhizal colonization measurements were collected. The roots were immediately

wrapped in aluminum foil and kept under ice-cold water until the beginning of the extraction of root membrane proteins. After that, we determined the hydrolytic activities of H$^+$-ATPase (PM H$^+$-ATPase) in enriched vesicles of plasma membrane and H$^+$-ATPase (V-ATPase) and pyrophosphatase (H$^+$-PPase) in enriched vesicles of tonoplast extracted from whole maize root systems. At 30 days after inoculation (d.a.i.), the same analysis was performed, but on the partial root system (20 plants per treatment), in order to evaluate the effect of mycorrhizal colonization under distinct regions of the maize root system (Fig. 1). We decided to use partial roots because it was observed an inhibition of the PM H$^+$-ATPase activity at 30 d.a.i. in the whole roots. This analysis allowed us to emphasize and detect which root region of the mycorrhizal colonization is affecting the ATP hydrolysis in the initial stages. The first root region, denominated mature region (MR), was characterized by the predominance of intense emergence of lateral roots and root hairs. On the other hand, the second one was denominated young region (YR), encompassing the root cap, meristematic, and elongation root zones. This model was considered as the standard for plant membrane vesicles preparation and also the determination of mycorrhizal colonization.
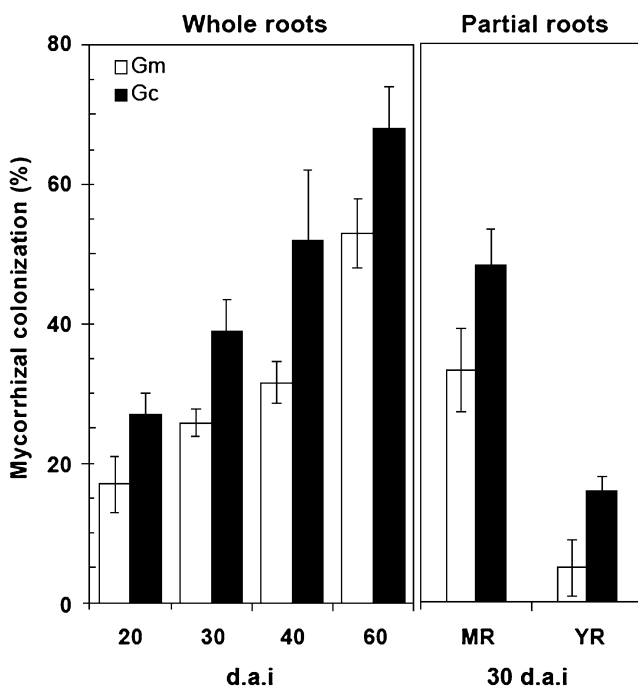

Fig. 1 Mycorrhizal colonization rate of maize plants inoculated with *G. margarita* (*Gm*) or *G. clarum* (*Gc*) measured in whole maize root system at 20, 30, 40, and 60 days after inoculation (*d.a.i.*) or in the partial root system at 30 d.a.i.. *Bars* represent the means±SD. In all periods, *G. margarita* had a lower colonization rate than *G. clarum* at the significance level of $p \le 0.01$. Data represent means from three independent experiments with five replicates per treatment

Determination of mycorrhizal colonization

Maize roots were collected and stained following the method of Phillip and Hayman (1970), and the AM colonization was quantified using the gridline insertion method (Giovannetti and Mosse 1980). Fine roots were washed thoroughly in running tap water and cut into 1-cm pieces, which were subsequently treated with 10% KOH solution for 1 h. Afterward, the root pieces were washed five times with sterilized distilled water, treated with 1% HCl for 3 min, and finally stained with 0.05% Trypan blue. The infected root pieces were examined under a dissecting microscope at 10–40× magnification.

Preparation of plasma membrane and tonoplast-enriched vesicles

Plasma membrane and vacuolar vesicles were isolated from roots using differential centrifugation essentially as described by De Michelis and Spanswick (1986), with modifications described in Façanha and De Meis (1995, 1998). About 100 g (fresh weight) of corn roots were homogenized using a mortar and pestle in 2 mL/g of ice-cold buffer containing 250 mM sucrose, 10% (*w/v*) glycerol, 0.5% (*v/v*) PVP (PVP-40, 40 kDa), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.5% (*w/v*) bovine serum albumin, and 0.1 M Tris–HCl buffer, pH 8.0. Just prior to use, 150 mM KI, 2 mM dithiothreitol (DTT), and 1 mM phenylmethylsulphonyl fluoride (PMSF) were added to the buffer. The homogenate was strained through four layers of cheesecloth and centrifuged at 8,000×*g* for 10 min. The supernatant was recovered and centrifuged at 100,000×*g* for 40 min. The pellet was resuspended in a small volume of ice-cold buffer containing 10 mM Tris–HCl, pH 7.6, 15% (*v/v*) glycerol, 1 mM DTT, 1 mM PMSF, and 1 mM EDTA. The suspension containing the root vesicles was layered over a 20/30/42% (*w/w*) discontinuous sucrose gradient that contained, in addition to sucrose, 10 mM Tris–HCl buffer, pH 7.6, 1 mM DTT, and 1 mM EDTA. After centrifugation at 100,000×*g* for 3 h in a swinging bucket, the vesicles that sedimented at the interface between 20/30 (tonoplast) and 30/42 (plasma membrane) sucrose were collected, diluted with 5.0 mL of ice-cold buffer containing 10 mM Tris–HCl, pH 7.6, 10% (*v/v*) glycerol, 1 mM DTT, and 1 mM EDTA, and immediately frozen under liquid N$_2$ and stored at −70°C until use. Protein concentrations were determined by the method of Lowry et al. (1951).

