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Pectin localization in the Mediterranean orchid *Limodorum abortivum* reveals modulation of the plant interface in response to different mycorrhizal fungi

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Abstract

In most mycorrhizal symbioses, phylogenetically distinct fungi colonize simultaneously the roots of individual host plants. A matter of debate is whether plants can distinguish among these fungal partners and differentiate their cellular responses. We have addressed this question in the orchid mycorrhizal symbiosis, where individual roots of the Mediterranean species *Limodorum abortivum* can be colonized by a dominant unculturable fungal symbiont belonging to the genus *Russula* and by more sporadic mycelia in the genus*Ceratobasidium* (form-genus *Rhizoctonia)*. The phylogenetic position of the *Ceratobasidium* symbionts was further investigated in this work. Both *Russula* and *Ceratobasidium* symbionts form intracellular coils in the cortical roots of *L. abortivum*, but hyphae are very different in size and morphology, making the two fungi easily distinguishable. We have used John Innes Monoclonal 5, a widely used monoclonal antibody against pectin, to investigate the composition of the symbiotic plant interface around the intracellular coils formed by the two fungal partners. Immunolabelling experiments showed that pectin is exclusively found in the interface formed around the *Ceratobasidium*, and not around the *Russula* symbiont. These data indicate that the plant responses towards distinct mycorrhizal fungal partners can vary at a cellular level.

Keywords

Orchid mycorrhiza *Ceratobasidium* Pectin Interface *Limodorum abortivum Russula*

Introduction

Plants interact in nature with a variety of mutualistic and pathogenic microorganisms, and individual plants can be colonized at the same time by multiple microbes. In endosymbiotic interactions, the microbial symbiont is accommodated inside the plant cell, separated from the plant cytoplasm by a specialized symbiotic compartment, the interface (Bonfante and Perotto *1995*). The formation, structure and composition of this interface has received much attention because all signalling molecules and nutrient exchanges (Harrison *2005*; Day et al. *2001*; Oldroyd et al. *2009*) must go across it during the intracellular stages. The organization and composition of the interface compartment have been compared in plants colonized by organisms as diverse as rhizobia and mycorrhizal fungi (Perotto et al. *1994*) or mycorrhizal and pathogenic fungi (Bonfante and Perotto *1995*). Similarities as well as notable differences have been found, which have been ascribed to the specific features of the symbiotic relationship.

In addition, individual plants can be colonized simultaneously by distinct microbial partners that demonstrate the same symbiotic phenotype. For example, multiple occupancy of plant roots by genetically distinct fungi has been established for most endomycorrhizal symbioses: arbuscular (e.g. Alguacil et al. *2009*; Jansa et al.*2008*; Opik et al. *2009*), ericoid (e.g. Bougoure and Cairney *2005*; Perotto et al. *1996*; Sharples et al. *2000*) and orchid (e.g. Bidartondo and Read *2008*; Huynh et al. *2009*; Roy et al. *2009*; Suarez et al. *2006*) mycorrhiza. The association of plants with a multitude of microbial partners has been interpreted as a way to widen the range of symbiotic functions, as the effectiveness of the symbiosis (that is the amount of benefit that the plant host derives from the microbial symbiont) varies among symbiont

species and genotypes in natural populations (Jansa et al. *2007*; van der Heijden et al. *1998*). In addition, multiple occupancy of plant roots by mycorrhizal fungi may also depend on the plant's inability to recognize and exclude certain fungi.

Although host plants can simultaneously interact with several mycorrhizal fungi, it is unclear whether they respond to each of them on a cell-specific basis. We have addressed this question by studying the intracellular symbiotic interface formed in orchid roots around hyphae of distinct mycorrhizal partners.

