Plant Science 263 (2017) 39–45

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

Cell-specific expression of plant nutrient transporter genes in orchid mycorrhizae

Valeria Fochi[a,b], Nicole Falla[a], Mariangela Girlanda[a,b], Silvia Perotto[a,b,**], Raffaella Balestrini[b,*]

[a] Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, Viale Mattioli, 25, 10125 Torino, Italy
[b] CNR-Istituto per la Protezione Sostenibile delle Piante (IPSP), Viale Mattioli, 25, 10125 Torino, Italy

ARTICLE INFO

Keywords:
Orchid symbiosis
Serapias vomeracea
Tulasnella calospora
Laser microdissection
Gene expression
Nitrogen

ABSTRACT

Orchid mycorrhizal protocorms and roots are heterogeneous structures composed of different plant cell-types, where cells colonized by intracellular fungal coils (the *pelotons*) are close to non-colonized plant cells. Moreover, the fungal coils undergo rapid turnover inside the colonized cells, so that plant cells containing coils at different developmental stages can be observed in the same tissue section. Here, we have investigated by laser microdissection (LMD) the localization of specific plant gene transcripts in different cell-type populations collected from mycorrhizal protocorms and roots of the Mediterranean orchid *Serapias vomeracea* colonized by *Tulasnella calospora*. RNAs extracted from the different cell-type populations have been used to study plant gene expression, focusing on genes potentially involved in N uptake and transport and previously identified as up-regulated in symbiotic protocorms. Results clearly showed that some plant N transporters are differentially expressed in cells containing fungal coils at different developmental stages, as well as in non-colonized cells, and allowed the identification of new functional markers associated to coil-containing cells.

1. Introduction

Orchids belong to one of the largest plant families [1] and an extraordinary floral diversification is correlated with the enormous number of species [2]. Their survival in nature is ensured by a complex symbiotic association with soil mycorrhizal fungi, which are particularly important during seed germination and early plant development. Orchid seeds are in fact very minute and without stored reserves, and germination and seedling development require therefore an external source of nutrients and organic C, provided by the mycorrhizal fungi [3,4]. Seed germination leads to the formation of a protocorm, a preseedling stage that generally lacks chlorophyll and is therefore heterotrophic and completely fungus-dependent for nutrients and organic C supply [5]. This peculiar ability of the plant to gain organic C from its mycorrhizal fungal partner is known as mycoheterotrophy [5,6]. Irrespective of the fact that orchid seedlings may then develop photosynthetic leaves or remain fully or partially mycoheterotrophic, mature roots of adult orchids are generally found to be colonized by mycorrhizal fungi [4].

Orchid mycorrhizal (OM) protocorms and roots are heterogeneous structures composed of different plant cell-types, where cells colonized by intracellular fungal coils (the *pelotons*) are close to non-colonized plant cells. Moreover, the fungal pelotons undergo rapid turnover inside OM cells, so that plant cells containing pelotons at different developmental stages can be observed in the same tissue section [7]. Differential expression of orchid genes in mycorrhizal and non-mycorrhizal protocorm regions has been reported in the model system formed by the photosynthetic Mediterranean orchid *Serapias vomeracea* and the basidiomycete *Tulasnella calospora* [8]. In this study, protocorms were manually dissected to separate the basal part, which contained the mycorrhizal tissue, from the apical region, featuring a plant meristem that is never colonized by the fungus [7]. However, the basal protocorm region is made of a heterogeneous cell population, featuring colonized and non-colonized orchid cells. Thus, localized expression of plant genes in specific cell-types may be masked when RNA is extracted from the whole tissue, as already suggested for the arbuscular mycorrhizal (AM) symbiosis [9,10].