Vesicles sidedness controls were performed by comparing the inhibitor-specific ATPase latency in detergent-permeabilized and non-permeabilized vesicles. The sidedness of tonoplast vesicles was quite stable, consisting of 80–85% of right-side-out vesicles independently of treatments.

Immediately after isolation, about 60–70% of the plasma-lemma vesicles were right-side-out according to vanadate-sensitive ATPase latency. After two freezing and thawing cycles, however, plasmalemma vesicles reoriented (inside-out), while orientation of tonoplast vesicles remained unchanged as indicated by nitrate-sensitive ATPase latency.

ATPase and pyrophosphatase hydrolytic activity

The ATPase and pyrophosphatase activities were determined by measuring the release of P_i, either colorimetrically (Fiske and Subbarow 1925). The reaction was started by addition of 0.03 mg L^{-1} vesicle protein to the reaction medium and stopped with ice-cold 5% trichloracetic acid after 30-min incubation at 35°C. Before the hydrolysis assay, vesicles were always frozen twice. The reaction medium for the PM H^+-ATPase contained 50 mM HEPES-KOH (pH 6.5), 5 mM $MgSO_4$, 100 mM KCl, and 1 mM ATP. For tonoplast H^+-ATPase and H^+-PPase, the same protocol was used, but modifying the reaction medium pH to 7.0 and the substrate to pyrophosphate (0.1 mM PP_i) for H^+-PPase. In all experiments, the ATPase activities were measured in the absence and presence of their inhibitors (0.2 mM vanadate for PM H^+-ATPase; 100 mM KNO_3 for V-H^+-ATPase) obtaining the vanadate-dependent and nitrate-dependent activities. For potassium-dependent H^+-PPase, the activity was measured in the reaction medium with or without 100 mM KCl.

Light and electron microscopy

Root samples from the different treatments were harvested and immediately freehand cut in segments of 0.3 to 0.7 cm of length onto a paraffin plate, followed by immersion in the fixative solution containing 2.5% glutaraldehyde and 4.0% formaldehyde in 0.05 mM phosphate buffer (pH 7.0; James et al. 1994; Olivares et al. 1997). Fixed samples (12–24 days) were subsequently washed three times in the same buffer and post-fixed with a 2.0% osmium tetroxide solution in water at room temperature for 2 h. The post-fixed samples were washed three times as described above and dehydrated in a graded series of ethanol solutions (30, 50, 70, 90, and 2×100%; 20 min each). The material was infiltrated and embedded in LR white acrylic resin (Agar Scientific, Stansted, UK) for 7 days and polymerized in gelatin capsules filled with the same resin for 16 h at 60°C (James et al. 1994). Semi-thin sections (0.8 to 1.0 μm) for light microscopy (LM) were obtained using a glass knife on a Reichert Ultracut Ultramicrotome and stained with 0.1% toluidine blue in an aqueous solution of 1.0% sodium tetraborate. The slides were examined and the digital images were captured with an Axioplan ZEISS optical microscope coupled with ZVS-47EC camera using the program Analysis®. Ultrathin sections (50 to 70 nm) for transmission electron microscopy were obtained using a diamond knife as described above for LM. The sections were collected on formvar-coated copper grids (300 mesh) stained in 5% uranyl acetate aqueous solution for 30 min followed and 0.2% lead citrate solution in NaOH 0.01 N for 4 min, left to dry for 1 h, and viewed under a transmission electron microscopy Zeiss EM 900 under 80 KV.

Measurements of the root H^+ efflux rates

A detailed description of the experimental setup of the H^+-selective vibrating probe technique used for H^+ efflux measurements was published recently by Ramos et al. (2008b). Further details regarding the vibrating probe system are available in Feijó et al. (1999) and Kunkel et al. (2006).

Maize seeds were superficially sterilized with 5% sodium hypochlorite (v/v) for 15 min, rinsed with five changes of sterile water, and plated in Petri dishes filled up (23.5×23.5 cm) with 80 mL of modified Clark solution at one fourth strength (Clark 1975) in 0.5% (w/v) Phytagel (Sigma-Aldrich, Gillingham, UK). After 4 days, 20 aseptically germinated spores of *G. clarum* were placed around the roots. These were left for 25 days in a controlled-environment growth chamber, with 16 h of light (26°C, 350 μmol m^{-2} s^{-1}) and 8 h of dark periods, for fungal colonization purposes. Subsequently, 100 mL of the same liquid medium was added to the dishes, and the proton flux measurements were performed in roots of non-mycorrhizal or mycorrhizal intact maize plants. In addition, pieces of root system were washed and samples subsequently collected for microscopic evaluation of mycorrhizal colonization. Lateral roots were used as a model for H^+ flux measurements, as they showed to be more accessible than primary roots in microscopic assays. Readings were taken in five defined root zones of mycorrhizal and non-mycorrhizal plants, i.e., apex (0–150 μm); elongation (600–1,200 μm); root hairs (zone with major presence of these structures); and finally mature zone (posterior to root hair zone). The vanadate-sensitive H^+ flux rate was obtained by subtracting the fluxes from roots without incubation with 50 μM vanadate from those left untreated. The analysis of fungal exudates and Fusicoccin (FC) effects on H^+ flux rate were performed in similar conditions. Therefore, in these experiments, 0.3 mL of fungal exudates (extracted from *G. clarum* germinated spores) was added to the liquid medium, roots were incubated for 60 min, and the measurements were restarted. For Fusicoccin experiments, 10 nM Fusicoccin was added and incubated for 30 min prior to analysis.

