Universidade do Minho
Escola de Ciências

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UNIVERSIDADE DO PORTO

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Cell to cell communication in plants: ions take charge

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Tese de Mestrado em Fisiologia Molecular de Plantas

Trabalho efectuado sob a orientação do
Professor Doutor José Alberto B. de Magalhães Feijó

e do orientador interno
Doutor Rui Manuel Peixoto Tavares

Setembro de 2009

DECLARAÇÃO

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Título da Tese de Mestrado:

Cell to cell communication in plants:
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Orientador:

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"There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved."

Charles Darwin, 1859

Aos meus pais

I. ACKNOWLEDGMENTS

No effort of this nature is feasible without assistance and supervision of others, directly or not related to the work itself. I feel I couldn´t have been more or better supported than I was, and that is reflected in the smile I wore along the journey herein reported.

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Dear JF, peer of the science Gods realm: thanks for receiving me in the lab, asking nothing in exchange except for rigorous and honest work. The pleasure of working in such environment, both physical and intellectual, goes much beyond words can convey. Thanks for all the support, for showing me ways to look at life and science, for giving me the opportunity to think and work freely, and for being a GREAT friend! You once advised me not to follow leaders. I totally agree, but as Dylan says: "You don't need a weather man to know which way the wind blows"... Obrigado Professor, até já! (can I have the same desk?)

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For giving me so so much, and for the brilliant future ahead of us, thank you dear Sara.

II. General Abstract

In the words of the german mycologist Anton de Bary, symbiosis is defined as the living together of dissimilar organisms. The not unusual establishment of gradual physiological and/or developmental interdependence among organisms underlies a selective force attributable to symbiotic phenomena along extended co-evolutionary time. The commonly cited example of flowering plants´ major radiation events in the Cretaceous stands as an impressive case of adaptive speciation linked to mutualistic co-evolution with insects. The existence of a whole biological Kingdom of photosynthetic organisms is regarded as the evolutionary derivation of a successful adaptation of the ancestral chloroplast as an endosymbiotic entity. Understanding the basal features of symbiotic systems stands as a fundamental topic in Biology, as it encompasses mechanisms that might have played roles in the evolution of major biological themes, such as multicellularity and immunity. Plants establish widespread, both phylogenetically and geographically speaking, mutualistic symbiotic interactions with large groups of Fungi. Among other important differences, according to whether the fungal hyphae penetrate or not the plant cell wall during proximate cellular interactions these relationships are classified as endo- or ectomycorrhizas. These comprise some of the most ubiquitous symbioses on Earth, with a large impact on agriculture and in shaping ecological niches. Addressing inter-specific cell-cell communication in these systems is crucial in order to properly understand its mechanistic basis and evolutionary paths. In the first chapter we address the hypothesis of the existence of a putative role played by ion dynamics in cell signaling between symbionts, as it has been shown to be the case for well characterized plant-microbe interactions. We describe an original process of ion flux modulation in ectomycorrhizal roots, elaborating on its relevance for plant nutrition and ontogeny. In the second chapter, we theoretically address the evolution of root endosymbioses, introducing useful evolutionary developmental biology concepts, such as homology and modularity, aiming at further clarifying the emergence of these systems. The transversal relevance of ionic behaviour in the establishment of biological connections stands out as the main conclusion of the overall work. Playing a crucial role in morphogenesis, modulation of ion fluxes and bioelectricity can provide an additional layer of regulation and fine-tunning of biological processes, particularly in information flow and processing during the establishment of symbiotic crosstalks.

III. Resumo Geral

De acordo com o micologista alemão Anton de Bary, o termo "simbiose" pode ser definido como "a vida em comum de diferentes organismos". O vulgar estabelecimento de interdependência fisiológica e/ou ontogénica entre organismos evidencia uma força selectiva que pode ser atribuída a fenómenos simbióticos, operando durante o tempo co-evolutivo. O exemplo comunmente citado dos grandes eventos de radiação das angiospérmicas durante o Cretáceo destaca-se como um caso de especiação adaptativa estreitamente relacionado com interacções mutualísticas com insectos. A própria existência de todo um Reino biológico composto por organismos fotossintéticos é vista como tendo resutado da derivação evolutiva da adaptação do cloroplasto ancestral ao ambiente endossimbiótico. A compreensão de aspectos basais do funcionamento de sistemas simbióticos destaca-se como um tema fundamental em Biologia, uma vez que relaciona mecanismos que poderão ter actuado ao longo da evolução de grandes inovações biológicas, como a multicelularidade e a imunidade. As plantas estabelecem relações mutualísticas com um variado número de grupos de fungos, com enorme dispersão geográfica e filogenética. Entre outras diferenças importantes que as distinguem, estas podem ser classificadas como endo- ou ectomicorrizas consoante a capacidade exibida pelas hifas dos fungos penetrarem ou não a parede celular vegetal. Correspondem a algumas das mais ubíquas simbioses na Terra, com grande impacto na agricultura e na modificação de nichos ecológicos. O estudo da comunicação interespecífica célula-célula nestes sistemas é fundamental, de forma a que possamos melhor entender as suas bases mecanísticas e percursos evolutivos. Assim, no primeiro capítulo debruçamo-nos sobre a hipótese da existência de um putativo papel desempenhado pela dinâmica iónica celular em fenómenos de sinalização entre simbiontes, tal como demonstrado em exemplos bem caracterizados de interacções planta-microorganismo. Descrevemos um processo de modulação de fluxo iónico em raízes ectomicorrizadas, elaborando em torno da sua relevância para a nutrição e desenvolvimento da planta. No segundo capítulo apresenta-se uma abordagem teórica à evolução de endossimbioses radiculares, introduzindo conceitos úteis provenientes da biologia evolutiva e do desenvolvimento, tais como os de homologia e modularidade, com o objectivo de melhor clarificar a emergência destes sistemas. A relevância transversal do comportamento iónico

no estabelecimento de conecções biológicas destaca-se como conclusão central do trabalho aqui descrito. Apresentando um papel crucial em fenómenos de morfogénese, a modulação de fluxos iónicos e bio-electricidade providenciam uma camada adicional de regulação e afinação de processos biológicos, particularmente no fluxo e processamento de informação durante a formação de interações simbióticas.

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1. ION CHOREOGRAPHIES IN ECTOMYCORRYZAL ROOTS

Work published under the title:

A pH signaling mechanism involved in the spatial distribution of calcium and anion fluxes in ectomycorrhizal roots (2009) *New Phytologist* 181: 448–462

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Key Words: protons, anions, calcium, ectomycorrhizae, ion-selective vibrating probe, pH signaling, *Pisolithus microcarpus*

1.1 ABSTRACT

Mycorrhization is a typical example of a host-microorganism symbiotic interaction where the symbiont cell biology and the host immune response co-evolved several functional links. Here we address the role played by ion fluxes across the root, concerning nutrient uptake, osmoregulation, growth and signaling events. An ion-selective vibrating probe system was used to assess the net fluxes of protons (H^+), calcium (Ca^{2+}) and anions (A^-) along non-mycorrhizal and ectomycorrhizal (ECM) roots of *Eucalyptus globulus* colonised with *Pisolithus* sp. Our data showed that, from five root zones analysed, the main effect of fungal colonization was localised to the elongation zone, where strong variations in ion dynamics and rhizophere acidification capacity were observed. Additionally, ion fluxes exhibited periodic fluctuations. To verify whether these fluctuations sustained oscillations, we applied continuous wavelet time spectrum analysis and determined that H^+ and A^- fluxes from ECM roots show longer periods than non-mycorrhizal roots. In contrast, Ca^{2+} oscillations are completely abolished following fungal interaction. These results are interpreted in light of a working model in which nutrient uptake and stimulation of growth are mediated by ECM fungi and may be pH-dependent. Furthermore, the variations detected in ECM roots for H^+ and A^- fluxes suggest a main contribution from the plant, while the results obtained for Ca^{2+} suggest a significant involvement of the fungus.

1.2 INTRODUCTION

Establishment of an effective ectomycorrhizal symbiosis encompasses a progression of complex and overlapping developmental processes in both the colonizing mycelium and the roots of host trees (Martin *et al.*, 2007). During mycorrhizal symbiosis, host plants show enhanced growth and increased soil nutrient uptake ability, which are believed to be promoted by the fungal partner (Taylor & Peterson, 2005). The mechanisms by which this occurs are poorly understood, although a number of anatomical and physiological factors are clearly involved, namely an increase in the absorbing surface area promoted by the extraradical mycelium (Marchner & Dell, 1994; Gobert & Plassard, 2002); the synthesis and exudation of organic compounds (Ahonen-Jonnarth *et al.*, 2000; van Scholl *et al.*, 2006) and exoenzymes (Pasqualini *et al.*, 1992; Courty *et al.*, 2006) to the soil in order to solubilize nutrients; and the regulation of host root proteins involved in the nutrient transport across the plasma membrane (PM) (Lei & Dexheimer, 1988; Javelle *et al.*, 2003; Muller *et al.*, 2007).

Changing ion fluxes across the root plasma membrane implies alterations of transmembrane electrical potential, contributed by the electrogenic proton (H^+) pumps, which in turn controls ion transport systems (Tazawa, 2003). High H^+- ATPase activities were found in the PM of external hyphae and sheaths of ectomycorrhizal (ECM) fungi (Lei & Dexheimer, 1988). This enzyme was also found to be stimulated by external anion concentrations (Churchill & Sze, 1984; Ullrich & Novacky, 1990) and inhibited by Ca^{2+} (Lino *et al.*, 1998). In this context, an induction in the uptake has been demonstrated for *Pinus pinaster* ECM roots (Gobert & Plassard, 2002; Plassard *et al.*, 2002; Boukcim & Plassard, 2003; Hawkins *et al.*, 2008). This supports the notion that H^+ transport, PM H^+-ATPase activity and root surface acidification work together in order to promote uptake (Ullrich & Novacky, 1990; Glass *et al.*, 1992; Forde, 2000). Positive effects on ion uptake during mycorrhizal symbiosis have been described for nitrogen, phosphate and some tracer elements such as copper and zinc (Marchner & Dell, 1994), though previous results for calcium have been limited and difficult to interpret (Bucking *et al.*, 2002). For example, in the root cortex almost 100% of the cell wall calcium content can be easily exchanged for an external ^{44}Ca label (Peterson & Enstone, 1996; Kuhn *et al.*, 2000). Similarly, studies of nutrient mobilization in ECM symbiosis have been performed by radioisotope coupling with laser microprobe mass analysis (LAMMA), energy-dispersive X-ray spectroscopy (EDXS) and secondary ion mass spectroscopy (SIMS) (Peterson

& Enstone, 1996; Bucking & Heyser, 2000; Bucking *et al.*, 2007). However, very few detailed studies aiming to determine the regulation of ion dynamics in the ECM symbiosis have been carried out.

There is a profound effect of pH in several biological processes, including nutrient uptake, cell growth and plant–microbe interactions (Feijó *et al.*, 1999; Felle, 2001; Michard *et al.*, 2008). Recently, we showed that extracellular H^+ fluxes are involved in both presymbiotic and symbiotic development of arbuscular mycorrhizal symbiosis (Ramos *et al.*, 2008a,b). By contrast, the possible impact of pH changes was not yet established for ECM associations. Proton fluxes presumably generated by the PM H^+-ATPase activity can modify the root surface pH in ways that may trigger, for instance, modifications in the availability of free extracellular Ca^{2+} or anion transport. As a first step to test the role of ion fluxes in ECM associations, we performed a systematic analysis of the different root ion fluxes in the presence and absence of fungal colonization. We measured these fluxes by means of ion-specific vibrating probes. Major alterations were observed in the growing zone of the root, and are compatible with the notion that pH modulates nutrient uptake. Furthermore, the major alterations detected in ECM roots for H^+ and A^- seem to be associated with root-specific fluxes, while the results for Ca^{2+} suggest a significant contribution of the fungus in the overall ion choreography.

1.3 RESULTS

1.3.1 ECM COLONIZATION EFFECTS ON PLANT GROWTH PARAMETERS

For ion flux analysis purposes (Fig. 1b), formation of ectomycorrhizas was performed under *in vitro* conditions (Fig. 1a) in order to produce *E. globulus* with a high degree of colonization by *P. microcarpus* isolate 90A (Fig. 1c,d). During the experiments, plants presented 78.3% of ECM root colonization (Table 1; Fig. 1d). In addition, significant and positive effects of ECM colonization were found both on plant height and on shoot and root fresh weights ($P < 0.05$; Table 2). No changes in the number of root tips, at the time of the analysis, were detected. A significant decrease in the length of root hairs was found in ECM roots (Table 1). Plant growth was strongly correlated with ionic fluxes as significant Pearson's correlation coefficients were found between H^+ fluxes and plant growth parameters (0.78; $P < 0.008$), root surface pH

(–0.82; $P < 0.0001$) and anion fluxes (–0.59; $P < 0.002$). Moreover, we also found significant correlation coefficients between root surface pH and plant growth parameters (–0.72; $P < 0.0102$).

Table 1| Average values of fungal and plant growth parameters analyzed in nonmycorrhizal (control) or mycorrhizal roots of *Eucalyptus globulus* colonized by *Pisolithus microcarpus* (ECM), 10 d after transplanting to hydroponic conditions ($n = 35$)

PARAMETER ANALYZED	CONTROL	ECM
Fungal colonization (%)	nd	78.3
Plant height (cm)	14.38	17.61*
Shoot fresh weight (mg/plant)	33.94	45.33**
Root fresh weight (mg/plant)	12.2	15.75*
Root hair lenght (μm)	386.52	152.39**
Root tips (n°)	12	17

Significantly different by Student's *t*-test (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). For root tips, $P = 0.051$. nd, not determined.

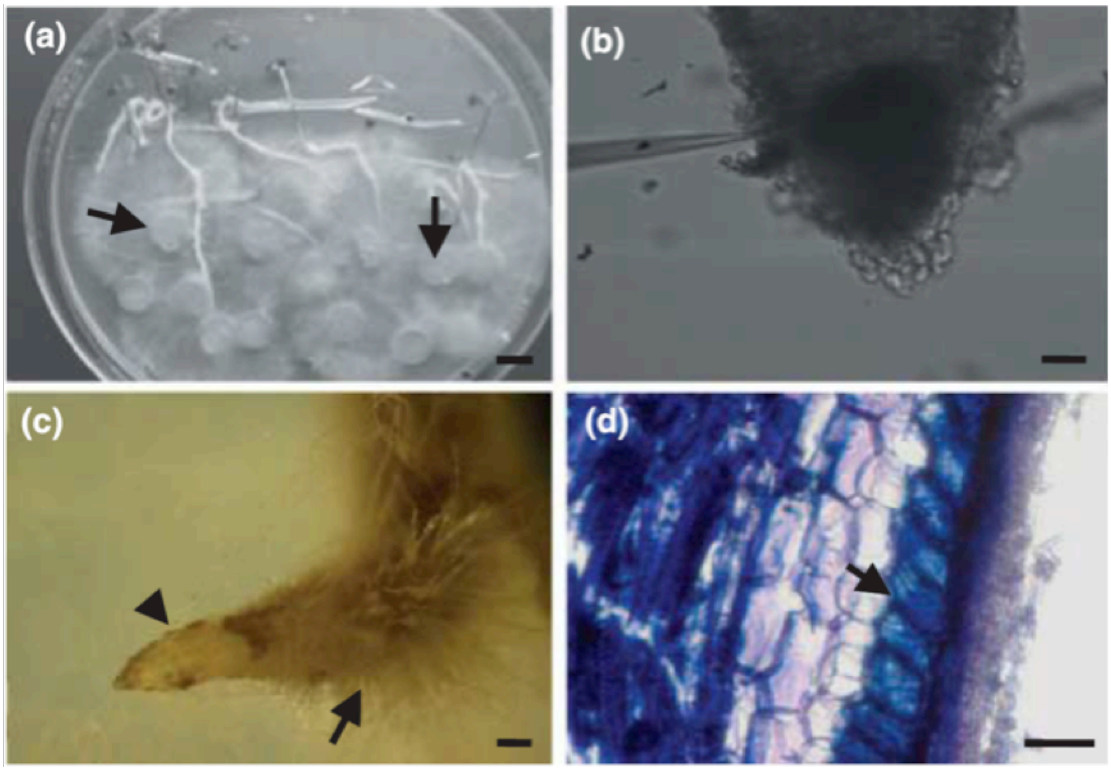

Figure 1| (a) Ectomycorrhiza formation under *in vitro* germination conditions of *Eucalyptus globulus* seedlings and *Pisolithus microcarpus* before transplanting to hydroponic settings. The arrows show inoculum discs containing MNM medium and fungal mycelium. Bar, 9 mm. (b) Representation of a root apex during measurements with an ion-selective vibrating probe. Bar, 170 μm. (c) Representation of a lateral root (arrowhead) of *E. globulus* around *P. microcarpus* mycelium (arrow) under our experimental conditions. Bar, 450 μm. (d) Cross-section of *E. globulus* roots colonized by *Pisolithus microcarpus.* The arrow indicates the fungal colonization. Bar, 50 μm.

