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Review

Laser microdissection as a tool to study fungal gene expression in mycorrhizal endosymbioses

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Abstract
Laser microdissection (LMD) is a microscopy technique that, through the collection of specific cell-type populations from sections of heterogeneous tissues, allows the subsequent extraction of nucleic acids as well as primary and secondary metabolites. In plants, LMD was widely used to study cell-specific gene expression during symbiotic interactions with other organisms, including mycorrhizal fungi. In particular, LMD was extensively used to study cell-specificity in gene expression profiles in arbuscular mycorrhizal (AM) and orchid mycorrhizal (ORM) interactions. These earlier studies were mainly focused on the identification of functional markers in plant cells containing intracellular fungal structures, i.e. arbuscules, the typical structures in AM, and coils, typical of ORM. Several plant and fungal genes coding for nutrient transporters were identified in these cells thanks to LMD, suggesting that symbiotic nutrient exchange is cell specific. In the absence of a stable transformation protocol for the expression of tagged genes in the mycorrhizal fungal partner, LMD protocols represent a useful tool to study fungal gene expression in specific cell-type populations inside symbiotic plant tissues.

Keywords
AM symbiosis, cell-specificity, LMD, gene expression, nutrient exchange, orchid symbiosis

Introduction
Mycorrhizal fungi are symbiotic soil fungi that assist plants in nutrient uptake since land colonization. Endosymbiotic mycorrhizal fungi, such as arbuscular mycorrhizal (AM) or orchid mycorrhizal (ORM) fungi, form specialized structures inside the host cells of colonized roots, which represent a heterogenous landscape of different cell-type populations, expected to play different functions in the symbiosis. Since several years, researchers have tried to characterize the different responses in these different cell-types (Fiorilli et al., 2019) as well as to identify molecular markers related to the development of the typical symbiotic fungal structures, such as arbuscules in the AM and coils in the ORM symbiosis. Most attention has been focused on the AM symbiosis because AM fungi establish mycorrhizal interactions with the roots of most crop species and play an important role as bio-fertilizers. As reported by Rouphael et al. (2015), the development of more sustainable horticultural practices would greatly benefit from the biostimulant functions displayed by AM fungi. The success of the AM symbiosis is mainly due to the nutritional benefits to the host plant: AM fungi absorb inorganic phosphate (Pi) and water from the soil, as well as other macro- and micronutrients, delivering them to the host plant. In turn, the fungus receives photosynthesis-derived carbon compounds, such as carbohydrates and lipids (Balestrini and

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Lumini, 2018). The use of AM fungi has been largely explored as an environmentally friendly strategy to improve plant nutrition and growth and to enhance plant tolerance and resilience to different abiotic and biotic stresses, although the impact and the reliability of these strategies across diverse environmental conditions and production systems must be evaluated before practical recommendations can be provided. Additionally, the exploitation of AM fungi in agricultural programs requires the identification of the events that lead to the establishment and functioning of the symbiotic interaction, including the mechanisms involved in nutrient exchange and water transport. Root colonization by AM fungi occurs through the formation of inter- and intracellular hyphae and culminates, in the inner root cortical layers, with complex and highly branched intracellular fungal structures called arbuscules. Arbuscules are surrounded by a plant derived membrane and, because of the huge increase in the contact surface between the fungus and the plant, are considered to be key structures in the AM symbiosis, where most of the nutrient exchange between the two partners is likely to occur (Balestrini and Bonfante, 2005). Once the first arbuscules are formed in the root cortex, all developmental stages of the symbiosis can be found in a mycorrhizal root. Therefore, a mycorrhizal root is a heterogeneous assortment of different cell types, including colonized and non-colonized epidermal and cortical plant cells. The presence of multiple cell types, some of which involved in the different stages of the symbiotic interaction, makes the root gene expression profiling very complicated.

A similar situation is also found in other endomycorrhizal symbioses, such as orchid mycorrhiza (Balestrini and Lumini, 2018). In nature, members of the large plant family Orchidaceae establish a characteristic symbiotic interaction with some soil fungi that induce seed germination and development of adult plants. The minute orchid seeds, called "dust seeds", lack the endosperm and contain little stored food reserves. Colonization by a compatible fungus is essential for their germination and for the provision of major nutrients such as carbon and nitrogen (Balestrini and Lumini, 2018). Germinated orchid seeds develop into tuber-like structures named protocorms that finally produce leaves and roots. All stages of the orchid life cycle are mycorrhizal, and orchid mycorrhizal protocorms and roots are heterogeneous structures made of different cell-types, where plant 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. Thus, a serious drawback in the investigation of the molecular aspects of mycorrhizal interactions in both AM and ORM is that the development of the symbiosis is not a synchronous process and that colonized and non-colonized cells occur in the same tissues. As a consequence, the transcriptional profile of whole mycorrhizal organs (e.g. roots and/or protocorms for orchids) represents the simultaneous gene expression of multiple cell types and fungal structures involved in the interaction (Balestrini and Fiorilli, 2020) and could mask cell type-specific differences. In the last fifteen years, protocols based on the use of laser microdissection (LMD) were widely applied to plant tissues and provided novel information on the role of different cell-type populations in pathogenic (Tang et al., 2006; Chandran et al., 2010; Hacquard et al., 2010) and symbiotic plant-fungal interactions that included mycorrhizal associations such as ectomycorrhizal, ORM and AM symbioses (Fochi et al., 2017a, 2017b; Hacquard et al., 2013; Fiorilli et al., 2019; Balestrini and Fiorilli, 2020). In particular, LMD technology was successfully applied to investigate the gene expression profiles of both plant and fungal partners during different colonization stages in the AM symbiosis, but also to study proteomic and metabolomic profiles in arbuscule-containing cells (Gaude et al., 2012, 2015). The main objective of this review is to highlight the instrumental role of LMD to study fungal gene expression in mycorrhizal endosymbioses.