Fungal exudates extraction

About 500 spores of *G. clarum* were surface-sterilized as described by Bécard and Fortin (1988). Afterwards, they were placed in glass tubes filled up with 5 mL of sterile water, pH 5.7, and then incubated in darkness at 26°C for 8 to 10 days. Then, the incubation media were collected and centrifuged in centricons (Vivascience, 5 kDa).

Statistical analysis

We used two-way analyses of variance (ANOVAs) to compare the temporal mean profiles of the groups in terms of plant growth parameters, mycorrhizal colonization, and hydrolytic activity of proton pumps. The two factors in these analyses were "fungal treatment" and "day after inoculation". We also performed one-way ANOVAs to compare "fungal treatment" means in each time point. To compare mycorrhizal plants in mature and young regions, we used again one- or two-way ANOVAs when appropriate, considering "fungal treatment" and "root regions" as factors. All analyses were validated by convenient residual analyses that did not show departure from the normal distribution according to the Kolmogorov–Smirnov test and showed homocedasticity among factors (data not shown). The *t* test was used to compare the fungal treatments for two independent samples and calculated confidence intervals for the mean difference with Bonferroni correction for multiple comparisons in order to guarantee a global 95% confidence level. Pearson's coefficient was used to test the correlation between different variables. Enzymatic activity data represent means from three independent experiments with five replicates per treatment. All statistical analyzes were conducted in R program, and the level of significance was set up at 5% (Ihaka and Gentleman 1996).

Results

Plant growth and mycorrhizal colonization

Changes in plant growth were influenced by the species of AM fungus and the stage of colonization. At 20 d.a.i., plants inoculated with *G. margarita* showed the same shoot fresh weight (SFW; Table 1) and shoot height (SH; Table 1; $p=0.09$ and 0.99, respectively) when compared to control, but displayed a decrease in root fresh weight (RFW; Table 1; $p<0.001$). On the other hand, in the same period, inoculation with *G. clarum* promoted a decrease in the three plant growth parameters when compared to control plants (p values for principal effects ≤ 0.001; Table 1). At 30 d.a.i., all plant growth parameters remained under inhibition mainly in those plants inoculated with *G. clarum*, with the

Table 1 Growth parameters of shoot and root fresh weight, and height of non-inoculated (C) or inoculated maize plants with *G. margarita* (Gm) or *G. clarum* (Gc) at 20, 30, 40, and 60 d.a.i.

d.a.i.	Treatments	Shoot height (SH) cm	Fresh weights	
			Root (RFW) g plant−1	Shoot (SFW)
20	C	34.42 a	0.43 a	0.38 a
	Gm	36.62 a	0.25 b	0.32 a
	Gc	26.52 b	0.21 b	0.23 b
30	C	42.65 a	0.38 a	0.42 a
	Gm	46.20 a	0.29 b	0.33 b
	Gc	33.68 b	0.24 b	0.32 b
40	C	50.54 b	0.33 b	0.53 b
	Gm	61.35 a	0.67 a	0.91 a
	Gc	60.98 a	0.73 a	0.95 a
60	C	84.50 c	1.04 b	1.38 c
	Gm	93.16 b	1.12 b	1.84 b
	Gc	110.3 a	1.45 a	2.28 a

Data were analyzed by ANOVA combined with Tukey's test. Averages followed by the same lowercase letter, in a same d.a.i., are not significantly different by Tukey's test at $p<0.05$. Data represent means from three independent experiments with ten plants analyzed per treatment. Exclusively at 60 d.a.i., 20 plants were analyzed

exception of *G. margarita* for SH ($p=0.07$; Table 1). At 40 d.a.i., mycorrhizal plants exhibited higher growth parameters when compared to non-mycorrhizal counterparts ($p<0.001$). The same occurred at 60 d.a.i., except for plants with *G. margarita* that have RFW in the borderline of statistical significance ($p=0.09$; Table 1).

Both AM colonization rates enhanced with the time, but the increase was more pronounced in plants inoculated with *G. clarum* (Fig. 1; $p<0.001$). We performed quantification of fungal colonization at 30 d.a.i. using partial root systems, which showed a clear-cut differential distribution of the fungal colonization along two sections of root system (Fig. 1). The MR contained many root hairs and exhibited a higher fungal colonization rates than YR (Fig. 1). The results showed no statistical interaction ($p=0.76$) between the two fungal treatments and root regions but indicated that *G. margarita* has a lower colonization rate than *G. clarum* ($p<0.001$).