1.3.2 H^+ FLUX AND SURFACE pH SIGNATURES FOR ECM ROOTS

A differential pattern of H^+ fluxes was observed along the zones of eucalyptus roots (Fig. 2a). In both nonmycorrhizal and ECM roots, the apex, meristematic and elongation zones were characterized as domains of significant H^+ efflux. By contrast, root hair and mature zones were characterized as domains of H^+ influx (Fig. 2a). A sixfold stimulation on H^+ effluxes was observed at the elongation zone in the presence of colonizing *P. microcarpus* ($P < 0.001$). As expected, surface pH values along the root system showed a pattern consistent with the flux profile, and equally affected ECM colonization (Fig. 2b). The two domains described for H^+ fluxes along the roots corresponded to patches of variable acidity, ranging from 5.56 in the meristematic region to 5.68 in the apex. In ECM roots, significant acidification was observed in the apex, meristematic and, most notably, elongation regions. The lowest pH value (< 5.4) was observed in the elongation zone. In root hairs and mature zones, pH values were found to be 5.6 and 5.8, respectively. These results support an ECM-driven increase in overall H^+ influx. All regions showed significant differences in the surface pH after the establishment of ECM. The global extracellular pH gradient increased by approx. 0.12 pH units in the control (Me vs Mat) to 0.4 pH units after ECM (Elong vs Mat) (Fig. 2b).

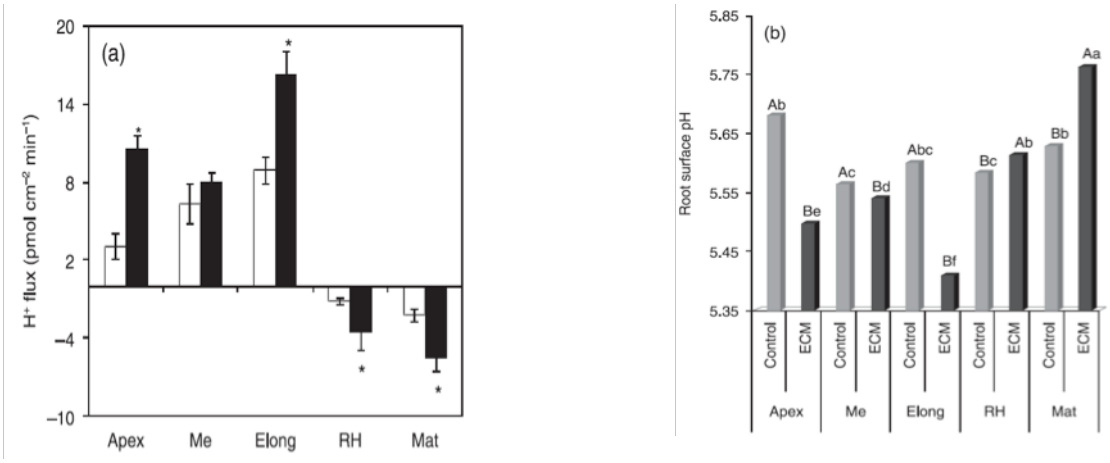

Figure 2| Proton fluxes (a) and root surface pH (b) along nonmycorrhizal (control, open bars) and ectomycorrhizal (ECM) roots of *Eucalyptus globulus* colonized by *Pisolithus microcarpus* (ECM, closed bars). Apex, meristematic (Me), elongation (Elong), root hairs (RH) and mature (Mat) indicate the zones analyzed. Bars represent the mean values ± SE of five independent experiments (*statistical difference at $P < 0.01$). Negative values correspond to ion influx and positive values to effluxes. For H^+ fluxes and surface pH, by two-way ANOVA combined with Duncan's test, the results showed that there was significant interaction between fungal treatment and root zones ($P < 0.0001$). For H^+ fluxes, we found no statistically significant difference with fungal inoculation at the meristematic zone. For pH data interpretation, bars followed by the same capital letter, in the same root region, are not significantly different by Duncan's test at $P < 0.05$. Bars followed by the same lower-case letter, in different root regions, are not significantly different at $P < 0.05$ ($n = 5$).

1.3.3 Ca^{2+} AND ANION FLUX PROFILES

Interestingly, the patterns of the Ca^{2+} and anion fluxes in control and ECM roots revealed a quite different scenario. In all zones analyzed, the inoculation of eucalyptus plants induced an inhibition of the magnitude of Ca^{2+} fluxes (Fig. 3a). Furthermore, an inversion of flux direction (efflux to influx) was observed in the elongation zone. On the other hand, a significant increase of anion influx was observed primarily at the elongation zone ($P < 0.001$) and, to a lesser extent, at the root hair zone ($P < 0.01$, Fig. 3b). The results also showed a significant inhibition of the anion influx at the meristematic zone ($P < 0.01$), while no significant changes were observed at the apex and mature zones (Fig. 3b).

1.3.4 TIME-COURSE VARIATION OF EXTERNAL Ca^{2+} AND ANION CONCENTRATIONS

Analysis of the time-course variations in Ca^{2+} and anion concentrations in the medium with nonmycorrhizal (control) and ECM roots after a 5 min exposure to the nutrient medium is presented in Fig. 3(c) and (d). These results indicate that ECM roots were more efficient than the control in taking up Ca^{2+} ions from the external medium (Fig. 3c). By contrast, control roots seem to take up anions less efficiently than ECM (control change is nonsignificant) (Fig. 3d). This correlates well with the root surface pH values, since ECM roots showed a superior capacity to acidify the medium compared with the control (Fig. 2b).

1.3.5 PHARMACOLOGICAL ASSAYS ON H^+, Ca^{2+} AND ANION FLUXES

Highly significant changes in the ion fluxes were observed in the root system of *E. globulus* in the presence of *P. microcarpus* ECM fungus, notably in the elongation zone (Figs 2, 3). We further investigated the various fluxes in this region by detailed temporal analysis and pharmacological inference of the putative entities involved in their generation. All fluxes showed a clear oscillatory behavior in the elongation zone, irrespective of the conditions assayed. Changes in the oscillatory components of the ion fluxes were also induced by fungal colonization, mainly in the case of H^+ and anion fluxes (Fig. 4; wavelet spectral analysis in Fig. 5). The addition of 100 μm orthovanadate, a P-type PM H^+-ATPase inhibitor (Bowman, 1982; Bowman *et al.*, 1983), strongly inhibited all effluxes at the elongation zone (Fig. 4a). Ca^{2+} and anion fluxes were differentially inhibited by 100 μm gadolinium and 50 μm DIDS, respectively (Fig. 4b,c). Gadolinium (Gd^{3+}) is a widely used inhibitor for Ca^{2+} channels (Yang & Sachs,

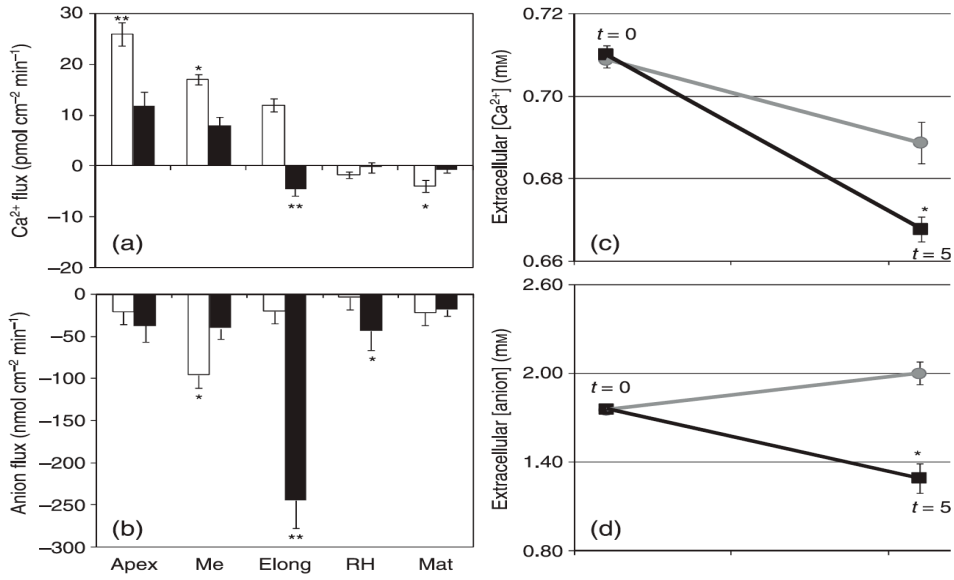

Figure 3| Fluxes of calcium (a) and anions (b) along nonmycorrhizal (control, open bars) and ectomycorrhizal (ECM) roots of *Eucalyptus globulus* colonized by *Pisolithus microcarpus* (closed bars). Apex, meristematic (Me), elongation (Elong), root hairs (RH) and mature (Mat) refer to the root zones analyzed. Negative values correspond to ion influx and positive values to effluxes. Bars represent mean values ± SE of five independent experiments. (c, d) Fluctuations on external Ca^{2+} (c) and anion (d) concentrations in nonmycorrhizal (control, circles) and ECM (squares) roots. For uptake analysis, roots were exposed for 5 min to Clark solution containing 0.2 mM Ca^{2+} (c) and 1.5 mM anions (d). Scale bars represent the mean values ± standard error (n = 5). *Means significantly different by Student's t-test at $P < 0.001$. For Ca^{2+} and anion fluxes, by two-way ANOVA combined with Duncan's test, the results showed that there were significant interactions between fungal treatment and root zones ($P < 0.0001$). There were no significant effects of fungal inoculation for Ca^{2+} fluxes at the root hair zone, and for anion fluxes at the apex and mature zones. *, $P < 0.01$; **, $P < 0.001$.

Table 2| inhibition of H^+, Ca^{2+} and anion fluxes (%) following the respective pharmacological assays

	% INHIBITION		
TREATMENT	Orthovanadate	Gadolinium	DIDS
Nonmycorrhizal	97.72	81.65*	100.00*
Ectomycorrhizal	82.6	75.41	73.83

For H^+ fluxes, orthovanadate was applied to the final concentration of 100 μM. For Ca^{2+} and anion fluxes, 100 μM gadolinium and 50 μM DIDS, respectively, were applied. n = 5. *At the same column, the mean values are significantly different by Student's t-test at $P < 0.01$.

1989; Hedrich *et al.*, 1990; Klusener *et al.*, 1995; Caldwell *et al.*, 1998; Antoine *et al.*, 2000, 2001) and DIDS is a commonly used Cl^- blocker (Schroeder *et al.*, 1993; Zonia *et al.*, 2001, 2002; Messerli *et al.*, 2004). Vanadate treatment led to an almost complete blockage of H^+ fluxes (Fig. 4a, Table 2), and the observed differences between nonmycorrhizal and ECM roots were not significant ($P > 0.05$), suggesting that all effluxes detected were the result of the

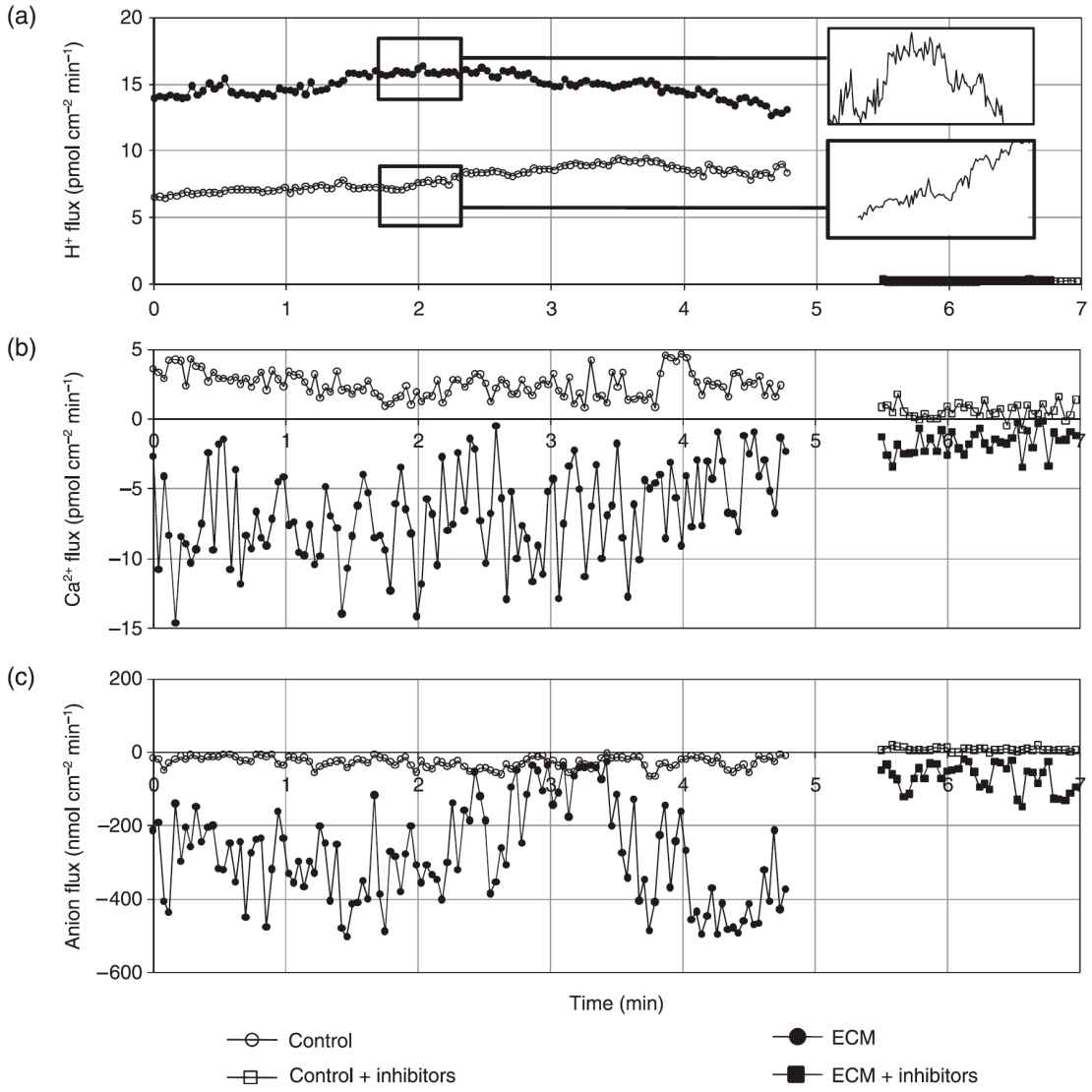

Figure 4| A representative graphical display of the standard output showing the oscillations in ion fluxes at the elongation zone of nonmycorrhizal (control) or mycorrhizal roots of *Eucalyptus globulus* colonized by *Pisolithus microcarpus* (ECM). (a) H^+ flux oscillations in the absence and presence of 100 μM orthovanadate (VO_4^{3-}). (b) Ca^{2+} flux oscillations in the absence and presence of 100 μM gadolinium (Gd^{3+}). (c) Anion flux oscillations in the absence and presence of 50 μM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). Negative values correspond to ion influx and the positive values to effluxes.