Mycorrhizal fungal diversity has been investigated in several orchid species (reviewed in Taylor et al. *2002*; Rasmussen *2002*; Dearnaley *2007*). *Limodorum abortivum* is a common orchid species in the Mediterranean region. Although capable of photosynthesis, in the adult stage *L. abortivum* is partially mycoheterotrophic, i.e. it is nutritionally dependent upon the mycorrhizal fungal symbionts for organic carbon (Leake *1994*). As shown for other fully and partially mycoheterotrophic orchids, this trophic strategy is mirrored in *L. abortivum*by a specific signature in the natural abundance of stable isotopes (Gebauer and Meyer *2003*) and by high mycorrhizal specificity (Girlanda et al. *2006*). We have previously identified basidiomycetes in the species complex *Russula delica–Russula chloroides* as being the largely dominant fungal partners of *L. abortivum*(Girlanda et al. *2006*). More sporadically, roots of *L. abortivum* can also harbour culturable fungi assigned by morphological criteria to the form-genus *Rhizoctonia* (Girlanda et al. *2006*).

The aims of the present work were to better define the phylogenetic position of the *Rhizoctonia* symbiont(s) of *L. abortivum* and to investigate the composition of the plant interface formed around the two different mycobionts by using immunolabelling with the anti-pectin John Innes Monoclonal (JIM) monoclonal antibody JIM 5 (Knox et al. *1990*). This antibody binds specifically to non-esterified pectin, and it has been used to characterize the plant–microbe interface compartment (Bonfante and Perotto *1995*; Balestrini et al. *1996*).

Materials and methods

Sampling

Plants were collected with a soil pad at Monte San Giorgio, near Turin (Piemonte, Italy). A previous survey (Girlanda et al. *2006*) had identified in this population of *L. abortivum* the consistent co-occurrence of mycobionts identified in the *R. delica/R. chloroides* species complex and culturable *Rhizoctonia*. Five plants were sampled, carried to the laboratory and processed within 36 h. Roots were rinsed extensively with tap water and brushed gently to remove remaining soil debris. Root colonization was evaluated on hand sections by light microscopy.

Fungal isolation

One or two roots from each plant specimen were surface-sterilized with 30% H_2O_2 (Carlo Erba) for 1 min and rinsed three times with sterile water. At least 15 sections per plant, obtained with a sterile blade, were plated onto malt extract agar and modified Melin–Norkrans agar media, both with or without chloramphenicol (50 mg/kg). Petri dishes (each containing four to five root sections) were kept at room temperature for up to 3 months in order to allow the development of slow-growing mycelia.

Electron microscopy and immunogold labelling with JIM 5 monoclonal antibody

For transmission electron microscopy (TEM) observation, segments from mycorrhizal *L. abortivum* roots were fixed in 2.5% (*v*/*v*) glutaraldehyde in 10 mM Na-phosphate buffer (pH 7.2) overnight at 4°C. After

washing in the same buffer, the samples were post-fixed in 1% (*w*/*v*) osmium tetroxide in water for 1 h, washed three times with water and dehydrated in an ethanol series [30%, 50%, 70%, 90% and 100% (*v*/*v*); 15 min each step] at room temperature. The samples were infiltrated in 2:1 (*v*/*v*) ethanol/LR White (Polysciences, Warrington, PA, USA) for 1 h, 1:2 (*v*/*v*) ethanol/LR White resin for 2 h, 100% LR White overnight at 4°C and embedded in LR White resin, according to Balestrini et al. (*1996*). Semi-thin sections (1 µm) were stained with an aqueous 1% (*w*/*v*) solution of toluidine blue for morphological observations.

Thin sections were post-stained with periodic acid thiocarbohydrazide-silver proteinate test (PATAg test) for visualization of polysaccharides or treated according to the immunolocalization protocol. Immunogold labelling with the monoclonal antibody JIM 5 (dilution 1:5–1:10) was performed on thin sections as described by Balestrini et al. (*1996*) and observed with a Philips CM10 transmission electron microscope. Labelling specificity was determined by replacing the primary antibody with buffer.