Structural analyzes using light and transmission electron microscopy revealed a relationship between timescale plant growth parameters and colonization rates over the time of interaction. Micrographs shown in Electronic supplementary material are from roots colonized with *G. clarum* at 30 d.a.i (earlier phase, micrographs a and b) and at 60 d.a.i (later phase, micrographs c and d). Based on mycorrhizal cross sections obtained at 30 d.a.i., it was possible to observe the intraradical hyphae colonizing intercellular parenchymatic cells and penetrating through cell wall mainly at the outer cortical hypertrophy cells (S1A in Electronic supplementary material). At advanced harvest time, a greater proportion of

cortical cells were infected by hyphae in agreement with the colonization rates (Fig. 1 and Electronic supplementary material). In addition, there was a clear relative predominance of structures involved in bidirectional exchange of nutrients (arbuscules) compared to penetration structures (infective hyphae), which was compatible with the biomass pattern.

Mycorrhizal colonization effects on the PM and vacuolar H$^+$-ATPases and H$^+$-PPase activities on the whole root system of the maize host

Hydrolytic activities of proton pumps from whole maize roots were analyzed in four harvesting times after inoculation with AM fungi (*G. margarita* or *G. clarum*). ATP hydrolysis of PM fractions was highly sensitive to 0.2 mM vanadate and insensitive to 100 mM KNO$_3$, indicating low contamination by tonoplast membranes (data no shown). After 30 d.a.i., PM-enriched vesicles from roots inoculated with *G. margarita* presented a vanadate-sensitive H$^+$-ATPase activity (PM H$^+$-ATPase) higher than non-mycorrhizal plants (Fig. 2, $p<0.001$). This was also observed in plants inoculated with *G. clarum*, but only at 40 and 60
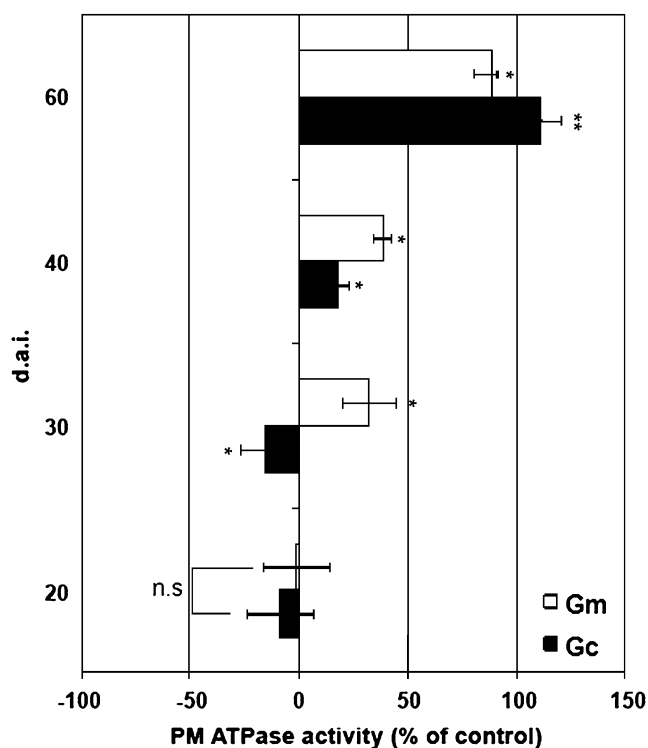

Fig. 2 Stimulation of vanadate-sensitive H$^+$-ATPase activity of plasma membrane vesicles (PM H$^+$-ATPase) isolated from non-inoculated (C) or inoculated maize roots with *G. margarita* (*Gm*) or *G. clarum* (*Gc*) during 60 days time course experiment (*n*=4). The activity is expressed as percentage (%) of the control. Data represent means from three independent experiments with five replicates per treatment. *Bars* represent the means±SD. *Asterisk* and *two asterisks* represent significant difference when compared to the control group at the significance levels of $p\leq0.01$ and $p\leq0.001$, respectively

d.a.i ($p<0.001$), while a significant inhibition in enzymatic activity was detected at 30 d.a.i. ($p<0.01$, Fig. 2). At 20 d.a.i., there was no statistical difference between control and inoculated plants (Fig. 2, $p>0.50$).

Tonoplast vesicles exhibited a V-ATPase activity that was stimulated by fungal inoculation only at 40 and 60 d.a.i. ($p<0.001$ for Gc and 0.01 for Gm), since at 20 and 30 d.a.i. there were no statistical changes induced by the mycorrhizal colonization (Fig. 3, $p>0.70$). There was no statistical significance among the stimulations promoted by Gm or Gc at 40 d.a.i ($p<0.65$). On the other hand, H$^+$-PPase activity exhibited a strong induction at the early stages of colonization (30 d.a.i., $p<0.001$), mainly in *G. clarum* treatment (Fig. 4), in parallel with a decrease of PM H$^+$-ATPase activity ($p\leq0.01$).

Effects of mycorrhizal colonization on H$^+$-ATPase and H$^+$-PPase activities on the partial roots

It is well known that young fast growing meristematic regions of a root system displayed highest H$^+$ pump activities and abundance than older mature regions (Jahn et al. 1998). Measurements of colonization rates also revealed that root YR are much less colonized than MR regions that exhibited a clear hyphal abundance. Thus, in order to explore the effects of this spatial segregation of mycorrhizal colonization on H$^+$ pump activities, membrane vesicles were isolated from these two regions of colonized maize roots (Fig. 5).