plasma membrane H^+-ATPase activity. Considering the stronger values of H^+ effluxes in ECM roots and the presence of different H^+-ATPase isoforms in the fungal hyphae, presumably with different sensitivities to vanadate, this degree of inhibition came as a surprise. Taken literally, one possible hypothesis is that the major proportions of these fluxes are actually generated by the root epidermis. The Gd^{3+} inhibition of Ca^{2+} fluxes showed a more complex pattern than that of vanadate on H^+ fluxes (Fig. 4b; Table 2). As previously mentioned, ECM reverses the efflux to

influx in the elongation zone, a result which is difficult to interpret, as it implies a shift in the balance of functional carriers for Ca^{2+}, which are presumably derived from different equilibrium conditions. In the context of ECM, Gd^{3+} inhibits close to 80% of the Ca^{2+} influx, a result consistent with the hypothesis that the majority of Ca^{2+} is taken up via Gd^{3+}-sensitive channels, some of which could be the result of fungal Ca^{2+} channels. This conclusion is supported by the observation that there is an almost total inhibition of Ca^{2+} channels in nonmycorrhizal roots (Figs 4b, 5). However, it should be pointed out that inhibition of an efflux by Gd^{3+} is not a straightforward interpretable result, and calls for further study. Anion influxes seem to be proportionally inhibited by DIDS in the same way in both control and ECM roots. This supports the notion that most anion fluxes are root-generated (Fig. 4c; Table 2). Furthermore, it was observed that all inhibitors performed more effectively in control conditions, supporting the hypothesis that in ECM roots there is a greater variety of ion transporters, some of which being refractory to the broadband inhibitors used.

1.3.6 SPECTRAL ANALYSIS OF THE ION FLUX OSCILLATIONS

As illustrated in the traces presented in Fig. 4, most of the continuous time-course measurements of fluxes showed components that were suggestive of sustained periodicity. To the extent that the spectral properties of these temporal variations could enlighten aspects of their regulation, we employed continuous wavelet time–frequency spectrum coupled to Fourier analysis to further dissect these properties. In all cases analyzed, we found evidence for underlying oscillations, sometimes with one single component, and in others with more than one component (Fig. 5a,c,e). More interestingly, they all showed some degree of modification upon colonization of *Eucalyptus* roots with the ECM fungus *P. microcarpus* (Fig. 5b,d,f). Results shown in Fig. 5 reveal that, in the control H^+ flux oscillations, there is one dominant period of c. 3.1 min, which lengthens to 5.3 min in the presence of the ECM fungus (Fig. 5a,b). This broadening of the major components of the oscillations were confirmed by Fourier analysis ($P < 0.05$; Fig. S1a,b). In addition, no significant oscillations were found in controls without a biological sample (not shown). By contrast, Ca^{2+} flux oscillations seem to show an opposing trend after ECM (Fig. 5c,d). Firstly, they seem to have two major components in the control condition: a dominant one of c. 5.3 min ($P < 0.01$) and a second of c. 1.5 min. However, both disappeared in the presence of the fungus (Fig. 5c,d), giving rise to a number of small periods at the borderline of the S/N ratio of the system. The Fourier analysis confirmed

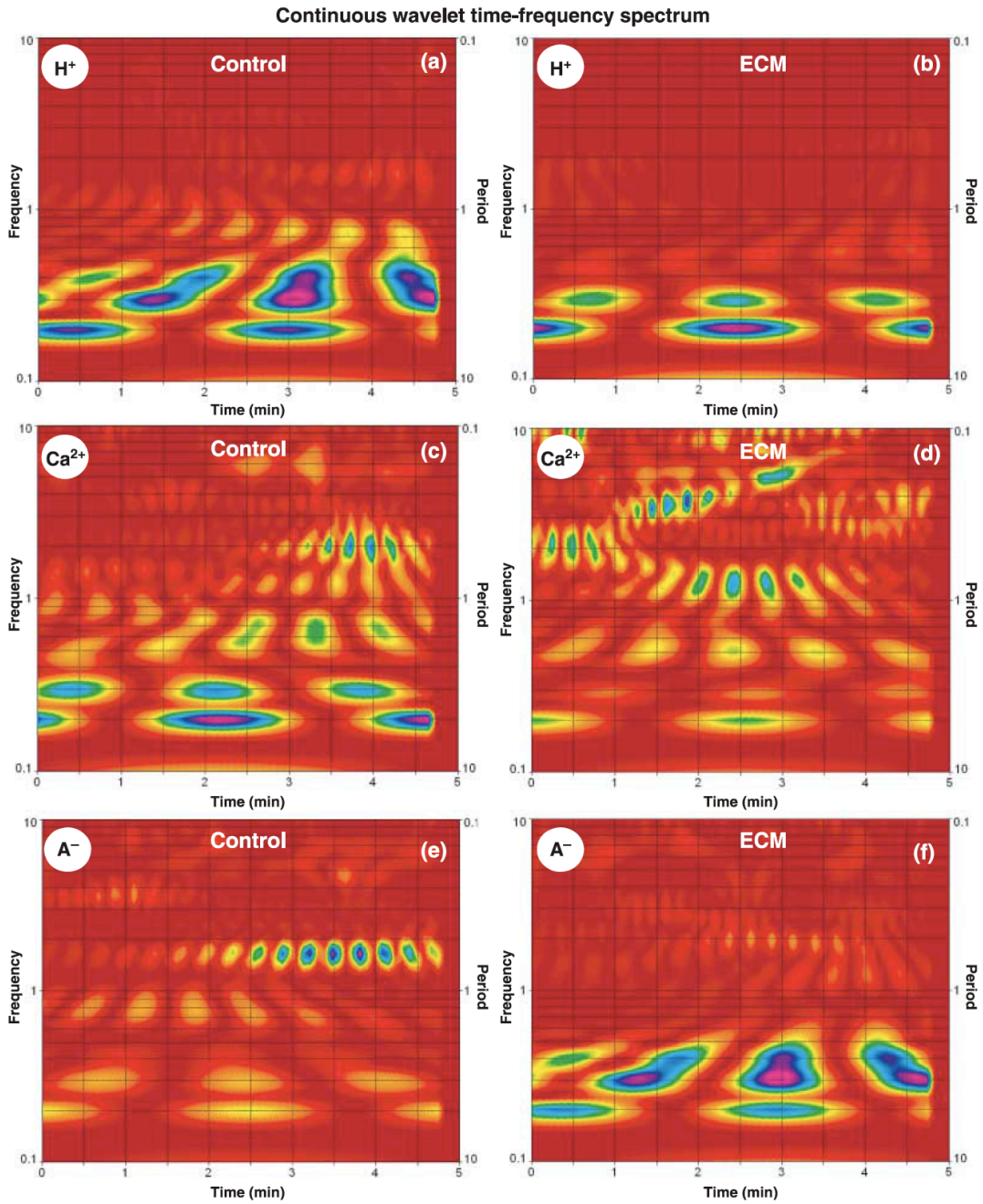

Figure 5| Continuous wavelet time spectrum analyses of the H^+ (a, b), Ca^{2+} (c, d) and anion flux oscillations in the elongation zone of nonmycorrhizal (a, c, e) and mycorrhizal roots (ECM) of *Eucalyptus globulus* colonized by *Pisolithus microcarpus* (b, d, f), as presented in Fig. 4. The frequencies are represented in min^{-1} and the periods in min. Wavelet analysis was coupled to Fourier analysis in order to dissect the frequency components, and shows the oscillatory pattern of the ion fluxes. No significant periods of ion fluxes were found in the medium without any biological sample (see also Fig. S2).

these results and showed that some of the high frequencies detected by continuous wavelet time–frequency spectrum analysis were not statistically significant at $P < 0.05$ (Fig. S1c,d). Finally, anion fluxes showed a third and different scenario. Control roots had at least one significant oscillation period of c. 0.6 min, thus characterized as being very fast, together with

others considered nonsignificant by Fourier analysis at $P < 0.05$ (Fig. 5e, S1e). In the presence of the ECM fungus, however, there was a drastic change of behavior, giving rise to a longer period of c. 3.0 min (Fig. 5e,f, S1f). Fourier analysis revealed the same short period of 0.6 min in ECM roots as above the level of system noise, but with a much-reduced significance. These results demonstrate that the ECM colonization changes the H^+ and anion flux oscillations, by increasing their periods by approx. double and sixfold, respectively, while for Ca^{2+} flux the oscillations are completely disrupted in the presence of the fungus. In addition, all ion flux oscillations were fully inhibited by the respective inhibitors such as orthovanadate, gadolinium and DIDS (data not shown).

1.3.7 A DUAL EFFECT OF THE EXTERNAL pH AND Ca^{2+} CONCENTRATION ON EXTRACELLULAR ION FLUXES

In systems showing prominent pH and Ca^{2+} dependency, the homeostasis of these ions seems to be closely interrelated. We tested whether this was also the case at the elongation zone, by growing *E. globulus* roots in medium with three different Ca^{2+} concentrations (0, 0.5, 1 mm) for 5 d, and analyzing the H+ fluxes and root surface pH after that period (Fig. 6). The results showed that an increase in Ca^{2+} availability provoked a significant inhibition on the H^+ effluxes in the root elongation zone (Fig. 6a). Likewise, the root surface pH increased with the Ca^{2+} concentration. Also, at 0.5 mm Ca^{2+}, almost all H^+ effluxes were inhibited (Fig. 6a). pH also induced some changes on Ca^{2+} efflux at the elongation zone, since under acidic conditions (pH 5.3) there was a significant increase in Ca^{2+} efflux (Fig. 6b). By contrast, under basic conditions, a significant inversion of the Ca^{2+} efflux to one of influx was observed, suggesting the presence of a pH-sensitive Ca^{2+} transport at the elongation zone.

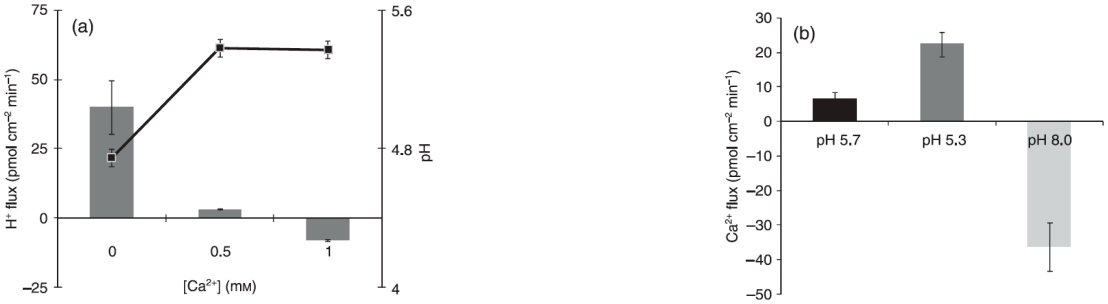

Figure 6| (a) Extracellular H+ fluxes (bars) and root surface pH values (squares) at the elongation zone of *Eucalyptus globulus* roots under three calcium concentrations (CaCl2). (b) Extracellular Ca2+ fluxes at the elongation zone with three different medium pH values. In this experiment, pH 5.7 was used as the control, since this value was used for all experiments of this work. The remaining pH values were obtained by growing roots for 2 d in the same medium used for ion flux analysis, to which was added 50 μM Tris-HCl, pH 5.3, or 50 μM Tris-base, pH 8.0. The negative values correspond to ion influx and the positive values to effluxes.

1.4 Discussion

This study presents the novel observation that different *Eucalyptus* root zones experience a differential modulation in their ion fluxes by the colonization of the ECM fungus *P. microcarpus*. Our experimental approach was efficient to produce plants with a high degree of fungal colonization at the stage of analysis. Thus, despite the inhibition of root hair growth, positive effects of ECM fungus on plant growth were observed (Table 1). This is a new aspect of host-pathogen interaction during ECM that reveals a potentially important aspect of coevolution between the fungal cell biology and the plant immune system, and one that may open for new paradigms of cell–cell communication through ion signaling pathways.

1.4.1 The control of root surface pH in ECM roots by extracellular H^+ fluxes is linked to PM H^+-ATPase activity

In comparison with control uninfected plants, the highest rates of H^+ efflux and acidic surface pH were located at the elongation zones of ECM roots (Fig. 2). These effluxes are dependent on the PM H^+-ATPase, as they were inhibited by 100 μm orthovanadate (inhibitor of P-type plasma membrane H^+-ATPase), a result which is conceptually sound since the elongation zone is a specialized growing zone (Winch & Pritchard, 1999). In fact, it has been shown that this zone shows notably higher immunolocalization and higher activity levels of PM H^+-ATPase than the apical and meristematic zones (Jahn *et al.*, 1998; Palmgren, 2001; see details in Enriquez-Arredondo *et al.*, 2005). Using immunocytochemical approaches, Lei & Dexheimer (1988) found strong PM H^+-ATPase labeling in root cortical cells of *Pinus sylvestris- Laccaria laccata*, in external hyphae sheaths and Hartig nets. This localization supports the concept of a coupling mechanism between fungal and host H^+ pumps in ECM roots (Fig. 2, Table 2). Indeed, it has been demonstrated that for arbuscular mycorrhizal associations, some host PM H^+-ATPase isoforms show increased activity and gene expression after fungal colonization (Ferrol *et al.*, 2002; Ramos *et al.*, 2005; details in Rosewarne *et al.*, 2007). The H^+ efflux mediated by the PM H^+-ATPase is important for the regulation of cytoplasmic pH (Felle, 2001; Palmgren, 2001; Tazawa, 2003) and the activation of cell wall-loosening enzymes and proteins through

acidification of the apoplast (Hager, 2003). This effect is closely related to auxin-induced cell growth as proposed by the 'acid-growth theory' by Rayle & Cleland (1992). This implies that enhanced H^+ efflux in ECM roots (Fig. 2a) results in an acidification of the apoplastic/ external pH (Fig. 2b). Moloney *et al.* (1981) demonstrated that pH changes in the apoplast are crucial for root growth, since acidic buffering conditions act as stimulators whilst neutral or basic pHs act as inhibitors. Our results clearly show that when the H^+ flux rate (Fig. 2a) and surface pH values (Fig. 2b) are combined, highly significant Pearson's correlation coefficients are obtained (–0.82; $P < 0.0001$). Other candidates that contribute to the control of extracellular H^+ flux in ECM are the presence of anions in the growth medium. These are reported to act as stimulators of the PM H^+-ATPase (Churchill & Sze, 1984; Ullrich & Novacky, 1990; Glass *et al.*, 1992; Forde, 2000; Garnett *et al.*, 2001). This concept is especially appealing taking into account the observed oscillatory behavior (Figs 5, S1), where the ECM colonization induced changes in the flux oscillations, leading to their increased periods. For Ca^{2+} flux oscillation, ECM colonization abolished all significant periods observed in control roots (Figs 5b,c, S1). Combined with the reversion from efflux to influx in the elongation zone, this result could be interpreted as showing that the fungus contributes to the majority of the Ca^{2+} influx through specific channels. These different activities would produce intricate temporal patterns impossible to synchronize on an organized oscillatory pattern. This being the case, the prediction would be that ectomycorrhizal plants should have an improved efficiency of Ca^{2+} uptake from the soil, a result partly confirmed in Fig. 3(c).