Sequence analysis

DNA was extracted from the roots and fungal isolates using Qiagen plant mini kit, amplified with fungal specific primers (ITS1F/ITS4) and cloned as indicated in Girlanda et al. (*2006*). Sequences of the fungal ribosomal DNA (rDNA) region were obtained from mycelia after isolation in culture as well as after direct root amplification with fungal specific primers (ITS1F/ITS4) and cloning. To better define the taxonomic position of the *Rhizoctonia* mycobiont, the sequences were aligned with reference sequences from Genebank using CLUSTALX 2.0 (Larkin et al. *2007*) with default conditions for gap opening and gap extension penalty. The alignment was then imported into MEGA 4.0 (Tamura et al. *2007*) for manual adjustment. The best-fit models (HKY+G) were estimated by the Akaike Information Criterion using jModelTest 0.1.1 (Posada *2008*). Phylogenetic analysis was performed using the Bayesian Inference (BI) approach. BI of phylogeny using Monte Carlo Markov Chains (MCMC) was carried out with MrBayes 3.1.2 (Huelsenbeck and Ronquist *2001*). Four incrementally heated simultaneous MCMC were run over 10,000,000 generations, under model assumption, using random starting trees and default starting values of the models. Trees were sampled every 1,000 generations resulting in an overall sampling of 10,001 trees. The first 2,500 trees were discarded as “burn-in” (25%). For the remaining trees, a majority rule consensus tree showing all compatible partitions was computed to obtain estimates for Bayesian Posterior Probabilities (BPP). Branch lengths were estimated as mean values over the sampled trees. Such a Bayesian analysis was repeated three times, always using random starting trees and random starting values for model parameters to test the independence of the results from the revisiting of the prior topologies during chain growth (Huelsenbeck et al.*2002*). Only BPP values over 50% are reported in the resulting tree.

Results and discussion

Plants of *L. abortivum* display a rhizome densely covered with thick roots that are easily detached (Fig. 1). Observation of resin-embedded root sections (Fig. 2a) showed that most cortical cells were colonized by fungal pelotons formed by narrow septate hyphae (2.5–5.5 µm diameter) lightly stained by toluidine blue. This dominant fungal symbiont of *L. abortivum* could not be isolated in pure culture and was identified by molecular methods as belonging to the *R. delica/R. chloroides* species complex (Girlanda et al. *2006*).