Membrane vesicles isolated from MR of maize roots inoculated with *G. clarum* (Fig. 5a) showed a significant inhibition of V-ATPase at early stages of colonization (30 d. a.i., $p<0.01$), but without any significant effect on both the PM H$^+$-ATPase and H$^+$-PPase ($p=0.68$ and 0.36, respectively). On the other hand, PM H$^+$-ATPase activity was found to be inhibited in YR colonized with *G. clarum* (Fig. 5a, $p<0.001$), whereas it was stimulated by *G. margarita* ($p<0.01$). In contrast, inoculation with *G. margarita* stimulated the V-ATPase activity, while the same activity was inhibited with *G. clarum* (Fig. 5b, $p<0.01$).

Divided root systems revealed an apparent down regulation of the H$^+$-ATPase activity at early stages of the mycorrhizal colonization in YR, localized straight below the major colonization sites (Figs. 1, 5b). An opposite behavior was observed for the H$^+$-PPase activity, since both fungal species promoted a strong activation of the PP$_i$ hydrolysis in the same region (Fig. 5).

H$^+$ efflux rate in mycorrhizal roots and the effect of fungal exudates

Analysis in vitro of the root H$^+$ flux rate using noninvasive technique revealed high vanadate-sensitive H$^+$ efflux and
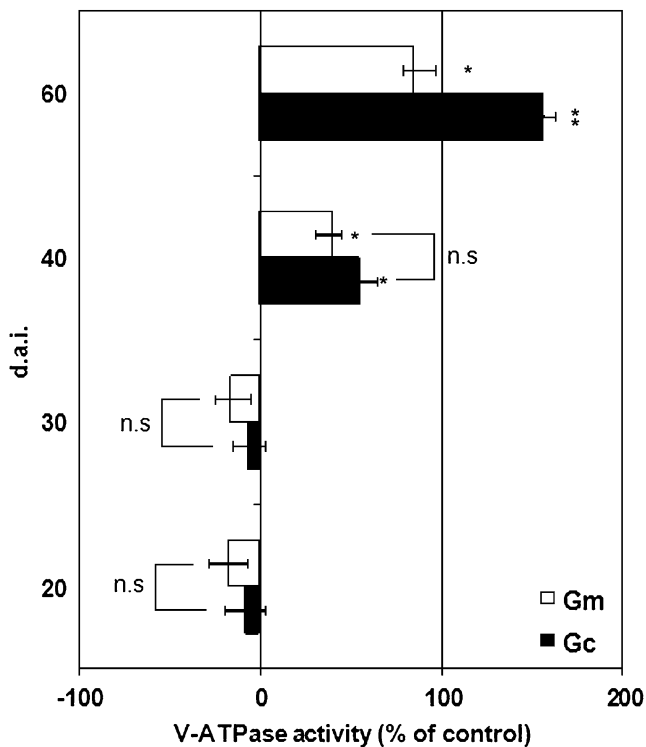
Fig. 3 Stimulation of nitrate-sensitive H⁺-ATPase activity of tonoplast vesicles (V-ATPase) isolated from non-inoculated (C) or inoculated maize roots with *G. margarita* (*Gm*) or *G. clarum* (*Gc*) during 60 days time course experiment ($n=4$). The activity is expressed as percentage (%) of the control. Data represent means from three independent experiments with five replicates per treatment. *Bars* represent the means±SD. *Two asterisks* represent significant difference when compared to the control group at the significance level of $p \le 0.001$

mycorrhizal colonization (about 80%) in roots inoculated with *G. clarum* during the establishment phase of AM interaction (Fig. 6a). Interestingly, those stimulations were found at the elongation zone and root hairs, while there were no significant changes at the apex and mature zones ($p=0.51$, Fig. 6a). This measurement of the vanadate-sensitive H⁺ fluxes demonstrate that even in the young root segments, where indeed a lower H⁺-ATPase activity was found in AM-colonized roots (Fig. 5), this enzyme can be strongly activated in specific cells of physiologically strategic root regions (Fig. 6).

In order to verify if signaling molecules exudated by AM fungi could affect directly the host root P-type H⁺-ATPase, maize root H⁺ fluxes were measured in the presence of fungal exudates isolated from *G. clarum* (Fig. 6b). The results show that the H⁺ pumping activity can be inhibited by specific AM fungal signals, and this activity is clearly related to the root plasmalemmal H⁺-ATPase, which is inhibited by vanadate and stimulated by fusicoccin (Fig. 6b).

Discussion

In this work, a set of experiments were performed to confirm and expand the notion that the effects of arbuscular mycorrhizal colonization on plant growth involve a differential modulation of the H⁺ pumps in major root cells. Positive and significant Pearson's correlation coefficients were found between the PM H⁺-ATPase activity and mycorrhizal colonization (0.68; $p \le 0.01$). Moreover, V-ATPase activation was also correlated with the mycorrhization process (0.89; $p \le 0.01$). A PM H⁺-ATPase activation (vanadate-sensitive) was also found in microsomal membranes from colonized roots (McArthur and Knowles 1993; Bago et al. 1997; Benabdellah et al. 1999).