1.4.2 Ca^{2+} efflux suppression and increase upon Ca^{2+} uptake in ECM roots

Calcium has a paradoxical effect on PM H^+-ATPase, as it has been reported to be an inhibitor via a Ca^{2+}-dependent phosphorylation pathway (Lino *et al.*, 1998; Tazawa, 2003) and an activator in guard cells (Assmann *et al.*, 1985). An inhibition of the PM Ca^{2+} influx channels in both animal (Yang & Sachs, 1989) and plant cells (Allen & Sanders, 1994; Klusener *et al.*, 1995; Knight *et al.*, 1996; Antoine *et al.*, 2000, 2001) occurs by the addition of extracellular Gd^{3+} in a micromolar range. Despite its use for detection of Ca^{2+} stretch-activated channels (Caldwell *et al.*, 1998), Gd^{3+} is likely to inhibit other cationic channels as well, because of its relatively broad effect. Our pharmacological analysis suggested that the Ca^{2+} influx in the elongation zone of ECM roots is the result of the activity of Gd^{3+}-sensitive calcium channels

(Figs 3a, 4; Table 2). However, as the Ca^{2+} effluxes are largely governed by the chemical potential gradient of Ca^{2+} generated by the PM Ca^{2+}-ATPase, we hypothesized that the suppression of effluxes in the control roots could represent an indirect dissipation of the Ca^{2+} gradient, as promoted by Gd^{3+} treatment (Fig. 4, Table 2). In addition, similar flux inhibition profiles were obtained by Nemchinov *et al.* (2008) in *Nicotiana benthamiana* leaves. The authors proposed a model in which Gd^{3+}-sensitive Ca^{2+} influxes and Ca^{2+} pumps are involved in the signal transduction pathways of the hypersensitive response mechanisms (Nemchinov *et al.*, 2008). As a passive Ca^{2+} efflux from the cell cytosol is thermodynamically improbable (Shabala & Newman, 2000), an active mechanism must be involved. Two possible mechanisms of Ca^{2+} efflux might occur, one through Ca^{2+} release from the cell wall and the other by Ca^{2+} extrusion via the PM Ca^{2+}-ATPase (Lecourieux *et al.*, 2006; Nemchinov *et al.*, 2008). It remains to be determined which of these two mechanisms is responsible for this event to occur. Alternatively, an increase in the activity of Ca^{2+} influx in ECM roots could reflect an increased cytosolic concentration of this ion. Indeed, it has recently been demonstrated that the exposure of *E. globulus* root hairs to hypaphorine (an indole alkaloid secreted by *P. microcarpus*) led to an elevation of cytoplasmic Ca^{2+} concentration (Dauphin *et al.*, 2007). Thus, hypaphorine led to a reduction of the Ca^{2+} gradient across the plasma membrane, which was correlated with the arrested growth of root hairs (Béguiristain & Lapeyrie, 1997; Dauphin *et al.*, 2007). These results seem similar to our own observations (Table 1), where root hair length was reduced in ECM roots. Recently, Martin *et al.* (2008) published the genome of the ECM fungus *Laccaria bicolor*, in which numerous and diverse Ca^{2+} channels are found to be encoded (see details at http:// genome.jgi-psf.org/Lacbi1/Lacbi1.home.html). Accordingly, we found ECM roots to have a higher uptake capacity of Ca^{2+} from the external medium (Fig. 3c). In itself this would not necessarily lead to a major accumulation of Ca^{2+} in ECM of whole plants, but clearly suggests a higher potential for ion uptake and storage in the cell wall (Peterson & Enstone, 1996; Kuhn *et al.*, 2000) promoted by the fungus. In ECM associations, such as *Suilus bovinus-Pinus sylvestris*, an exposure to Ca^{2+} also led to an accumulation of this ion in the interfacial apoplast in between symbionts and in the fungal sheath (Bucking *et al.*, 2002). Depending on the fungal species, Ca^{2+} can also accumulate as calcium oxalate in the fungal hyphae (Malajczuk & Cromack, 1982). In the light of this, calcium dynamics in ECM interactions needs to be more carefully investigated, not just using radioisotopes, but also by

means of an integration of techniques such as ion-selective vibrating probes, patch-clamp and imaging analyses.

1.4.3 Activation of anion uptake by ECM fungus

It is well known that an increase in the root surface concentration of H^+ generates a proton-motive force, which is necessary to drive the secondary transport of , $SO4^{2+}$, Cl^-, Ca^{2+} and K^+ (Portillo, 2000; Palmgren, 2001). Accordingly, we found that the changes in H^+ efflux attributable to ECM fungal infection in the elongation zone were strictly correlated to the root surface pH values (–0.82; $P < 0.0001$), and, significantly, correlations of root surface concentrations of H^+ were found with both Ca^{2+} (–0.78, $P < 0.001$) and anion fluxes (0.66; $P < 0.006$). The correlation between Ca^{2+} and anions at the elongation zone (0.99, $P < 0.001$) raised the possibility of an activation of anion influx by Ca^{2+}, as demonstrated in other cells (Hedrich *et al.*, 1990). Since plant cells have adapted to low anion concentrations, anion uptake is generally coupled to the electrochemical gradient generated by the PM H^+-ATPase activity (Evans *et al.*, 1980; Zimmermann *et al.*, 1994; Garnett *et al.*, 2001). Consequently, ECM roots possess strong anion influxes and H^+ effluxes primarily at the elongation zone (Fig. 3b). Consistent with this, we observed high H^+-ATPase activity in this root zone. It has been reported that this enzyme is stimulated by anions in plant (Churchill *et al.*, 1983; Churchill & Sze, 1984; Zimmermann *et al.*, 1994) and animal cell membranes (Vieira *et al.*, 1995). The induction of uptake in *P. pinaster* ECM roots, even at low external concentrations, was previously shown by Gobert & Plassard (2002). The H^+ efflux and consequent root surface acidification are necessary for the uptake mechanism to operate (Ullrich & Novacky, 1990; Glass *et al.*, 1992; Forde, 2000), as this occurs via PM cotransporters (nH^+/NO^{3-}) (Crawford,1995). This was already demonstrated for *Eucalyptus nitens*, where large H^+ effluxes were found in medium with NO^{3-}. However, fluxes were quantitatively linked to H^+ fluxes (Garnett & Smethurst, 1999; Garnett *et al.*, 2001, 2003). In addition, according to Garnett *et al.* (2003), negative correlation coefficients can be obtained between and H^+ fluxes. Nitrate is thus a strong candidate to be a component of the anion fluxes we observed, but unfortunately the technical limitations of the electrodes used do not warrant a straightforward conclusion in this respect (see the Materials and Methods section and Fig. S2a,b), with chloride probably playing also an important role. In normal conditions, the maintenance of the electrical membrane potential depends on the H^+ efflux and influxes of anions and potassium (Felle,

2001; Tazawa, 2003). In ECM symbiosis, fungi have a high capacity to uptake potassium in their external hyphae (Rygiewicz & Bledsoe, 1984). One possible molecular basis for this was recently discovered in the same type of hyphae, where Corratgé *et al.* (2007) cloned the HcTrk1 transporter from *Hebeloma cylindrosporum*, and demonstrated it to encode for a single-file pore channel that cotransports Na^+/K^+ into the hyphae. Pharmacological analyses suggested the presence of anion channels at the elongation zone, since the influxes were sensitive to DIDS. In guard cells, DIDS also inhibits anion uptake (Schroeder *et al.*, 1993; Schwartz *et al.*, 1995) similar to what was observed in this study (Table 2). Further studies should be focused on the proper discrimination of the specific anions involved on the observed response at the elongation zone of ECM roots.

1.5 CONCLUDING REMARKS

Based on our results, we propose a model for pH signaling in ECM roots, which is directly linked to nutrient uptake and plant growth (Table 1, Fig. 7). ECM fungi induce positive modulation of the H^+ efflux rates and rhizosphere acidification, mediated by PM H^+-ATPase activities from both host and fungal partners. In turn, this stimulation triggers a pH signal that modulates Ca^{2+} transport and, indirectly, anion uptake (Hedrich *et al.*, 1990). This hypothesis is supported by our observation that external Ca^{2+} acts as a strong inhibitor of the H+ efflux and root surface acidification in the elongation zone of eucalypt roots. By contrast, Ca^{2+} fluxes were also affected by the medium's pH, as has previously been reported in other plant cells (Foster, 1990). An increase in anion uptake and lower concentrations of external Ca^{2+} will thus occur, which are reflected both in the promotion of plant growth and in PM H^+-ATPase activity (Zimmermann *et al.*, 1994). The spectral analysis of the ion flux oscillations revealed itself to be an efficient parameter to compare biophysical effects of the ECM fungus in the fast oscillation components. This analysis can be used as an additional tool during the study of ion dynamics using the ion-selective vibrating probe technique, on the assumption that shifts in the main components of oscillations correspond to the activation/shift of a variety of molecular transporters.

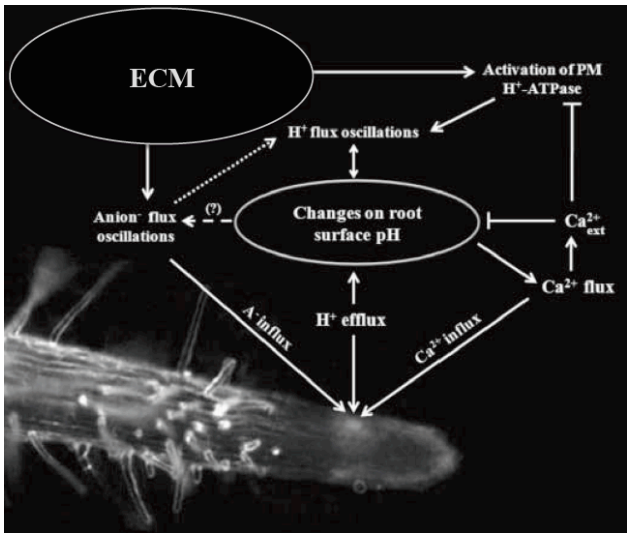

Figure 7| Proposed model for the pH signaling mechanism in ectomycorrhizal (ECM) roots and the differential modulation of anion (A^-) and calcium (Ca^{2+}) uptake

1.6 METHODS

1.6.1 BIOLOGICAL MATERIAL, INOCULUM PRODUCTION AND *IN VITRO* SYNTHESIS OF ECTOMYCORRHIZAS

Three agar discs containing mycelium of the ECM gasteromycete *Pisolithus microcarpus* isolate PT 90A were inoculated onto Petri dishes containing 20 mL of modified MNM (Marx, 1969) medium and incubated for 28 d at 28°C. From the resulting colonies, 9 mm agar discs were cut off from the edge of actively growing colonies. *Eucalyptus globulus* Labill. Seeds were superficially sterilized with 5% sodium hypochlorite (v/v) for 15 min, rinsed with five changes of sterile water, and plated on modified Clark solution at quarter-strength (Clark, 1975) to which was added 2.9 μM thiamine-HCl and 1% sucrose in 0.5% (w/v) Phytagel (Sigma-Aldrich, Gillingham, UK). The use of Phytagel produced a clear and colorless medium, which is excellent for imaging and ion flux measurement with reduced electrical noise (Ramos *et al.*,

2008a). After 7 d, aseptically germinated seedlings were placed on the edge of 10-d-old ECM fungal mycelium grown on the same medium used for seedlings. These were left for 15 d in a controlled environment growth chamber, with 16 h of light (26°C, 350 μmol m^{-2} s^{-1}) and 8 h of dark, for ectomycorrhiza formation. ECM plants were later transferred to hydroponic conditions in the same solution and growth chamber settings for 10 d. Subsequently, ion fluxes measurements were performed in secondary roots of intact plants. In addition, pieces of root system were washed and samples were subsequently collected for microscopic evaluation of mycorrhizal colonization, as described by Brundrett *et al.* (1996).

1.6.2 Measurements of H^+, Ca^{2+} and anion fluxes and currents using the ion-selective vibrating probe system

A detailed description of the experimental setup of the ionselective vibrating probe technique utilized in this study has been well described (Kochian *et al.*, 1992; Feijó *et al.*, 1999; Shipley & Feijó, 1999; Zonia *et al.*, 2002; Kunkel *et al.*, 2006; Ramos *et al.*, 2008a). In short, *E. globulus* plants colonized or not by ECM fungus *P. microcarpus* isolate PT 90A under hydroponic conditions, were placed in plastic Petri dishes (140x140 mm) filled with 30 mL of modified Clark solution at quarter strength, except for Ca^{2+} measurements, where 100 μM Ca^{2+} was used. Visual Minteq analysis was performed according to Parker *et al.* (1995) using the ion concentrations of the modified Clark solution applied in this study. We focused on secondary roots, as they are biologically and physiologically more significant than primary roots for nutrient supply to the plant. The volume occupied by secondary roots in the soil can reach 30–40% more than primary ones. Readings were taken in five defined root zones of nonmycorrhizal (control) and mycelium-covered roots: apex (tip), meristematic (100–150 μm); elongation (300–800 μm); root hairs (major presence of these structures); and finally mature zone (posterior to root hair zone). Ion-specific vibrating microelectrodes were produced as described by Feijó *et al.* (1999). Micropipettes were pulled from 1.5 mm borosilicate glass capillaries and treated with dimethyl dichlorosilane (Sigma-Aldrich). After silanization, they were backfilled with a 15–20 mm column of electrolyte (15 mM KCl and 40 mM KH_2PO_4, pH 6.0, for H^+; 100 mM KCl for anions; 100 mM $CaCl_2$ for Ca^{2+}) and then frontloaded with a 20–25 μm column of the respective ion-selective liquid exchange cocktail (Fluka, Milwaukee, WI, USA). We

used Cl^- electrodes to measure the anion fluxes given that this electrode has poor selectivity for Cl^- under our experimental conditions (Supporting information, Fig. S2a,b). Firstly the measurement of chloride activity in the medium is slightly affected by the presence of other ions (Fig. S2a), but these changes should be expressed below noise level within the microvolt range usually measured on vibrating conditions for cellular fluxes. More importantly, the Cl^- electrode calibration with different anions showed that this electrode responds with a Nernstian slope to chloride and nitrate, and while sub-Nernstian to sulfate and phosphate, also exhibits a significant response within the concentrations used in this study (Fig. S2b). Last but not least, the background concentrations in the medium of the individual anions span various orders of magnitude, likewise affecting the signal-to-noise (S/N) ratio measurement of the fluxes in a way that is inversely proportional to the concentration. Taken together, these considerations make it almost impossible to discriminate the individual activities of every single anion, and therefore we have opted to refer to these fluxes as reflecting the global 'anionic' concentration rather than Cl^- proper fluctuations. An Ag/AgCl wire electrode holder (World Precision Instruments, Sarasota, FL, USA) was inserted into the back of the microelectrode and established electrical contact with the bathing solution. The ground electrode was a dry reference (DRIREF-2, World Precision Instruments) that was inserted into the sample bath. The microelectrodes were calibrated at the beginning and end of each experiment using standard solutions covering the experimental range of each ion, in order to obtain a calibration line. Both the slope and intercept of the calibration line were used to calculate the respective ion concentration from the mV values measured during the experiments.