Laser microdissection (LMD) has been used over the last years to study cell-specificity in gene expression profiles in the AM interaction, where particular attention has been paid to the root cortical cells containing the arbuscule, the fungal structure typical of this symbiosis. The results indicate that LMD can be a powerful tool for the identification of plant genes involved in specific stages of fungal colonization [9–17], or to verify the expression of fungal genes during the symbiotic

* Corresponding author.
** Corresponding author at: Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università degli Studi di Torino, Viale Mattioli, 25, 10125 Torino, Italy
E-mail addresses: silvia.perotto@unito.it (S. Perotto), raffaella.balestrini@ipsp.cnr.it (R. Balestrini).

http://dx.doi.org/10.1016/j.plantsci.2017.06.015
Received 17 May 2017; Accepted 14 June 2017
Available online 11 July 2017

stage [9,14,18]. In AM, information derived from these studies has led to the identification of functional genetic markers associated with arbusculated cells. Among them, several plant and fungal transporter genes have been identified, suggesting that functions like nutrient exchange are cell-specific, and confirming the hypothesis that arbuscule-containing cells represent the core of a functional AM symbiosis [16,10].

LMD has been successfully applied to study cell-type specific gene expression in orchid mycorrhiza for both plant [19] and fungal [20] genes. Here, we have used LMD to dissect the complexity of OM tissues and to investigate cell-type specific expression of plant genes related to nitrogen (N) transport. In fact, field (see [6]) and laboratory [21,22] studies provide evidence that orchids receive nitrogen (N) from their mycobionts, and the expression of fungal and plant genes potentially involved in N transport and assimilation has been recently investigated in *S. vomeracea* protocorms colonized by *T. calospora* [20]. The results suggest that organic N may be the main form transferred by the fungus to the orchid host. They also suggest that the intracellular fungus takes up ammonium from the symbiotic interface, indicating for the first time a flow of nutrients back to the fungal partner from the non-photosynthetic orchid. Based on these and on previous data, Dearnaley and Cameron [23] have proposed a model for mycorrhizal nutrient exchange in non-photosynthetic orchid protocorms, where the plant receives P, N and C (the latter two nutrients as amino acids) from the fungal partner and exports NH_4^+. Nutrient transfer in OM cells is thought to occur across the symbiotic interface, an apoplastic matrix between the intracellular fungal hyphae and the orchid cell membrane. However, it is still debated whether fungal nutrients are transferred to the plant across an intact fungal membrane by viable intracellular hyphae [21–24], or whether nutrients are released to the plant by digestion of degenerating fungal pelotons [22,25], a strategy called tolypophagy [3].

We have applied a LMD approach to investigate accumulation of specific plant gene transcripts in different cell-type populations collected from OM protocorms and roots of *S. vomeracea*. In particular, we addressed the following questions: (i) can we identify, among the N transporters described as being up-regulated in mycorrhizal protocorms by Fochi et al. [20], some genetic markers of peloton-containing orchid cells, and (ii) is the accumulation of transcripts coding for these N transporters modulated during the different stages of fungal peloton development?

2. Materials and methods

2.1. Biological materials

Symbiotic protocorms were obtained by co-inoculation, in 90 mm Petri dishes, of the mycorrhizal fungus *Tulasnella calospora* (isolate AL13/4D) and seeds of the Mediterranean orchid *Serapias vomeracea*, as described in [8]. Briefly, seeds of *S. vomeracea* were surface sterilized in 1% sodium hypochlorite and 0.1% Tween-20 for 20 min on a vortex, followed by three 5-min rinses in sterile distilled water. Seeds (re-suspended in sterile water) were dropped on strips of autoclaved filter paper (1.5 × 3 cm) previously positioned on solid oat medium (0.3% milled oats, 1% agar). A portion of actively growing mycelium of *T. calospora* was then placed in the centre of each Petri dish and the plates were incubated at 20 °C in full darkness for 30 days. Mycorrhizal seedlings of *S. vomeracea* were obtained as described in Balestrini et al. [26]. Briefly, after symbiotic protocorms had developed the first leaf primordium, plates were moved to light for about 20 days in a growth chamber (23 °C for 16 h light/21 °C for 8 h dark) until the leaf primordia turned green. At this stage, protocorms were transferred to magenta jars containing fresh OA medium (7,5 g/L agar and 3 g/L oat flour) and were maintained in the growth chamber with the same parameters described above until a radicle was formed at the protocorm base. At this point, they were transferred to new magenta jars containing a fresh OA medium (5 g/L agar and 3 g/L oat flour) supplemented with previously sterilized soil. Seedlings higher than 2 cm were finally transferred to new magenta jars containing twice-sterilized natural soil, which was watered with a sterile solution of water and oat flour (3 g/L), and maintained with the same photoperiodic parameters as above.