In fungi and plants, cellular nutrition and growth are dependent on H⁺ electrochemical gradients that are primarily generated by the PM H⁺-ATPases (Portillo 2000; Palmgren 2001; Sondergaard et al. 2004). In maize roots, this enzyme is asymmetrically localized within both epidermal and outer cortical cells (Jahn et al. 1998). On the other hand, ultrastructural studies on mycorrhizal roots have revealed interesting changes in PM H⁺-ATPase distribution at the internal hyphae and periarbuscular membranes (Marx et al. 1982; Gianinazzi-Pearson et al. 1991). The PM H⁺-ATPase, low-affinity phosphate trans-
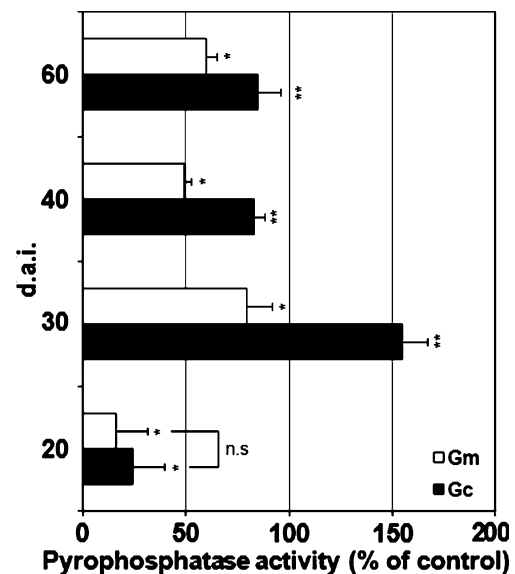

Fig. 4 Stimulation of potassium-sensitive H⁺-PPase activity of tonoplast vesicles (H⁺-PPase) isolated from non-inoculated (C) or inoculated maize roots with *G. margarita* (*Gm*) or *G. clarum* (*Gc*) during 60 days time course experiment. The activity is expressed as percentage (%) of the control. Data represent means from three independent experiments with five replicates per treatment. *Bars* represent the means±SD. *Asterisk* and *two asterisks* represent significant difference when compared to the control group at the significance levels of $p \le 0.01$ and $p \le 0.001$, respectively
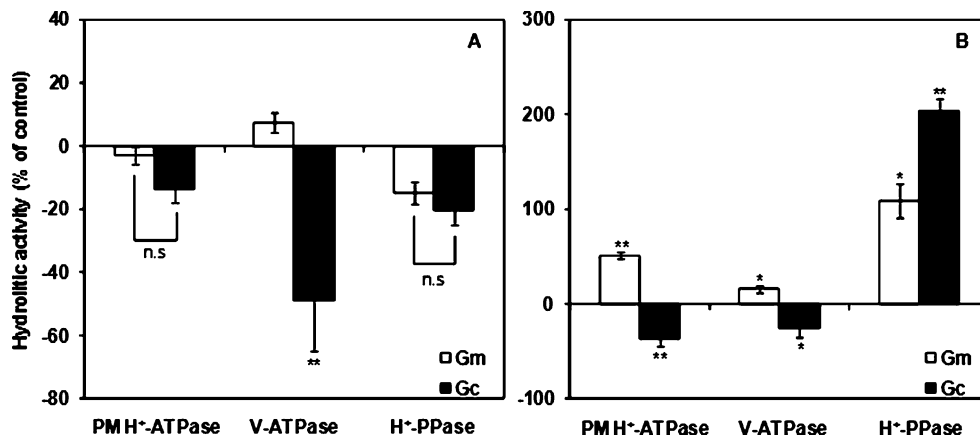
Fig. 5 Vanadate-sensitive PM H$^+$-ATPase, nitrate-sensitive vacuolar H$^+$-ATPase and potassium-sensitive vacuolar H$^+$-PPase activities of purified membranes isolated from mycorrhizal and non-mycorrhizal maize partial roots. **a** Enzymatic activities in mature regions (*MR*) and **b** in young regions (*YR*) of the maize root system expressed as percentage of the control. Data represent means from three independent experiments with four replicates per treatment. *Bars* represent the means±SD. *Asterisk* and *two asterisks* represent significant difference when compared to the control group at the significance levels of $p\leq 0.01$ and $p\leq 0.001$, respectively