1.6.3 Inhibition with vanadate (VO_4^{3-}), gadolinium ($GdCl_3$) and 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)

Inhibitor treatments were performed in *Eucalyptus* roots after determination of each ion flux at the elongation zone (n = 5). The data acquisition was stopped and the respective inhibitors (Sigma-Aldrich) were added in the Petri dishes with the following concentrations: plasma membrane H^+-ATPase (100 μM orthovanadate), calcium channels (100 μM gadolinium) and chloride channels (50 μM DIDS). Five to 10 min later, a background reference was taken and ion fluxes were again recorded. Interference caused by the inhibitors was controlled for by

direct incubation with ionophore-loaded probes. No significant interference of the inhibitors was found to occur for H^+ and anions. For Ca^{2+}, the interference was more pronounced with high levels of gadolinium, which in the present study was used at lower concentrations (100 μM; Fig. S3).

1.6.4 Ion flux oscillation analysis

Frequency analyses were performed using AutoSignal v1.7 (Systat Software, Inc.). For each set of flux oscillations to be analyzed, a data trend removal was applied, consisting of a linear least-squares fit subtraction to remove the very low frequency trend of the data. Two distinct methods were then used to assess the frequency components of the oscillations: Fourier and Wavelet analyses. For Fourier analysis, a fast Fourier transform Radix 2 algorithm was used, ensuring that each data set was a continuous acquisition without breaks and with a constant sampling rate. Peaks were detected by a local maxima detection algorithm and considered relevant according to their significance levels (the higher the significance level, the less likely it is that a detected spectral signal will arise from random noise). Significance levels are given in the Results section. For wavelet analysis, a continuous wavelet time– frequency spectrum was obtained with a noncomplex Morlet wavelet (wave number, 12). A peak-type critical limit was used instead of the traditional confidence levels, as implemented in the software.

1.6.5 Statistical analysis

All data was analyzed by one-way or two-way ANOVA in order to compare the mean values (considering 'fungal treatment' and 'root region' as factors), which were validated by convenient residual analyses and, when necessary, combined with Duncan's test for multiple comparisons. To compare the control and fungal treatment (Table 1), we applied Student's *t*-test for two independent samples and calculated confidence intervals for the mean difference, in order to guarantee a global 95% confidence level. The results are expressed as means with respective standard error, and the numbers of repetitions are given in each figure legend. All statistical analyses were conducted using the R program and the level of significance was set up at 5% (Ihaka & Gentleman, 1996).

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1.8 SUPPLEMENTARY DATA

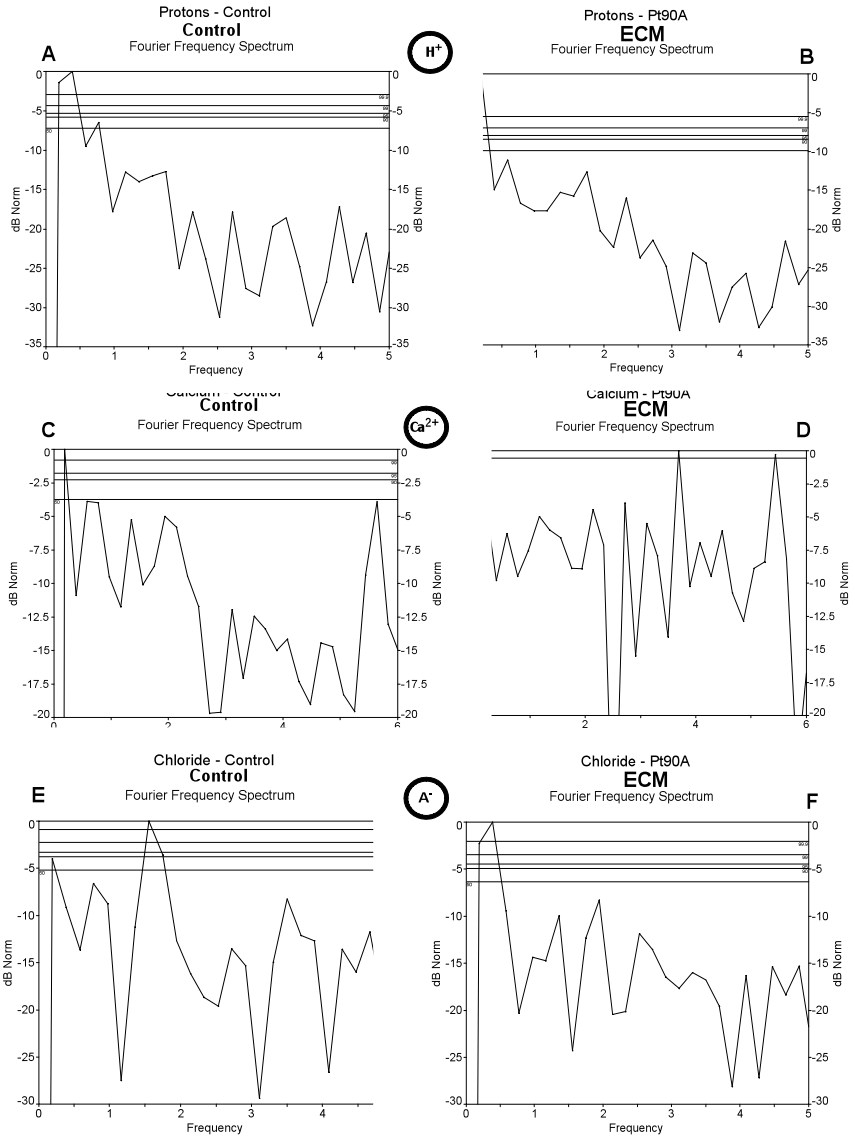

Figure S1| Fourier frequency spectrum analysis of H^+ (A, B), Ca^{2+} (C, D) and anion flux oscillations in the elongation zone of non-mycorrhizal (A, C and E) and mycorrhizal roots (ECM) of *E. globulus* colonized by *P. microcarpus* (B, D and F). Frequencies are represented in min^{-1}.

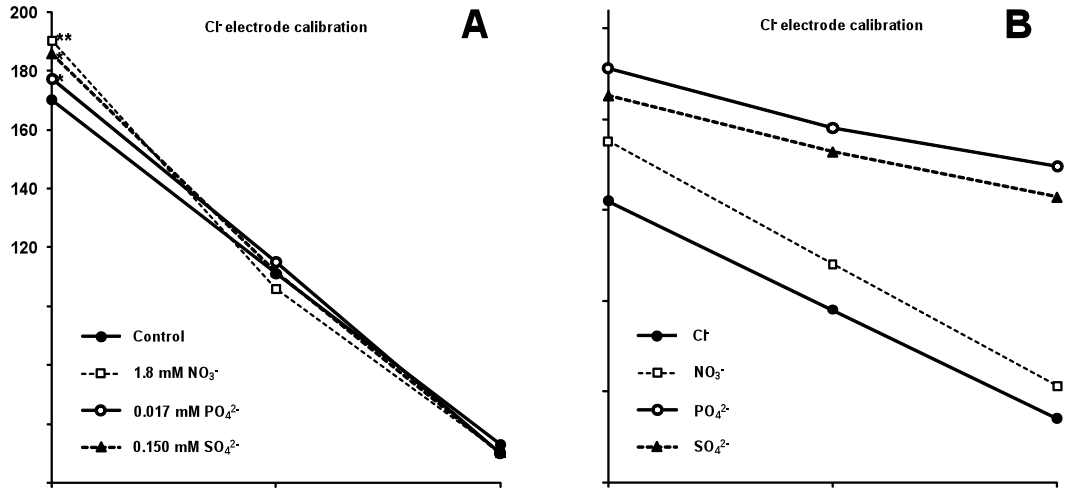

Figure S2| (A)- Interference on Cl^- activity detection by the Cl^- electrode (Control) by further anions (NO_3^-, SO_4^{2-} and PO_4^{2-}) present in ¼ strength Clark`s solution. The interference was more pronounced in lower Cl^- concentrations, especially for NO_3^- and SO_4^{2-}. Yet, in real vibration measurement conditions, where differences are usually measured in the microvolt range, this effect is minimal. * *P*-value < 0.1 and ** *P* -value < 0.01. (B)- Calibration of Cl^- electrodes with different anions. Nitrate and Chloride show almost perfect Nersntian responses. Phosphate and Sulphate, while clearly sub-Nersntian, show significant responses on the interval measured.

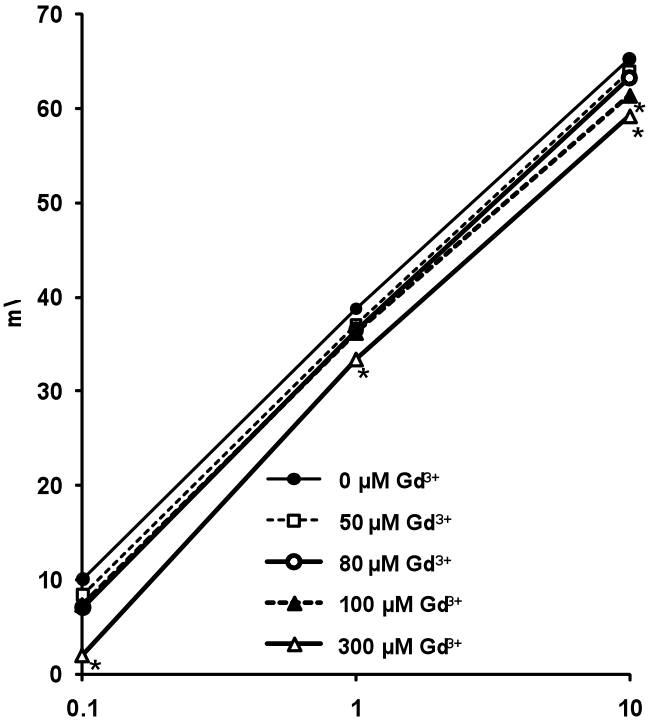

Figure S3| Effect of different concentrations of Gadolinium (Gd^{3+}) on the sensitivity of Ca^{2+} electrodes (n = 5). * *P*-value < 0.01.

2. ON THE EVOLUTION AND DEVELOPMENT OF ROOT SYMBIOTIC SYSTEMS

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Plant-microbe symbioses: new insights into common roots (2009) *BioEssays* **31** (11): 1233-1244

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Key Words: Mycorrhiza, Nodulation, Symbiosis, Modularity, Glomeromycota, Evo-Devo

2.1 ABSTRACT

Arbuscular mycorrhiza (AM), a type of plant-fungal endosymbiosis, and nodulation, a bacterial-plant endosymbiosis, are the most ubiquitous symbioses on earth. Recent findings have established part of a shared genetic basis underlying these interactions. Here, we approach root endosymbioses through the lens of the homology and modularity concepts aiming at further clarifying the proximate and ultimate causes for the establishment of these biological systems. We review the genetic structures that underlie interspecific signaling and its concomitant shift in genetic programs for either partner. Also, through the comparative analysis of AM and nodulation symbioses shared genetic modules, we identify the fundamental nodes in these networks, suggesting the elemental steps that permitted symbiotic adaptation. Here, we show that this approach, allied to recent technical advances in the study of genetic systems architecture, can provide clear testable hypotheses for the advancement of our understanding on the evolution and development of symbiotic systems.

2.2 INTRODUCTION

As early as 1905, Mereschkowsky stated that chloroplasts might have evolved from free-living cyanobacteria, which were engulfed by a protozoan host cell (Mereschkowsky, 1905). The adaptation of the ancestral chloroplast as an endosymbiont was so successful that entire new forms of life and a new kingdom arose from the evolution of this interaction (Kutschera & Niklas, 2005). The establishment of symbioses between distantly-related species recurred in this clade (Smith & Read, 2008), being the most ubiquitous result of such an event the arbuscular mycorrhizal (AM) mutualistic association (Smith & Read, 2008). This interaction is established between fungi belonging to the monophyletic phylum Glomeromycota (Schüssler *et al.*, 2001) and the roots of most vascular plants (Smith & Read, 2008), suggesting an ancient evolutionary origin (Parniske, 2008). Arbuscular mycorrhiza-forming fungi (AMF) are incapable of accomplishing their life cycle in the absence of a host plant (obligate biotrophy) (Harrison, 2005; Reinhardt, 2007). The AMF dependence on this symbiotic relationship is consistent with the expected progressive loss of functional redundancy over extended co-evolutionary time (Moran, 2002). Further support comes from both fossil (Remy *et al.*, 1994; Taylor, 1995) and genetic (Simon *et al.*, 1993) evidence of ancient arbuscular mycorrhizal-like associations, which suggest that these predate the colonization of land by plants around 450 million years ago, and thus might have played a role in the subsequent radical change in the biosphere. Despite this potentially pivotal role in the history of life on earth, the AM genetical system has only recently started to be dissected. However, most of the genetical analysis of AM establishment has focused on the plant symbiont alone (Smith & Read, 2008). This bias can be attributed to the tractability of the plant system, both for its capability to develop independently of the AMF, and for the development of plant genetics tools early in the history of genetics as an experimental scientific discipline.

Several plant genome sequences are already available, including AM-forming plants such as the model systems *Oryza sativa* (rice) (2005) and more recently *Zea mays* (maize), two of the most important crops for human nutrition. Moreover, most data concerning AM genetics has been generated in legumes, which are a relatively recent subgroup of the AM-forming plants (Markmann *et al.*, 2008; Parniske, 2008), constituting a further phylogenetic bias on our current knowledge of AM biology. Nevertheless, studies in legumes have provided some important insights such as that some nodulation mutants are unable to produce normal

AM phenotypes. We know of at least seven genes (common symbiosis genes) to be shared between plant-rhizobia and AM endosymbioses (Kistner *et al.*, 2005), one of which also plays a role in the establishment of symbioses with actinobacteria (Gherbi *et al.*, 2008; Markmann *et al.*, 2008). This is an invaluable cornerstone in our knowledge of the plant gene networks underlying AM evolution, that must be complemented with an approach focusing on the genetic bases underlying specificity in mycorrhizal endosymbioses. Recently, the first steps in this direction have been taken with the description of mutants for the establishment of AM symbiosis in non-legumes (Paszkowski *et al.*, 2006; Reddy *et al.*, 2007).