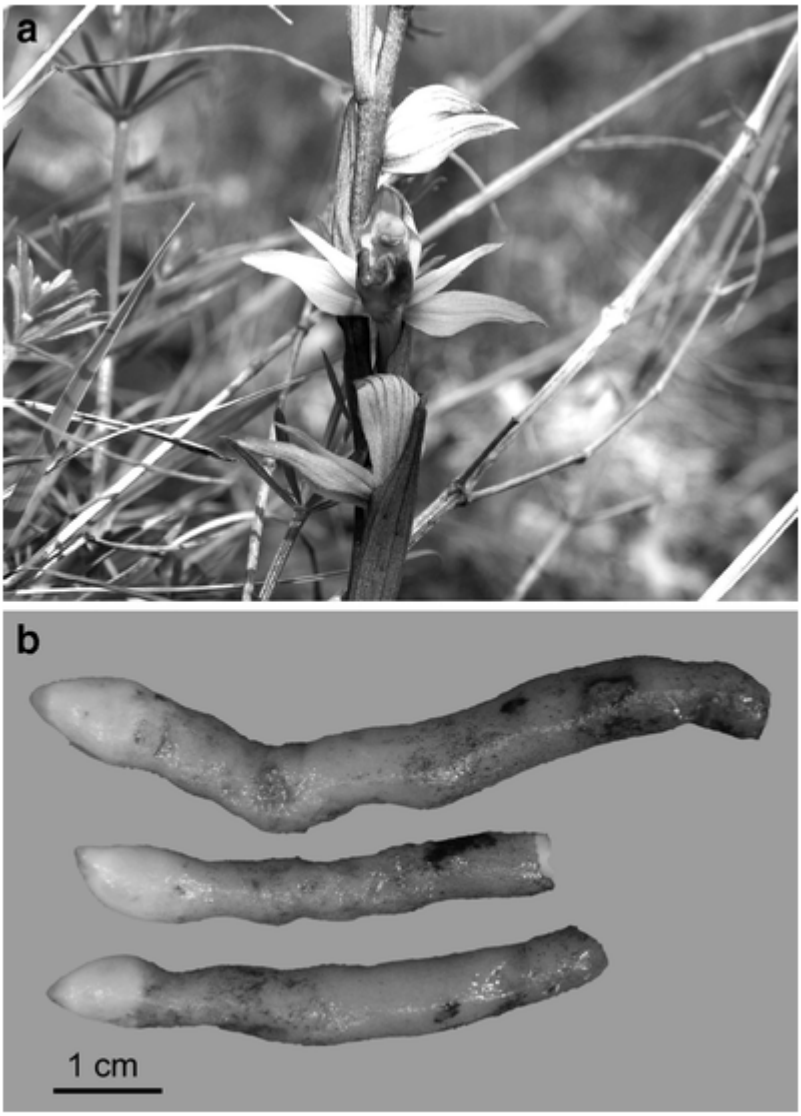

Fig. 1

Some morphological features of *Limodorum abortivum*. **a** Detail of the distinctive flowers. **b** Three large and fleshy roots of *L. abortivum*, showing the uncolonized meristematic apex on the *left*