2.2. Tissue preparation for LMD

For paraffin embedding, *S. vomeracea* symbiotic protocorms and mycorrhizal roots (cut into about 10 mm segments with a razor blade in fixative) were fixed in freshly prepared Farmer's fixative (absolute ethanol/glacial acetic acid 3:1) at 4 °C overnight. Samples were subsequently dehydrated in a graded series of ethanol (70%, 90% in sterile water and 100% twice) followed by two incubations in Neoclear (Merck, Darmstadt, Germany), with each step on ice for 30 min, and embedded in paraffin as described in Balestrini et al. [9,26]. Neoclear was then gradually replaced with paraffin (Paraplast Plus) and samples were embedded in paraffin in Petri dishes as described in Balestrini et al. [26]. Sections of 12 µm thickness were cut using a rotatory microtome, placed and stretched out on Leica RNase-free PEN foil slides (Leica Microsystems) with ddH_2O (filtered with a 0.2 µm filter). The sections were then dried on a 40 °C warming plate, stored at 4 °C and used within 2 days.

2.3. LMD

A Leica LMD 6500 Laser microdissection system (Leica Microsystems, Inc., Germany) was used to isolate the different cell-types from the prepared tissue sections. Just before use, the slides with the sections were deparaffinized with Neoclear for 8–10 min, rinsed in 100% ethanol for one minute and then air-dried. The deparaffinized slides were placed face-down on the microscope and two different cell-types were selected from symbiotic protocorms and roots, microdissected and collected separately (Fig. 1): (i) cells containing visible fungal coils (C), mainly selecting those in which the coil occupied the whole cell and (ii) non-colonized cells (NM). In a second experiment, a third cell-type was collected from protocorm sections, i.e. cells with more condensed central coils (CCC). Approximately 1500 cells for each cell-type population were collected for each replicate, and the pools were brought to a final volume of 50 µL with Pico Pure extraction buffer and processed for RNA extraction. For protocorms, at least three independent biological replicates of each cell type were collected for downstream gene expression analyses, while two independent biological replicates were used for roots.

2.4. RNA extraction and RT-PCR

RNA was extracted using the Pico Pure kit (Life Technologies, Carlsbad, CA, USA), without DNase treatment in the kit column. The RNA was eluted in 20–25 µL of Elution buffer and treated with RNase-free DNAse (TURBO™ DNase, Ambion), according to the manufacturer's instructions. RNA quantification was determined using a NanoDrop 1000 spectrophotometer. A One-Step RT-PCR kit (Qiagen, Valencia, CA, USA) was used for the RT-PCR experiments on RNA extracted from the different LMD samples. All RNA samples were checked for DNA contamination through RT-PCR analyses with specific primers for plant and fungal elongation factor genes, i.e. *SvEF1a* and *TcEF1a*, respectively (Table S1), without a previous retrotranscription step (RT-).

Specific primers for several plant genes involved in N metabolism (Table S1) were designed using *PRIMER3PLUS* (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and tested for their specificity with *PRIMER BLAST* (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The specificity of the primers was tested through PCR experiments on DNA from *S. vomeracea* seeds and *T. calospora* mycelium (data not shown).