There are recent and very comprehensive reviews dealing with the collection of AM genetic data (Parniske, 2004; Harrison, 2005; Requena *et al.*, 2007; Parniske, 2008), so this will not be the main topic of this paper. Here, we present an evodevo approach to root endosymbioses, namely through the concepts of modularity and Gene Regulatory Networks (GRNs), that can point the way to the most important features of these systems and prioritize an experimental program designed at determining their basal biological principles and mechanisms. We will develop the idea that the current knowledge of the plant genetics of nodulation further supports the hypothesis that at least part of this genetic network originated through co-option of AM symbiosis genes (Kistner & Parniske, 2002). We will also expand this notion by reviewing recent literature on the evolution of root-nodule symbioses and integrating in our analysis the concepts of homology and modularity (BOX 1).

2.3 FOLLOWING THE ONTOGENY OF AM

Plant mutualistic endosymbioses are characterized by the penetration of host cells by microbial symbionts, followed by a stage during which the symbiont lives partially or wholly within plant cells (Parniske, 2000). AM fungi receive plant-derived photosynthates from this association, in turn transferring to the host cells phosphate and other minerals scavenged from the soil (Harrison, 2005; Parniske, 2008). The development of AM fungi involves germination of an asexual spore, hyphal polarized growth and profuse branching (Akiyama *et al.*, 2005), which ultimately differentiate into appressoria. Appressoria develop on the root surface and functionally work as host cell penetration-helping structures (Figure 1) (Harrison, 2005; Parniske, 2008). Arbuscular mycorrhiza-forming fungi (AMF) are obligate biotrophs, i.e. they cannot complete their life-cycle without taking part in a symbiotic relationship with a plant

(Smith & Read, 2008). Despite this apparent limitation, these fungi have an initial asymbiotic stage characterized by a coenocytic complex of hyphae, occurring after the germination of a single spore containing up to 2000 nuclei (Bécard, 1993). An interesting observation is that they have the ability to, in the absence of host-derived signals, arrest and restart growth ("flickering") (Koske, 1981). AM fungal hyphae cross epidermal, sometimes hypodermal, and outer cortex layers through a host derived intracellular assembly apparatus which determines the future path of hyphal penetration (the prepenetration apparatus - PPA) (Genre *et al.*, 2005; Genre *et al.*, 2008) (Figure 1e) and re-enter the apoplast crossing the inner cortex intercellularly. Inner cortical cells are subsequently invaded and hyphae develop profuse ramifications, giving rise to tree-shaped structures termed arbuscules within their cytoplasm, surrounded by a plant-derived membrane (Parniske, 2008).

Mycorrhizal symbioses are multicellular complex systems in which a series of spatial and temporal tightly regulated events involving inter-organismic signaling lead to division of labour between the players. In particular, the obligate biotrophy of one of the players supports the notion that AM symbiosis can be looked at as a single (and singular) developmental program as typically applied to multicellular eukaryotes.

2.4 SETTING UP A *RENDEZ-VOUS*: DIFFUSIBLE SIGNALS IN PRESYMBIOSIS

A necessary step for the transition to the symbiotic stage is the pre-symbiotic recognition of the phototrophic partner by the AMF. It is known that fungi recognize compatible host roots with a certain level of specificity before undergoing hyphal branching (Requena *et al.*, 2007). Recently, it has been shown that hyphal branching in *Gigaspora margarita* is triggered by *Lotus japonicus* constitutively-secreted strigolactones, molecules belonging to the sesquiterpene lactones family (Akiyama *et al.*, 2005). These branching factors are now known to be recognized by phylogenetically distant fungal species (Besserer *et al.*, 2006) and, in an interesting evolutionary analogy, by some parasitic weeds among which are *Orobanche* and *Striga,* from whom its name derives (Cook *et al.*, 1966). Additionally, strigolactones have been recently appointed as novel endogenous plant hormones, acting in diverse angiosperms, including the non AM-forming *Arabidopsis thaliana*, as shoot branching inhibitor compounds (Gomez-Roldan *et al.*, 2008; Umehara *et al.*, 2008). The original role played by strigolactones

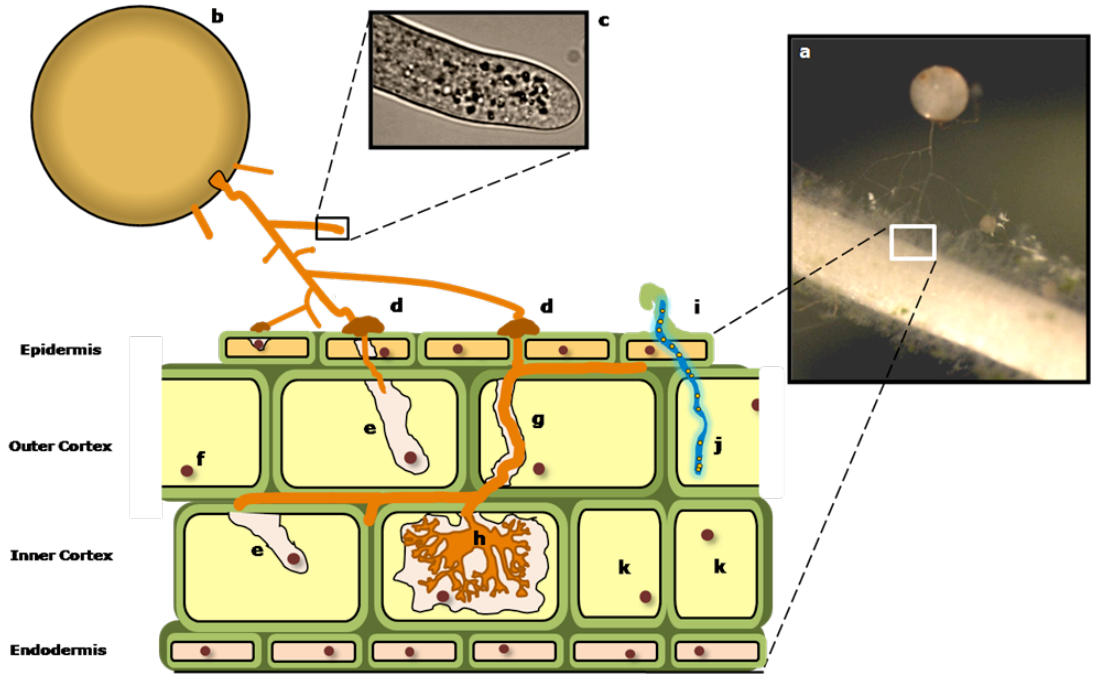

Figure 1| Schematic representation of plant root endosymbioses with AM fungi and nodulating bacteria. (a) A typical example of a leguminous plant root colonized by an AM fungus. The development of AM interactions starts prior to physical contact between symbionts: after spore germination (b), hyphal tip-growth (c) and profuse branching are induced by exudated root factors (namely strigolactones) (Akiyama *et al.*, 2005). Formation of an appressorium (d) on plant epidermal cells precedes the arrangement of the prepenetration apparatus (PPA) (e) sponsored by plant cell nucleus migration (Genre *et al.*, 2005; Genre *et al.*, 2008). (f) The PPA paves the way for the subsequent intercellular mycelium growth (g). Differentiation of intraradicular hyphae into an arbuscule (h) inside cortical cells constitutes the ultimate contact among symbionts with the formation of an interface, the "symbiotic synapse", where bilateral nutrient exchange takes place (Parniske, 2008). On the other hand, nodulating bacteria interrelate with host roots firstly by activating morphological changes on root hairs, which might curl (i), entrapping bacteria inside (Oldroyd & Downie, 2004; Oldroyd & Downie, 2008). A novel apparatus that might have evolved from the PPA, the infection thread (j), constitutes a plant-made invagination capable of transversing cell boundaries, carrying bacteria from infection foci into cortical cells (k) (Oldroyd & Downie, 2008).

during plant evolution is still unknown: if its original function was related to symbiosis signaling or organogenesis-related hormonal events. Addressing the existence of a putative role played by strigolactones in gymnosperms and non-vascular plants, currently missing, will be of extreme importance to address this question. Concurrently, the assessment of the role played by strigolactone in the root development of AM-forming angiosperms may bring further insight into the inference of the AM signaling evolutionary history.

One of the first fungal responses to strigolactone consists of an enhanced mitochondrial-related gene expression and respiratory activity (Tamasloukht *et al.*, 2003; Besserer *et al.*, 2006). Also, recent observations correlate an increased strigolactone biosynthesis with plant phosphate deprivation (Yoneyama *et al.*, 2007; Lopez-Raez *et al.*,

BOX1: MODULARITY AND HOMOLOGY: TOOLS FOR UNDERSTANDING DEVELOPMENT AND EVOLUTION

In a recent review on the developmental genetics of homologous structures, Wagner (2007) elaborates on the conceptual challenge of ascribing a common ground, at the genetic level, to characters known to be homologous. Such challenge stems from the realization that homologous genes do not necessarily encode for homologous structures, the same holding true for the reverse proposition. Epitomizing the elusiveness of the homology concept is the extreme example of the ey/pax6 genes, in which homologous genes were "independently recruited for superficially similar roles" (Abouheif *et al.*, 1997) in divergent phyletic lines, taking part in the development of the insect/vertebrate eyes, known to be independently derived structures that also share a dissimilar developmental genetic network (Abouheif *et al.*, 1997; Wagner, 2007). To overcome this conceptual hurdle, and elaborating on the concept of gene regulatory networks (GRNs), Wagner proposes that the genetic correspondence of the homology observed between two characters is to be ascertained through their "character-identity genetic networks", or ChINs. These are to be differentiated from the character-state genetic networks that encode for the often divergent shapes/sizes/colours (states) of a homologous character and are downstream of and activated by ChINs. This conceptualization predicts identical ChIN modules underlying homologous structures and, reversely, non-homologous characters diverging in their ChINs (Wagner, 2007). GRNs are modular, quasi-independent sets of genetic elements highly correlated in phenotype and seemingly unaffected by variation in elements of other modules (Davidson, 2001; Schlosser & Wagner, 2004). Genetic networks with a modular architecture permit that a mutation affecting one module will have a small-scale effect in other GRNs, permitting selective pressures to act on each individually. As ontogeny is characterized by a succession of GRNs in time and space, the aforementioned dissociative property implies that a change in the stereotypical order of events is not only easier in mutational terms, because it is facilitated by the reduced number of genes controlling module succession (inter-modular edges and respective nodes), but also carries with it less probabilities of whole-system breakdown. As such, for their particular ability to generate diversity, module connections are prime-targets for adaptive evolution (Davidson, 2001).

2008), in accordance with the crucial role of arbuscular mycorrhizal fungi in phosphate acquisition and delivery.

Fungal hyphae in turn produce unidentified "Myc-factors", which lead to the induction of cytoplasmic calcium oscillations in host root epidermal cells (Kosuta *et al.*, 2008) as well as to the transcriptional induction of symbiosis-related genes in the host root (Kosuta *et al.*, 2003). On a recent work that dealt with root exudate-induced gene expression in Glomeromycota, Tamasloukht and colleagues observed the up-regulation of 40 genes in *Gigaspora rosea* (Tamasloukht *et al.*, 2003). Twenty of these genes were found to have known functional homologues in other organisms, and were categorized in four classes: mitochondrial enzymes, DNA-synthesis, signal transduction and transcriptional regulation. Significantly, the later class includes the majority of the genes described. This is consistent with the gene expression profile shifts (activation of downstream gene regulatory networks - GRNs) that are expected with the progression of ontogenetic modules. The detected gene regulatory apparatus is expected to be involved in this transition from asymbiotic "flickering" to pre-symbiotic hyphal branching; as such, these gene products connect ontogenetic modules, thus constituting primary targets for adaptive evolution (Davidson, 2001). The description of this proposed GRN component constitutes a primary research target for the understanding of adaptive evolution in fungal development.

Indeed, information regarding this partner recognition module is scarce. One approach to filling this gap would consist of ascribing functional roles to the 18 yet-unknown described strigolactone responsive genes(Tamasloukht *et al.*, 2003). This description should further clarify the architecture of the putative ancestral module and/or reveal which specific additions of new genetic elements to the network contributed to its novel symbiosis-establishment role. Strigolactone recognition across Glomeromycota should be also assessed for further evolutionary considerations, and also in plant-pathogenic fungi. If a conserved genetic apparatus is confirmed, this module would become a focal point in the quest for basal symbiotic mechanisms.

2.5 APPRESSORIA FORMATION

After plant recognition and subsequent hyphal branching of the AMF, physical contact is established between the two symbionts (Figure 1d). Evidence shows that at this stage, AM fungi recognize plant thigmotropic signals (Giovannetti, 1993) and undergo an abrupt change in their ontogeny, developing a differentiated hyphal structure called appressorium (Harrison,

2005). Furthermore, this developmental module is shared with a number of known parasitic fungi (Bechinger *et al.*, 1999; Parniske, 2000), which is of relevance, as it may suggest the appearance of this feature before AM symbiosis. On the other hand, appressoria formation may have evolved more than once, representing convergent adaptations to the need of crossing plant outer tissues in different fungal lineages. Further genetic data is necessary to determine the evolutionary process resulting in this similarity.

Recent gene expression studies concerning appressoria formation in *Glomus mosseae* show an increased expression of a H^+-ATPase gene (Requena *et al.*, 2003) and several homologs of calcium-signaling related genes (Breuninger & Requena, 2004), indicating a significant role of calcium signaling in fungal response to plant stimuli. Interestingly, 63% of the clones identified in the latter expression study have no known homologues, "and may represent putative novel genes specific for mycorrhizal symbiosis" (reviewed in Balestrini & Lanfranco, 2006)). In an analogous manner to the 18 genes described by Tamasloukht and colleagues (2003) for the pre-symbiotic phase, this result may reflect the current lack of knowledge about AMF genetics and genomes.

2.6 PLANTS DETERMINE THE SHAPE OF HYPHAE TO COME

The development of appressoria, the ontogenic module preceding fungal entry into the root, elicits dramatic rearrangements of epidermal organization(Genre & Bonfante, 2007). In fact, recent ground-breaking studies (Genre *et al.*, 2005; Genre *et al.*, 2008) have shown that *Medicago truncatula* and *Daucus carota* host cells set up a novel subcellular milieu, guiding AMF hyphae towards the cortex. Following adhesion of the microorganism to the host, a repositioning of the nucleus has been observed, which first moves towards the future fungal entry point (Genre *et al.*, 2005; Parniske, 2008). The cytoskeleton is reorganized and, together with intense organelle movement across the cell, the formation of a transient tubular structure enriched in endoplasmic reticulum cisternae takes place (Genre & Bonfante, 2007). The resulting trans-cellular path, the prepenetration apparatus (PPA) (Genre *et al.*, 2005), constitutes an apoplastic column where the fungus is hosted after penetration (Genre & Bonfante, 2007).

It has been proposed that plants co-opted cellular response mechanisms to penetration of AMF from pathogenic fungi invasion-response strategies, or vice-versa, as a

number of elements appear to be common to both interactions (O'Connell & Panstruga, 2006; Parniske, 2008; Genre *et al.*, 2009). For instance, thigmo-stimulation per se can elicit nuclear repositioning in epidermal cells (Gus-Mayer *et al.*, 1998; Genre *et al.*, 2009), while cytoplasmic aggregation and nuclear movement are triggered after compatible pathogenic or mutualistic fungi interaction (Genre *et al.*, 2009). PPA formation, however, appears to be strictly correlated with AMF adhesion (Genre *et al.*, 2009), revealing a putative specific host developmental module responsible for the intracellular accommodation of mutualistic fungi.