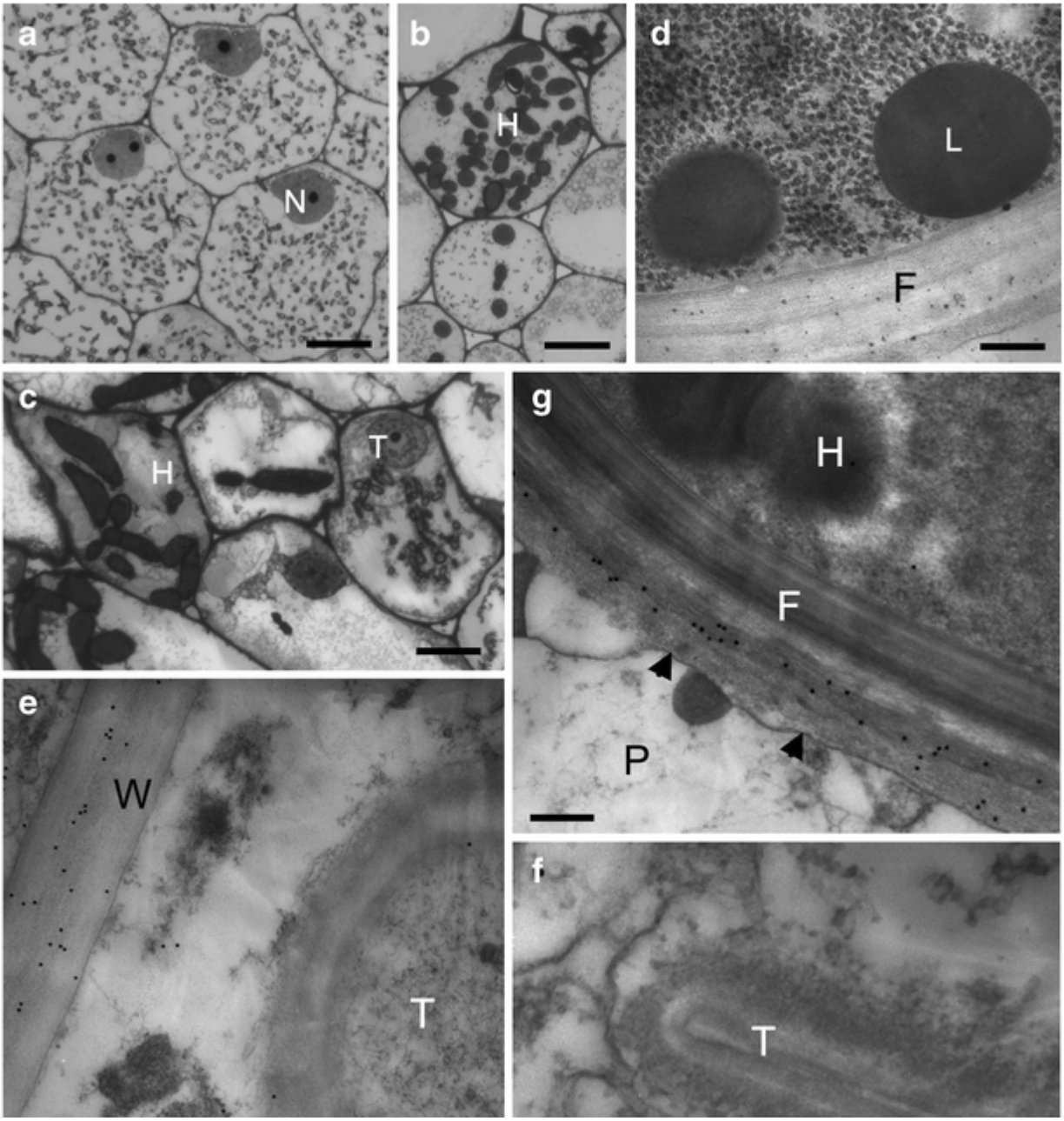

Fig. 2

Micrographs showing fungal colonization of *L. abortivum* roots and immunolabelling with the JIM 5 monoclonal antibody. **a**–**c** Semi-thin sections from a fixed and resin-embedded root, stained with toluidine blue. **a** Intense root colonization by thin fungal hyphae forming pelotons in the root parenchyma cells. *N*plant nuclei. *Bar* is 35 µm. **b** Sparse colonization of the root parenchyma cells by thick fungal hyphae (*H*).*Bar* is 60 µm. **c** Neighbouring root parenchyma cells colonized by fungal hyphae showing very distinct morphology. *T* plant cell colonized by thin hyphae, *H* plant cell colonized by thick hyphae. *Bar* is 30 µm. These morphological features correspond to those described by Girlanda et al. (*2006*). **d**–**g** TEM micrographs showing *L. abortivum* root parenchyma cells colonized by the two fungal types. **d** A detail of a thick hypha after PATAg staining, showing glycogen accumulation and lipid droplets (*L*) in the cytoplasm. *F*fungal wall. *Bar* is 0.25 µm. **e** After JIM 5 immunolabelling of cells colonized by thin hyphae,

gold granules are found on the peripheral plant cell wall (*W*), but not on the interface surrounding the intracellular hypha (T).*Bar* is 0.25 µm. **f** Gold granules are also not found on the interface material around a collapsed thin hypha (*T*) after immunolabelling with the JIM 5 antibody. *Bar* is 0.1 µm. **g** A thick hypha (*H*) surrounded by the fungal cell wall (*F*), by interfacial material and by the invaginated plant plasma membrane (*arrows*). After JIM 5 immunolabelling, gold granules are found on the interfacial material surrounding the hyphal wall. *Bar* is 0.25 µm

Much wider septate hyphae (8–14 µm diameter) were more sporadically observed in the root sections, where they formed sparse pelotons intensively stained by toluidine blue (Fig. 2b). Fungi with hyphal features resembling those observed in fresh and embedded *L. abortivum* root sections were isolated in pure culture and assigned by morphological criteria to the form-genus *Rhizoctonia* (Girlanda et al. *2006*).

Pelotons formed by *Rhizoctonia* and by *Russula* could be observed in neighbouring root cells (Fig. 2c), indicating that the two distinct fungal symbionts can co-occur in the same root region.

To better understand the phylogenetic position of the *Rhizoctonia* symbiont of *L. abortivum*, culturable fungi were isolated from a larger number of mycorrhizal plants and investigated by molecular methods. Isolation in pure culture yielded fungal mycelia with wide hyphae for all five *L. abortivum* plants collected at Monte San Giorgio. They were all assigned by morphological criteria to the form-genus *Rhizoctonia*. rDNA sequences obtained for fungi isolated from the five different plants were nearly identical (99–100% sequence identity), and BLAST searches identified them as belonging to the genus *Ceratobasidium*. The Bayesian tree in Fig. 3shows their position relative to other ceratobasidioid fungi. In this tree, they clustered together with*Ceratobasidium cornigerum*, a known symbiont of orchids (Hadley *1970*) and with fungal sequences obtained from other orchid species (Fig. 3).