Fig. 6 **a** Vanadate-sensitive H$^+$ flux activity in four regions of the maize root system non-inoculated (*open bars*) or inoculated (*closed bars*) with the AM fungus, *G. clarum* (*Gc*), under in vitro conditions (*n*=8). The analysis was done 10 days after spore germination. **b** Effect of water (control), fungal exudates from 500 germinated spores of *G. clarum*, 10 nM fusicoccin, and 50 μM vanadate on H$^+$ effluxes of elongation zone. A negative modulation of the H$^+$ flux by addition of fungal factors suggests a possible participation of those "myc factors" in the regulation of the PM H$^+$-ATPase, since the addition of FC stimulated the fluxes and vanadate inhibited. **c–e** Images of mycorrhizal maize roots, extraradical hyphae, and spores of *G. clarum* during the measurements of H$^+$ fluxes under in vitro conditions. *Bars* represent the means±SD. *Asterisk* and *two asterisks* represent significant difference when compared to the control group at the significance levels of $p\leq 0.01$ and $p\leq 0.001$, respectively
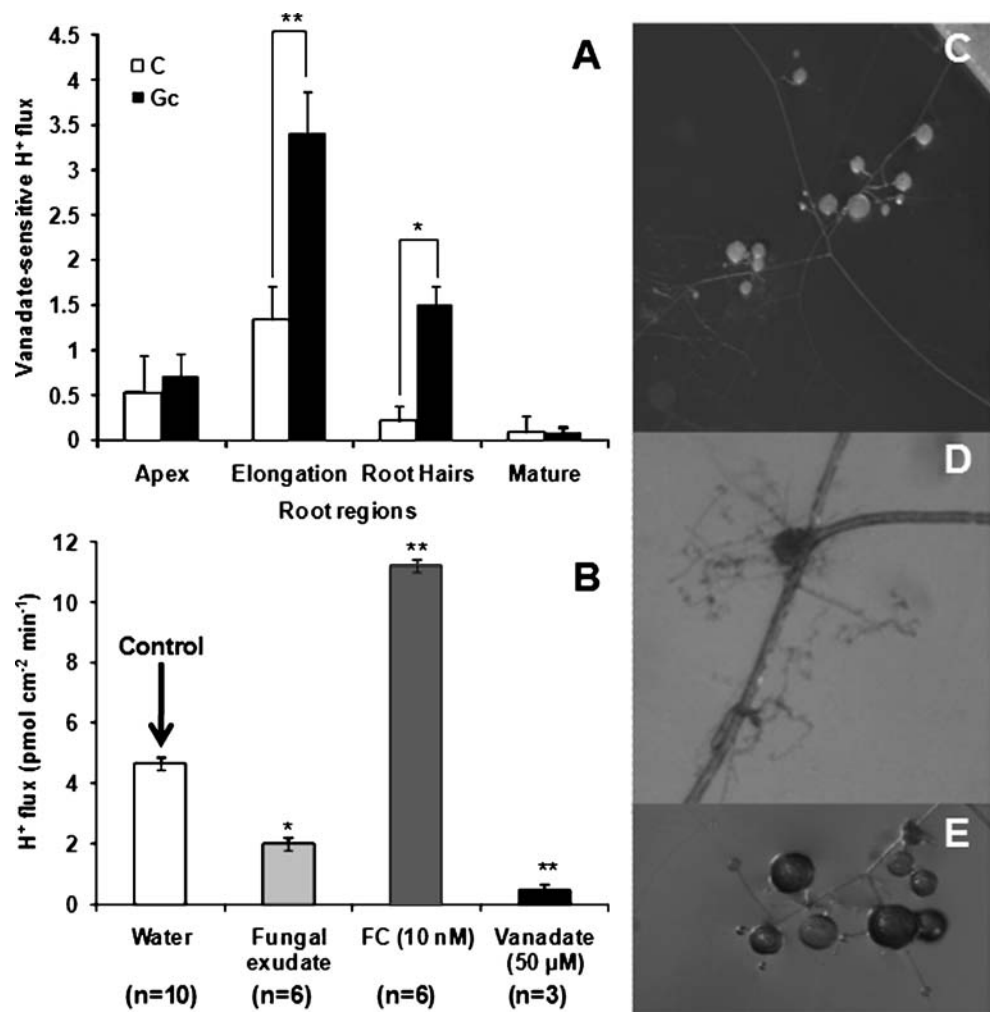
porter, and hexose transporters have increased expression in cortical arbuscule-containing cells (Gianinazzi- Pearson et al. 2000; Krajinski et al. 2002; Harrison 1996). This supports the notion that the H$^+$ electrochemical gradient generated by the H$^+$-ATPase provides a driving force for the flux of nutrients and solutes across symbiotic membranes (Marx et al. 1982; Smith and Smith 1990; Gianinazzi 1991; Guttenberger 2000).

Since in this work the PM H$^+$-ATPase activation was not restricted to the root regions containing higher abundance of arbuscular structures (MR), it is tempting to speculate that such activation may also represent an acidic mechanism by which the cell wall is plasticized in order to facilitate the penetration and differentiation of the fungal hyphae in the root apoplast during the establishment of mycorrhizal colonization. The acid growth theory proposed by Rayle and Cleland (1992) involves the activation of H$^+$ efflux via PM H$^+$-ATPase, resulting in acidification of the apoplast. This will consequently trigger expansin activity responsible for cell wall plasticity, which leads to cell expansion (Morsomme and Boutry 2000). Siciliano et al. (2007) characterized the transcriptome of the pre-penetration apparatus during AM interaction. They found that expansin-like genes were up-regulated in mycorrhizal plants. In agreement with this notion, we observed a high epidermal H$^+$ efflux in mycorrhizal roots leading to an increased capacity of these roots to acidify the local medium. These effluxes proved to be dependent on the PM H$^+$-ATPase given that they were sensitive to vanadate and activated by fusicoccin. This possibility is also in line with the results obtained by Bago et al. (1998) who showed that the pH of the medium decreased with the establishment of AM symbiosis, and high acidic pH values were found around extraradical hyphae and spores. It is worth noting that the H$^+$ flux activation reported here should also result in modulations of different ion channels and signal receptors sensitive to changes of the pH gradient and/or membrane potential.

Previously, it was reported that the mycorrhization process involves a long-distance signaling derived from the AM fungi, which results in a mycorrhizal membrane potential hyperpolarization (Fieschi et al. 1992). Here, we provided original evidences that signaling molecules exudated by AM fungi could act directly on the host root PM H$^+$-ATPase and its related H$^+$ fluxes. However, an inhibition of the rate of H$^+$ efflux was observed specifically at the elongation zone of non-colonized roots incubated with *G. clarum* fungal exudates. In addition, at the early stages of fungal colonization, the PM H$^+$-ATPase activity was inhibited in maize roots colonized by *G. clarum*. These inhibitory phenomena could be related to the fungal energetic cost that is proportionally higher in the first stages of colonization due to an increased fungal demand.