2.7 Establishing a "symbiotic synapse"

After passing through the outer cortex, supporting hyphae transverse inner cortical cells, inside which arbuscules are formed (Harrison, 2005). Similar to the events triggered on epidermal cells prior to fungal invasion, the proximity of hyphae elicit cell organization rearrangements in the inner cortex, including vacuole disruption and the assembly of PPA-like structures (Reinhardt, 2007; Genre *et al.*, 2008), predicting which cells will be colonized. Arbuscules are nutrient exchange structures. They are excluded from the plant cell cytoplasm, as they are enveloped by a host cell-derived membrane (the periarbuscular membrane - PAM), formed by the invagination of the host plasmalemma through delivery of membrane precursors (Harrison, 2005; Parniske, 2008) (Figure 1h). It is at this stage of development that the establishment of a functional "symbiotic synapse" (Figure 2) occurs, being formed by the fungal plasma membrane, the PAM and the space in-between the two (Parniske, 2008). This cortical cell-arbuscule synapse resembles, both in structure and function, the interface formed in legume-rhizobia (Harrison, 2005) complexes and, although only structurally, the pathogenic haustoria-plant interactions (Parniske, 2000). Interestingly, this synapse also shares similarities with the interface formed by the only known non-AM-forming genus of Glomeromycota (*Geosiphon*) and cyanobacteria (Box 2) (Schüssler, 2005). The fact that *Geosiphon* is derived from the AM-forming ancestral to all the Glomeromycota adds further interest to the study of the genetic basis behind the related diversity events, namely the hability to establish mutualistic interactions with cyanobacteria.

BOX 2. *Geosiphon pyriformis*, a unique glomeromycotan

Geosiphon pyriformis is a remarkable living system. Like all other known fungi of the phylum Glomeromycota, its asexual, it forms coenocytic hyphae and obtains at least part of the nutrients from an autotrophic symbiotic partner. What makes this species unique is that, unlike all the other known Glomeromycota, it does not enter into AM symbioses, associating instead with photoautotrophic and diazotrophic cyanobacteria that lie inside its own cells (Schüssler *et al.*, 2001; Schüssler, 2005). *Geosiphon* is able to obtain carbon from *Nostoc punctiforme, a cyanobacteria that can also be found as a free-living organism in freshwater.* After being invaginated, cyanobacteria become part of the symbiosome (the fungal intracellular compartment), along with a surrounding fungal membrane, thus turning into an organelle-like structure within the *Geosiphon*'s cytoplasm. The cyanobacteria grow in size and heterocysts are formed, where nitrogenase is protected from oxygen, allowing for N_2 fixation, which is provided along with carbohydrates to the fungus (Kluge, 1992). But nutrients flow both ways in the "symbiotic synapse": *Geosiphon* provides phosphate to its endosymbionts (Schüssler *et al.*, 2006) (Figure 2). Although the *Geosiphon-Nostoc* (G-N) and AM associations are fundamentally different in many aspects, the similarities between these symbiosis are noteworthy: both involve a glomeromycotan fungus that provides phosphate to its phototrophic symbiont, which in turn delivers carbohydrates to its partner (Schüssler *et al.*, 2006). At the micro-anatomical level, the resemblance is even greater: both symbioses involve a structurally similar symbiotic synapse, where metabolite exchange takes place (Figure 2) (Schüssler, 2002; Schüssler *et al.*, 2006). As for the mapping of ancestrality partnerships onto the glomeromycotan phylogenetic tree (Figure 3), the symbiosis with an autotrophic prokaryote is apparently a derived feature: *Geosiphon pyriformis* is an exception with respect to the choice of symbiotic partner. The most parsimonious explanation is that the ancestor to all Glomeromycota would be an intimate plant mutualist fungus (Schüssler, 2002). Recent molecular data support this hypothesis, in agreement with the whole fungal kingdom phylogenetic tree, thus suggesting that AM endosymbiosis represents a derived feature, acquired solely by the glomeromycotan phyletic line (James *et al.*, 2006).

Homology and modularity in AM/G-N symbioses

In light of the supracited phylogenetic, morphological and functional reasons, the AM and G-N symbiotic synapses appear to be homologous. The recognition of this homology entails the prediction of shared evolutionary mechanisms shaping symbiosis between fungi and plants/cyanobacteria. Describing the shared components, topology and interactions of and between

BOX 2 (cont.) modules can elucidate the characteristics of the most-recent common ancestor of the Glomeromycota, as much as delineate the basic characteristics of mycorrhizal symbiosis in extant species. More specifically, we can generate hypotheses regarding the major genetic steps allowing for the appearance of intracellular plant-fungal symbiosis. The genetics of symbiosis synapse establishment and maintenance are, as with the rest of AMF genetics, relatively obscure. The pervasive problem for evolutionary considerations on this phylum is that there is no study comparing gene expression patterns in the two types of glomeromycotan mycobionts. Gene-regulatory protein dynamics throughout fungal development should therefore constitute a major topic of future research.

2.8 SIGNALING IN ROOT ENDOSYMBIOSES: COMMON GENES, DIFFERENT OUTPUTS

AM symbiosis is widespread among land plants and examples are found in representatives of all major lineages. Nevertheless, these associations are promiscuous in that no specific pairwise association between plant and AM fungi lineages has been described so far (Figure 3). More recently in evolution, a group of plants restricted to four orders of the Eurosid clade I of dicots, comprising legumes (*Fabales*) and a few non-legumes, engaged in symbiotic relationships with dinitrogen reducing bacteria(Soltis *et al.*, 1995) (Figure 3). These species can consequently bypass the common nitrogen availability limitation in natural habitats, hosting bacteria inside specialized root organs, termed nodules, within which bacteria are provided with a carefully regulated oxygen and carbon supply, allowing for efficient nitrogen reduction to ammonia(Oldroyd & Downie, 2008). Legume roots interrelate with Gram-negative *Rhizobium* bacteria, whereas non-legume plants interact with the Gram-positive filamentous *Frankia* actinobacteria (Pawlowski & Bisseling, 1996) (Figure 3).

2.8.1 Nodulation at a glance

Rhizobia produce lipochito-oligosaccharides in response to plant-derived flavonoids signals which are recognized by NodD proteins. This recognition activates subsequent transcriptional reprogramming leading to activation of nodulation (*nod*) genes. Some of the resulting products of *nod* genes expression are called Nod Factors (NF) and represent the signal that trigger the inception of the nodulation process (Riely *et al.*, 2004; Oldroyd & Downie, 2008). NF act as morphogens, causing resumption of cell division in terminally

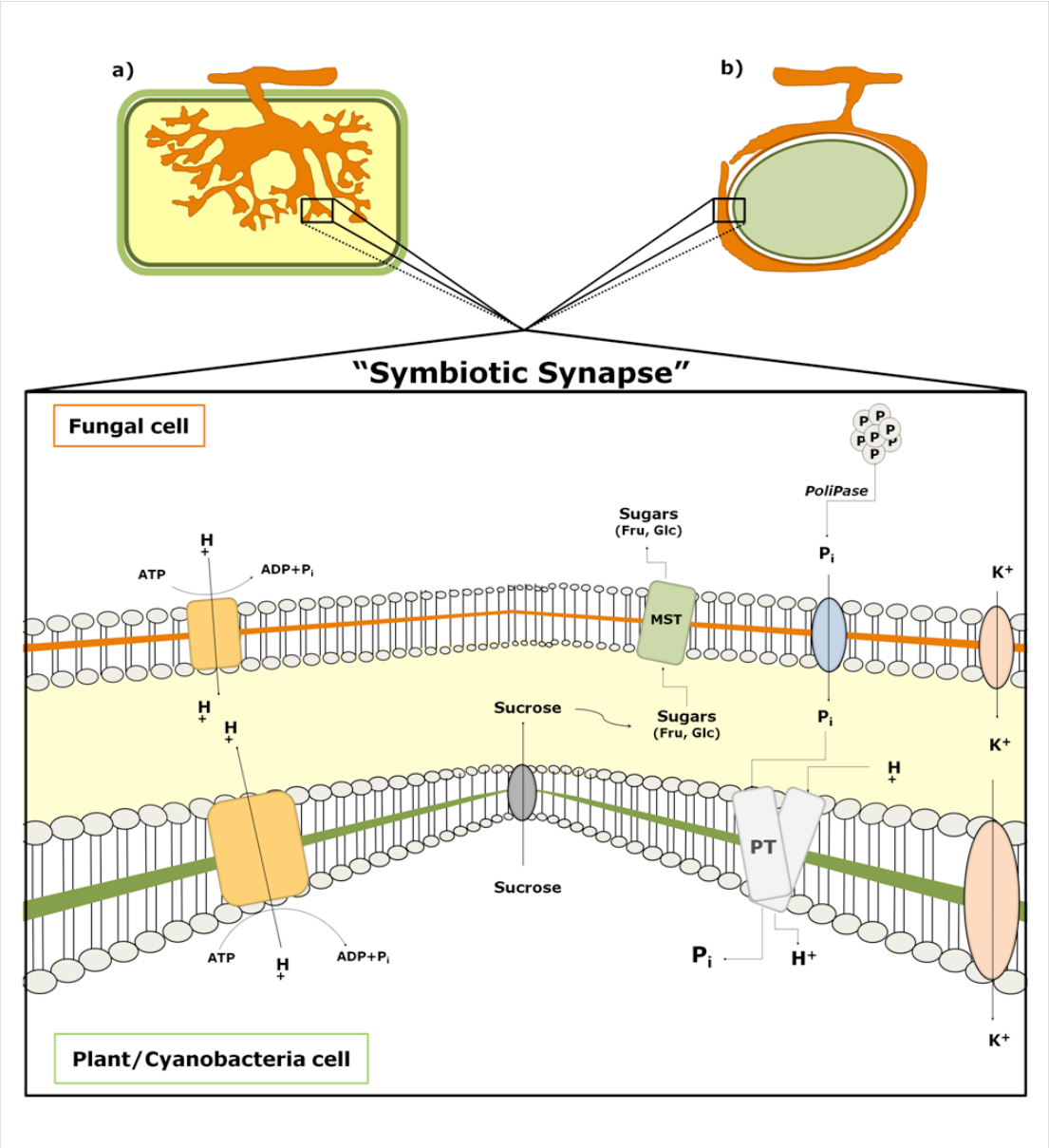

Figure 2| Schematic view of the "symbiotic synapse" in (a) AM and (b) *Geosiphon pyriformis*/ *Nostoc punctiforme* symbioses. Along the symbiotic synapse nutrients are bilaterally exchanged, most notably plant/cyanobacteria-derived photoassimilates and phosphate scavenged by fungi. Both fungal and plant plasma membrane H-ATPases extrude protons, creating an electrochemical gradient across the membranes, fueling the secondary active transport of inorganic phosphate (Pi), Sucrose, Glucose (Glc), Fructose (Fru) and further nutrients via membrane transporters (Schüssler *et al.*, 2006). Photosynthesized sucrose is hydrolyzed (probably by invertases (Schaarschmidt *et al.*, 2006)) and sugars are transported to fungal cells through monosaccharide transporters (MST). Also, nitrogen flows are opposite in these symbioses: they are directed from fungus to photobiont in AM (Govindarajulu *et al.*, 2005), while from photobiont to fungus in Geosiphon-Nostoc association. PT – phosphate transporter, PoliPase – poliphosphatase.

differentiated root cells (Yang *et al.*, 1994) and resulting in enlargement of root tissue into a nodule.

NF are thought to be perceived by the plant host probably by receptor-like kinases with *N*-acetylglucosamine–binding lysin motifs. In fact, two LysM domain receptor-like kinases, NFR1 and NFR*5* from *L. japonicus*, have been implicated in Nod factor recognition (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). The infection process starts when bacteria attach to root-hair

cells and induce their deformation, eventually getting entrapped within a curl. Concomitantly, mitosis is triggered in cortical cells below the epidermal infection foci, giving origin to a nodule meristem. Through a process mediated by actin cytoskeleton rearrangements(Yokota *et al.*, 2009), cylinder-like structures formed by invagination of the host plasma membrane and cell wall deposition, termed infection threads, elongate from the middle of the curl and transverse cells into the inner cortex (Gage *et al.*, 1996; Oldroyd & Downie, 2008) (Figure 1i). The mechanism through which plant cells establish infection threads is thought to have evolved from the pre-penetration apparatus (PPA) ontogenetical programme (Parniske, 2008), an interesting hypothesis still to be evaluated.

Once Rhizobia get inside specialized cells in the infection zone of the young nodule, close to the nodule meristem, they enlarge and differentiate into nitrogen fixing forms, called bacteroids (Gualtieri & Bisseling, 2000). These differentiated forms of bacteria residing inside the nodule, surrounded by a peribacteroid membrane are best known as symbiosomes (Oldroyd & Downie, 2004; Jones *et al.*, 2007).

2.9 Linking AM to Nodulation

The restriction of root nodulation endosymbioses (RNE) to a monophyletic group of four angiosperm orders, suggests that this phenomenon may constitute an evolutionary novelty at the base of this clade (Gherbi *et al.*, 2008; Markmann *et al.*, 2008). Phenotypic observations of legume mutants, more specifically the model plants *Medicago truncatula* and *Lotus japonicus* (Harrison, 2005), shed light into a fundamental link between RNE and AM symbioses. Mutant plants impaired in RNE symbiosis accomplishment are also unable to associate with Glomeromycota. This suggests a bond between plant-phosphate scavenging fungi and plant-nitrogen fixing bacteria symbioses genetic programmes (Kistner *et al.*, 2005). The link of plant–fungal and plant–bacterial endosymbioses in legumes, involving at least seven genes (Kistner *et al.*, 2005), best known as "common symbiosis genes" (Kistner & Parniske, 2002), inspired the idea that during the evolution of bacterial endosymbiosis, some genes were co-opted from the pre-existing AM genetic program (Parniske, 2004). This group of genes includes a leucine-rich-repeat receptor kinase (Endre *et al.*, 2002; Stracke *et al.*, 2002)