Fig. 3 Bayesian phylogram showing the phylogenetic relationships of fungal sequences obtained from *L. abortivum* mycorrhizal roots with other ceratobasidioid fungi. The sequence with accession number DQ061931 was obtained through direct PCR amplification of total DNA from *L. abortivum* roots, whereas the sequence with accession number HM117643 was obtained from culturable fungal isolates. The HKY+G substitution model was used. *Waitea circinata* (FJ755885) was used as an outgroup taxon. Bayesian posterior probabilities above 50% are indicated. *T* terrestrial orchid, *E* epiphytic orchid, *G* green orchid, *A* achlorophyllous orchid

Sequences assigned to the genus *Ceratobasidium* were also obtained from one out of the five individuals of*L. abortivum* after direct amplification of root DNA with specific fungal primers and cloning, the other plant individuals yielding solely *Russula* sequences. One of these *Ceratobasidium* sequences was previously deposited in Genbank (accession no. DQ061931). Surprisingly, the Bayesian tree in Fig. 3 shows that the sequence amplified from root DNA clustered separately from those obtained from the fungal isolates, together with fungi derived from environmental (mainly soil) samples. No other fungi identified in orchids were found in this cluster.

Thus, it is unclear whether both *Ceratobasidium* are involved in the mycorrhizal association with *L. abortivum*, and the results do not allow us to resolve the identification of the peloton-forming *Ceratobasidium*below the genus level. On the other hand, they reflect the difficulties in assigning a symbiotic role for fungi identified in mycorrhizal roots following different techniques (fungal isolation in culture or direct PCR amplification of root DNA). The analysis of individual pelotons may help in clarifying this aspect in *L. abortivum*.

Whatever the species identity of the *Ceratobasidium* symbiont(s), the hyphal size and cellular appearance allowed us to clearly distinguish the intracellular pelotons formed by *Ceratobasidium* from those formed by

the *Russula* symbiont. Apart from the smaller size, pelotons formed by *R. delica/R. chloroides* mainly showed an electron transparent content by TEM. By contrast, peloton hyphae formed by the *Ceratobasidium*symbiont were rich in cytoplasmic organelles and nuclei, and PATAg staining showed an abundant deposition of glycogen inside these hyphae (Fig. 2d). In both peloton types, hyphae were always separated from the root cell cytoplasm by an interfacial matrix and a perifungal membrane.

Pectins, a complex set of polysaccharides, are among the most abundant cell wall components and have been shown to vary in their localization patterns among different tissue types and developmental stages (Ridley et al. *2001*). Pectin has been commonly found in the interfacial matrix of endomycorrhizal associations (Bonfante and Perotto *1995*; Perotto et al. *1995*; Peterson and Massicotte *2004*). In orchids, the only previous study was carried out on the mycorrhizal protocorms of three orchid species colonized by*C. cornigerum* (Peterson et al. *1996*). Immunolocalization experiments showed that the interfacial matrix formed between invading hyphae and the invaginating plant plasmalemma was initially devoid of pectin. This plant wall component could be detected in the matrix of the orchid–fungus interface only when the hyphal coil began to degrade (Peterson et al. *1996*).

We have investigated the presence of pectin homogalacturonans in the interface matrix formed by *L. abortivum* around *Russula* and *Ceratobasidium* hyphal pelotons with the antibody JIM 5 (Knox et al. *1990*), the same used in the previous studies by Peterson et al. (*1996*). The epitope recognized by JIM 5 is typically found in the middle lamella and outer sections of walls near intercellular spaces (Knox et al. *1990*). Immunolabelling of thin sections of mycorrhizal *L. abortivum* roots showed intense deposition of colloidal gold granules on the plant cell wall near intercellular spaces (not shown). Labelling was specific as no gold granules were observed when the primary antibody was omitted.

Pectin was never detected by the JIM 5 antibody on the interface material surrounding *Russula* hyphae (Fig. 2e). In the same root cells, the peripheral wall showed intense gold labelling on the middle lamella (Fig. 2e). Pectin was absent from the interface matrix surrounding *R. delica/R. chloroides* also when hyphae collapsed and started to degrade (Fig. 2f).

In their study, Peterson et al. (*1996*) suggested that the lack of pectin in the interfacial material surrounding viable peloton hyphae could be the result of enzyme secretion by the intracellular hyphae that would degrade plant-derived cell wall components. According to these authors, the presence of pectin around senescing hyphae may thus depend on a termination of this activity following hyphal demise, allowing the plant-derived matrix to eventually build up (Peterson et al. *1996*).