Therefore, it is tempting to speculate that AM fungi can induce a down-regulation of the root main systems of ATP consumption as a preparatory stage for the root infection. Moreover, it seems likely that after the establishment of the AM colonization, the fungi can induce a reactivation of the pumps not only to facilitate the dynamic progression of the hyphal invasion through the cell wall but also for the reestablishment of a health root growth and metabolism. In fact, the H$^+$ flux was strongly stimulated at the elongation zone of maize mycorrhizae colonized with *G. clarum*.

Although it is very difficult to correlate data of gene expression with total enzymatic activities, our data are in a good correlation with molecular analyses performed by Rosewarne et al. (2007) showing a down-regulation of the main H$^+$-ATPase genes (*LHA1* and *LHA4*) in tomato plants during the initial stages of their interaction with *Glomus intraradices*. This reveals the necessity to perform detailed studies integrating enzymatic and molecular analyses in order to elucidate the functional participation of each H$^+$ pump isoform during plant colonization by mycorrhizae or pathogenic fungi. Actually, it has been postulated that regulation of H$^+$-ATPases plays an important role in the signaling processes involving pathogenic interactions (Schaller and Oecking 1999) or at the very least participates as an intermediate event in this signal transduction pathway (Xing et al. 1996).

During the same period (i.e., 30 d.a.i.), the potassium-sensitive H$^+$-PPase activity was found to be higher when the lowest levels of ATP hydrolysis and plant growth were detected. It is well known that at least for some plant species, it is common to observe inhibition of the plant growth during the initial stages of the mycorrhizal colonization (Graham and Eissenstat 1994; Wright et al. 1998a, b). This initial stress response seems to be promoted by some factors such as the intense AM fungal colonization, defense responses, or by the high consumption of plant photosynthetic carbon by fungal cells. Willians et al. (1987) reported that plant growth depression in mycorrhizal citrus plants might be due to the high cost of carbon provided by the plant for fungal growth and lipid storage in the vesicles of *Glomus* spp. On the other hand, *G. margarita* does not form intraradical vesicles but extensively forms extraradical storage auxiliary cells, which should also imply differences of energetic metabolism and/or carbon demand that, in turn, could account for the differences found in the balance of the pumps between the two fungi species.

Furthermore, along with these global observations in the roots, we also observed distinct local regions of differential regulation of proton pump expression and activity as well as distinct regions of fungal colonization. The root system was subdivided in two different areas to localize and reveal the negative modulation of ATP hydrolysis. We observed a

very low fungal colonization rate at the distal root regions (YR). These regions encompass the younger tissues of the roots and are located straight below the major fungal colonization zone. An intense drain of photoassimilates by the fungal cells affects strongly this zone (Bonfante-Fasolo and Perotto 1992). In contrast, high fungal colonization was exhibited in the mature root regions without any significant effect on both the PM H^+-ATPase and H^+-PPase; however, a significant inhibition of V-ATPase was observed at early stages of the colonization by *G. clarum* (30 d.a.i.). This suggests that the spatial distribution of the AM fungi may account for the decrease of the H^+-ATPase activities and the activation of the H^+-PPase observed in the young regions. This effect was also species-specific, and since the activity of the root meristems influences the root development and architecture (Berta et al. 1993), the energetic stress imposed on the root distal region by sugar availability could modulate the root morphology and thereby regulate the distribution of H^+ pumps along the root system. This is in agreement with previous reports that observed a down-regulation of the PM H^+-ATPase activity in plant cells undergoing sugar deprivation (e.g., Mito et al. 1996).

Our previous data suggested that the stimulation of H^+-PPase is an important part of the host response to the energy consumption by AM fungi (Ramos et al. 2005). There are already several reports indicating that the H^+-PPase may replace the role of H^+-ATPases under conditions of stress (Carystinos et al. 1995; Davies et al. 1997). This notion is supported by recent studies that also highlight the requirement of this pump during other stress responses mediated by plant cell (e.g., Nakanishi and Maeshima 1998; Park et al. 2005; Gaxiola et al. 2007). Taking together the present data, it seems clear that the energetic backup system represented by the H^+-PPase could be specially regulated in the young meristematic region due to its higher energy demand. The young root region of mycorrhizal roots should be more depleted of sugar drained by the AM fungi colonizing the above regions. Therefore, the activation of the PPi metabolism in this region would represent a critical energetic advantage, for instance, by saving the ATP needed to the cells with higher metabolic activity of physiologically strategic root regions such as the elongation zone and root hairs.

Conclusions

The activity of H^+ pumps in host root cells during colonization are differentially modulated by their interactions with AM fungi. These biochemical changes seem to influence the rate of host growth and that of fungal colonization. The lowest rates of ATP hydrolysis in the early stages of AM symbiosis might be compensated by

activation of the tonoplast H^+-PPase in those root regions that have the lowest available photoassimilates. Further studies are necessary to understand the dynamics of H^+-ATPase and H^+-PPase expression and activity in order to provide new insights into the role of H^+ pumps in such an important symbiotic interaction.

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