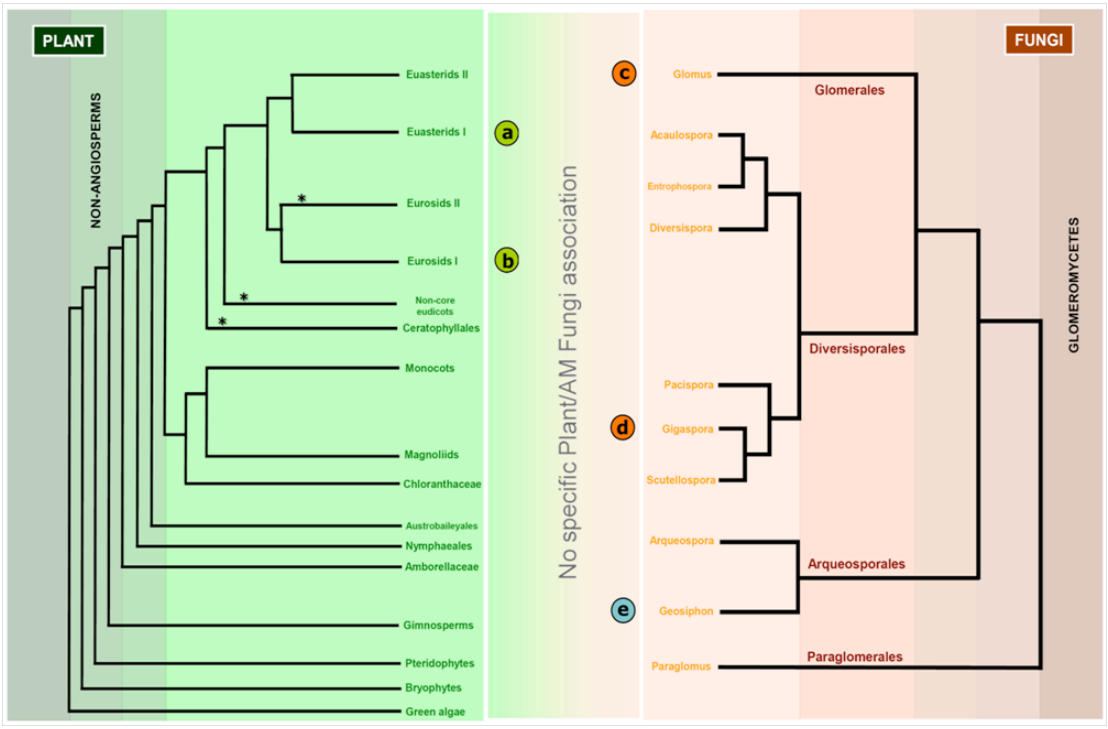

Figure 3| Inter Kingdoms symbiotic species relationships. Mycorrhizal fungi belonging to the phylum Glomeromycota (+- 200 species) can establish endosymbiotic relationships with the vast majority of plants roots (around 80%). As depicted in the cladograms, there is no exclusiveness of relations between groups of fungi and plants, as it is possible to find the same fungal species associated with a large number of plant species. (*) In some phylogenetic groups of plants one can find disperse families with no described link with AM fungi (e.g. *Brassicaceae*, *Caryophyllaceae*, *Amaranthaceae* or *Polygonaceae*). These groups present a great surplus value for the comparative study of symbiotic characteristics. Additionally, numerous bacterial groups are involved in this complex interactive system, like plant-interacting nodulating bacteria (green circles), bacteria associated with Glomeromycota (orange circles) and autotrophic bacteria with *Geosiphon* (blue circles): (a) *Frankia* and Rhizobia are the most commonly found in association with families included in the Eurosids I clade; (b) *Burkholderia* bacteria are found connected with *Rubiaceae* (Euasterids I), a quite well described symbiosis. Bacterial interactions with AM fungi where found to occur, namely within the (c) *Glomus*, (d) *Gigaspora* (e) and *Geosiphon* genera, the latter being established by the only known non-AM-forming species of the Glomeromycota and a cyanobacteria (*Geosiphon piriformis/ Nostoc punctiforme*). The cladogram representing plants was prepared according to the APGII system (2003) and Glomeromycota phylogeny was consulted in Schwarzott *et al.*, 2001). Nodulating bacteria and fungi-interacting bacteria species where consulted in Gualtieri & Bisseling, 2000 and Frey-Klett *et al.*, 2007, respectively.

(*Lotus japonicus SyMRK/ M. truncatula DMI2/ M. sativa NORK*), two channels permeable to cations (Charpentier *et al.*, 2008), (*LjCASTOR* and *LjPOLLUX/ MtDMI1*), two putative nucleoporines (LjNUP85 and LjNUP133) (Kanamori *et al.*, 2006; Saito *et al.*, 2007), a calcium and calmodulin-dependent protein kinase (Levy *et al.*, 2004; Mitra *et al.*, 2004; Gleason *et al.*, 2006) (*LjCCaMK/ MtDMI3*) and *LjCYCLOPS*, encoding a protein that physically interacts in vitro and colocalizes within the nucleus with CcaMK (Yano *et al.*, 2008).

More particularly, the *M. truncatula* DMI (DOESN´T MAKE INFECTIONS) proteins play pivotal roles in both early nodulation and mycorrhization signaling episodes and will serve as proof-of-concept for our approach.

2.10 How bacteria got *en route* to the root

Plants express and make use of leucine-rich-repeat receptor-like kinases (LRR-RLKs) for a variety of signal-transduction pathways. These receptors are structurally related to Toll receptors identified in *Drosophila melanogaster* and Toll-like receptors present in mammal cells, a family of important players in immunity (Leulier & Lemaitre, 2008). Leucine-rich repeats are usually involved in protein-protein interactions, which might be implicated in binding of signal molecules, whereas the intracellular kinase domain acts in protein phosphorylation. The *M. truncatula* DMI2/SYMRK (symbiosis receptor-kinase) can be included in this class of proteins. SYMRK takes an active part in the signaling pathway leading to the accommodation of both rhizobia (Endre *et al.*, 2002; Stracke *et al.*, 2002) and, as recently shown, of *Frankia* bacteria (Gherbi *et al.*, 2008; Markmann *et al.*, 2008). It acts near a point of convergence of AM and legume-rhizobia signaling pathways and exists in at least three different structural versions within angiosperms, particularly regarding its extracellular portion and exon number (Markmann *et al.*, 2008). This polymorphic extracellular domain is thought to recognize exogenous molecules related to symbiosis. Recently published data regarding the evolution of intracellular root symbioses with bacteria (Gherbi *et al.*, 2008; Markmann *et al.*, 2008), reflects the particular role of SYMRK in the evolution of nodulation in a modern group of legumes and close relatives, which present the version of the molecule with higher length (for details see Figure 1 of Markmann *et al.*, 2008)). De Mita and colleagues pointed out that adaptive changes reflecting positive selective constraints can be traced in LRR and extracellular regions of SYMRK genes from different *Medicago* species, but these do not correlate with shifts in rhizobial specificity (De Mita *et al.*, 2007). The functional adaptation of this specific receptor-kinase, correlating with a novel architecture acquisition, was possibly due to an increase in the number of exons in ancestral legumes (Markmann *et al.*, 2008).

The critical events that arose from the appearance of a novel type of receptor in the nodulating clade are yet unanswered, specifically those regarding the rearrangement of genetic

networks and developmental pattern shifts that allowed for the intracellular lodging of dinitrogen reducing bacteria. We propose that the GRN containing SYMRK/DMI2 constitutes a ChIN, an ancient network that lies at the origin of symbiosis formation. This ChIN feeds onto alternative modules for nodulation or mycorrhization leading to different character states present in extant species that establish these types of endosymbioses. Furthermore, a comparative approach should be directed at the *SYMRK* and downstream genes of non-nodulating species proximately related to legumes, shedding light over the genetic architecture of the proposed character states.

As is now evident, the establishment of symbiotic interactions involves specific developmental adaptations in both symbiotic partners and intricate coordination of their developmental programs. As gene networks provide a set of control systems for the course of development, they can provide not only an insight into the inner-workings of the ontogenic program but also establish the topology of genetic relationships shaped by selection (Davidson, 2001). This GRN topology can prove invaluable in the hierarchization of the successive steps that evolution has taken in the construction of their resulting biological features.

The similarities between Glomeromycota- and rhizobia- elicited symbiotic genetic programmes in legumes have allowed for the proposition of a ChIN, revealing the adequacy of the proposed comparative approach in the dissection of the genetic mechanisms underlying mycorrhizal endosymbioses. But bearing in mind the specificities of each symbiotic system, how can a basal common signaling structure transduce different incoming signals (e.g. NF and still unknown mycorrhization factors) into different developmental symbiosis modules?

2.11 Choosing between states: Ca^{2+} sets the frontiers

In fact, some advances have been recently made on the mechanisms underlying the choices between nodulation and mycorrhization through the study of the downstream events triggered by DIM2/SYMRK activation. At the top of the nodulation´s signal perception hierarchy are Nod-factor receptors, LysM type receptor kinases implicated in the binding of N-acetyl-glucosamine-containing molecules (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). Upon

Nod-factor perception, a transient increase in root hair´s cytosolic Ca^{2+} concentration is observed (Cardenas *et al.*, 1999; Felle *et al.*, 2000), followed by repetitive nuclear-associated oscillations (Ca^{2+} spiking) (Ehrhardt *et al.*, 1996; Oldroyd & Downie, 2006). The aforementioned *M. truncatula* DMI1 and DMI2/SYMRK are both required for calcium spiking (Endre *et al.*, 2002; Ane *et al.*, 2004). Downstream of calcium oscillatory phenomena, the CCaMK DMI3 putatively acts as a Ca^{2+} signal decoder, subsequently activating both AM and nodulation symbiosis-specific gene transcription (Levy *et al.*, 2004; Oldroyd & Downie, 2006).

The system through which CCaMK integrates ion concentration oscillations (at a frequency, amplitude or spike number level) remains to be clarified, though it has been shown that, specifically for nodulation symbiosis, DMI3-activated gene expression is regulated by Ca^{2+} spike number (Miwa *et al.*, 2006). The chimeric CCaMK has EF-hand Ca^{2+} binding sites and an autoinhibitory domain (Harper & Harmon, 2005). If the autoinhibitory sequence is absent from the DMI3 structure, nodule morphogenesis can be triggered in the cortex of mutant plants devoid of bacterial elicitation (Gleason *et al.*, 2006). This indicates that downstream of the calcium dynamical trajectory, the activation of this protein is sufficient to trigger the nodule ontogenetical programme. In a very recent paper, Kosuta and co-workers provide evidence for the occurrence of calcium oscillatory rhythms also involved in AM symbiosis, with a different signature from nodulation-associated spiking (Kosuta *et al.*, 2008). Also, by means of extensive mathematical analyses, these authors have shown that both [Ca^{2+}] trajectories are chaotic in nature. An intrinsic property of the common symbiosis pathway (ChIN) must be the plasticity to integrate signals from bacteria and AM fungi into precise outcomes (non-linear Ca^{2+} signatures) that can lead to differential DMI3 perception and consequent activation of symbiosis-type specific genetic networks (character states). The specific dynamic patterns of Ca^{2+} and other ions have long been related to nodulation (Cardenas *et al.*, 2000) and systematically observed in endo and ectomycorrhizal associations (Ramos, *et al.*, 2008a; Ramos, *et al.*, 2008b; Ramos *et al.*, 2009), thus allowing for the hypothesis that specific spatial and temporal coreographies of free ion concentration may play a role in the integration of these signals.

2.12 CONCLUDING REMARKS

The study of organismal ontogeny is becoming a major tool to understand evolutionary events (Gould, 1977; Raff, 1996; Carroll *et al.*, 2005; Muller, 2007). This research program stems from the realisation that among the rules of transformation maping genotype to phenotype in multicellular organisms, development stands as the generator of form, and thus a central target for adaptive evolution (de Beer, 1958; Lewontin, 1974).

Arbuscular mycorrhizae stand as a particularly successful and important symbiotic relationship, considering its ubiquity and putative importance in the colonization of land by plants. The knowledge of the fungal partner's ontogeny is still in its infancy, despite novel molecular genetics approaches (e.g. AM fugal genome sequencing projects, comparative transcriptomics). Such advances are expected to contribute to the disclosure of the poorly understood events leading to this intracellular tête-à-tête, providing a fertile, yet eminently descriptive dataset that calls for an analytical approach. For example, the release of the genome sequences of *Glomus intraradices* in the near-future and the possibility of a sequenced dikaryan outgroup can contribute significantly to generate handles into the description and comparison of the transcription profiles throughout fungal ontogeny. In addition, the gene expression in AMF and its dynamics throughout development are becoming clearer. For instance, there is a growing body of knowledge on the mechanisms through which diffusible molecules are perceived and decoded(Akiyama *et al.*, 2005; Besserer *et al.*, 2006), triggering gene expression reprogramming and ontogenetical shifts which are vital for life-cycle completion.

In fact, this specific developmental step illustrates how the modular nature of ontogeny can be helpful in guiding research on this symbiotic system. More specifically, strigolactone has been shown to act acts as the trigger for a central developmental transition in the presymbiotic phase. This particular transition between two developmental modules (groups of events with inner-cohesion in space and time) is, as other edges between modules, a major target for adaptive change, for its potential to generate diversity with seemingly low pleiotropic effects. As such, the extent of strigolactone-based communication across the *Glomeromycota* has to be quickly accessed. The comparative study of another developmental module, the intracellular "symbiotic synapse" (arbuscule/plant cell, siphonal bladder/cyanobacteria and

simbiosome/ plant cell interface in nodulation) can also be crucial for hypothesis-generation regarding the adaptive steps (putatively gene network rearrangements) that have allowed for symbiosis appearance and, in this case, diversification. As this intracellular contact between symbionts is also shared with plant-pathogenic fungi (Parniske, 2000), the knowledge of the degree and detail of homology between these groups of fungi can generate hypotheses regarding the stepwise genetic changes leading to the emergence of this mutualistic evolutionary novelty.

With respect to the AM plant partner, the aforementioned research program is already proving to be invaluable. A comparative approach has shown that some plant mutants were impaired in both RNE and AM formation, suggesting a common GRN referred to as "common symbiosis genes". Recent studies detailing the plant gene expression involved in root nodulation endosymbioses show that it parallels that of AM to a considerable extent (Kistner *et al.*, 2005). The phylogenetic mapping of this character points to the AM symbiosis as an ancestral condition, and as such, the derived character of nodulation is predicted to have arisen by a co-option event of some of the ancestral GRNs. This shared genetic network upstream of two dissociable states (RNE/AM) points to both homologous and modular properties of the underlying GRNs. We argue that these should be, as in the approach to AMF ontogeny, guiding standpoints to understand the plant genetic adaptations that enabled the establishment of AM mutualism.

The case-study of the *DMI* genes, identified in *Medicago truncatula*, serve as a proof-of-concept for our systematization. In this case, a RNE/AM-shared signal-integration cascade was shown to lay upstream of specialization GRNs. The homology and modularity concepts can thus enable the further exploration of the bordering nodes of this shared character-identity network, as for clarifying the genetic architecture behind each character state.

Although some transcription profiles are becoming available for different life-stages (Breuninger & Requena, 2004), the link between signal integration and shifts in gene expression is poorly understood. We propose an emphasis on the description of gene-expression regulation, particularly the mapping of transcription factor/effector gene *cis*-regulatory interactions. This emphasis relies on the fact that the progression of ontogenetic modules in both plant and fungal partners must rely on shifts in gene expression. These are, as stated earlier, particularly evolvable regions of genetic networks and thus prime candidates for the evolutionary bases of plant-fungal mutualism.

We propose that the comparative method and the concept of homology can provide the first level of systematization by mapping onto the phylogenies of plants and fungi the characters that make up the establishment of mycorrhizal endosymbioses. Furthermore, we consider that looking at comparative gene expression data through the eye of modularity and gene network concepts can provide the foundations for a research program aimed at advancing our understanding of the evolution and development of symbiotic systems.

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3. CONCLUSIONS

An integrated understanding of symbiotic events underlies the establishment of experimental paradigms that allow for the dissection of its proximate and evolutionary causes. Looking at these biological interactions from the point of view of not only co-evolution, but also of co-development, might help to properly formulate hypothesis and further address them in a systematic and coherent manner.

This work might contribute to the community in the sense that it provides 1) a physiological study based on the modulation of ion dynamics in ectomycorrhizal roots, where the first description of the molecular bases of cell-cell interactions are only now starting to appear, despite its major ecological relevance; 2) A collection of hopefully fresh ideas that might help the field addressing questions and formulating hypotheses regarding the evolution and development of plant endosymbioses.

Future studies based on hypothesis-driven approaches, supported on the ever-growing accumulation of data and knowledge, might reveal the fundamental steps that permitted the living together of the constantly evolving endless forms, whose beauty will always intrigue us in such a powerful way.

Do not feel absolutely certain of anything

Bertrand Russel, 1951