Interestingly, recent analysis of two ectomycorrhizal (ECM) fungal genomes (*Laccaria bicolor* and *Amanita sporigena*) showed that these fungi lack the potential to biosynthesize many of the major classes of secreted enzymes that depolymerize plant cell wall polysaccharides (Martin et al. *2008*; Nagendran et al.*2009*), and a correlation with the symbiotic ECM behaviour of these fungi was suggested. Fungi in the genus*Russula* are well known for their ECM phenotype on tree species, and the same *Russula* sequences found in*L. abortivum* were also found in ECM roots of neighbouring trees (Girlanda et al. *2006*). The unculturability of the *Russula* symbiont and its poor saprotrophic potential may indicate, as shown for other ECM fungi and suggested by Nagendran et al. (*2009*), a poor production of cell wall degrading enzymes in this symbiont. We therefore suggest that pectin is missing in the interfacial material surrounding the *Russula* pelotons not as a consequence of local degradation by the fungus, but because a pectin-containing interface is not built up around this fungal symbiont.

By contrast, intracellular *Ceratobasidium* hyphae were surrounded by a plant interface consistently labelled by JIM 5 (Fig. 2g). This observation further suggests that the lack/presence of pectin in the interface material is unlikely to be the result of fungal pectinolytic activity, as fungi in the genus *Ceratobasidium*secrete several isoforms of pectinolytic enzymes, at least *in vitro* (Sweetingham et al. *1986*).

Taken together, our observations suggest that a possible explanation for the different detection of pectin around coils formed by the *Russula* and *Ceratobasidium* symbionts is that the composition of the plant interface can be modulated when the plant cell is confronted with different fungal partners, and that pectin is a good marker to detect these changes. *R. delica/R. chloroides* is the ubiquitous and dominant fungal partner of *L. abortivum* over a wide geographic range (Girlanda et al. *2006*). Thanks to its double nature of ECM and orchid mycorrhizal fungus, it likely plays an important role in subsidizing the partially mycoheterotrophic host with organic carbon derived from autotrophic ECM plants, similar to other orchid symbionts (McKendrick et al. *2000*). By contrast, the *Ceratobasidium* symbiont is a sporadic and minor partner in mycorrhizal *L. abortivum* roots, and its role in plant nutrition is unclear. Similarly, specific fungi in the Ceratobasidiaceae have been reported to occur sporadically and at low abundance in the non-photosynthetic orchid *Hexalectris spicata* (Taylor et al. *2003*) suggesting that they are not critical mycorrhizal symbionts. Their occurrence in non-mycorrhizal rhizome tissue of *H. spicata* suggested the possibility of a pathogenic interaction with the orchid. Hadley (*1970*) also reported that some *Ceratobasidium* strains, when inoculated on orchid seeds, were quite aggressive when compared with other Rhizoctonias.

Pectin is an important source of molecular signals in plant–fungal interactions, and oligosaccharides produced by the action of pectin-degrading enzymes can induce defence responses in plants (Ridley et al.*2001*; D'Ovidio et al. *2004*). It is therefore tempting to speculate that its presence around the *Ceratobasidium*symbiont, an occasional fungal partner for *L. abortivum*, may represent a line of defence towards a less compatible fungus.

Ceratobasidium species are far more common fungal symbionts in terrestrial green orchid species (Stark et al. *2009*; Yukawa et al. *2009*; see also sequences in Fig. 3), such as those studied by Peterson et al. (*1996*). The absence of pectin observed in the viable stages of those interactions would agree with the hypothesis that the composition of the interface may somehow mirror plant–fungus compatibility. The mechanisms by which the orchid root cells can discriminate finely and locally between distinct mycorrhizal fungi—whether through recognition of the fungal surface or the release of soluble fungal effectors—are yet unknown.

The presence or absence of pectin on the plant–fungus interface may also reflect a different velocity in fungal growth within plant cells and tissues that would allow the deposition of pectin only around fungi that grow more slowly. We have no data on the growth rate of the *Russula* and the *Ceratobasidium* symbionts within the *L. abortivum* root tissues, although the latter is restricted to fewer cells. As fungal growth in endomycorrhizal symbioses appears to be mostly under the control of the host plant, as shown in legumes by mycorrhization mutants (Gianinazzi-Pearson et al. *1991*; Parniske *2004*), our results would again support the view that growth of the *Ceratobasidium* symbiont within *L. abortivum* roots is more carefully controlled.

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