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Application of LMD to study AM fungal functions in symbiosis

Since the first report by Balestrini et al. (2007), where LMD was applied to investigate gene expression in mycorrhizal roots, several studies have been using this technology in conjunction with RT-PCR and RT-qPCR. Many of these papers have described the results of targeted gene expression experiments on RNA extracted from different cell-types isolated by LMD from mycorrhizal and non-mycorrhizal roots, in particular cortical cells from non-mycorrhizal roots, cortical cells containing arbuscules and non-colonized cells from mycorrhizal roots (reviewed in Fiorilli et al., 2019; Limpens, 2019; Balestrini and Fiorilli 2020). Many LMD protocols have been successfully applied on different combinations of plant and fungal species, leading to the identification of plant and fungal functional marker genes associated with arbusculated cells in different plants (e.g. tomato, *Lotus*, *Medicago*). Concerning the host plants, new information was obtained mainly on genes involved in fungal accommodation and functioning of arbusculated cortical cells. Several genes coding for nutrient (and water) transporters were found to be expressed in arbusculated cells, suggesting that functions such as nutrient exchange are cell-specific and confirming the hypothesis that arbuscule-containing cells represent the core of a functional AM symbiosis (reviewed in Fiorilli et al., 2019, Limpens 2019, Balestrini and Fiorilli 2020). Interestingly, plant and fungal genes coding for proteins with the same function (i.e. phosphate and ammonium transporters) have been found to be both expressed in arbusculated cells, suggesting that plant and fungus may compete for the nutrient resources localized in the interfacial apoplast. A LMD-based technique was also used to evaluate, in mycorrhizal roots of bean plants, the local impact of water stress on gene expression (Recchia et al., 2018). In detail, gene expression data obtained for root cortical cells, containing or not fungal arbuscules and collected from water deficit treated plants, provided evidence that a cell type-specific modulation of the plant transcriptome occurs during symbiosis under stress. The use of LMD to collect several cell-type populations from mycorrhizal plants subjected to stressful conditions represents an additional advancement to understand the fine-tune regulation of plant responses to adverse conditions mediated by the AM fungus. The use of LMD also allowed an estimation of the percentage of fungal and plant transcripts in arbuscule-containing cells. Using specific primers for both fungal and plant housekeeping genes, it was estimated that, in this cell population, the plant transcripts represented about 80 % of the total transcripts, whereas the fungal transcripts accounted for about 20% (Fiorilli et al., 2013).

Most reports on the use of LMD to investigate the AM symbiosis have been focused on the expression of plant genes, whereas few reports tried to unravel specific changes in gene expression in the different fungal structures. The first experiments on an AM fungus consisted on studying the expression of fungal genes coding for nutrient transporters in arbuscules (Balestrini and Fiorilli, 2020). Tisserant et al. (2012) performed a non-targeted transcriptomic analysis on LMD-microdissected arbuscule-containing root cells to investigate the fungal transcriptome in the arbuscules. These data were validated by means of one-step RT-PCR on RNA extracted from AM fungal spores, extraradical mycelium and arbusculated LMD-microdissected cells, and represented a starting point for further studies on the expression of fungal genes in arbusculated cells (e.g., Belmondo et al., 2014). Unlike plant genes, which were investigated by LMD in several AM host plant species, most results derived by the use of LMD to study the fungal genes were obtained, with few exceptions, on *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler, which represents a model system for studies on AM fungi. A list of the fungal genes reported to be expressed in arbusculated cells can be found in Table 1.

Additionally, Zeng et al. (2018) recently combined LMD of mycorrhizal root sections with RNAseq analysis in order to gain novel insights into stage-specific fungal gene expression, focusing the analysis on fungal genes coding for secreted proteins (SPs). It is well known that AM fungi secrete an array of (small) proteins that may act as effector proteins (Kloppholz et al., 2011; Tisserant et al.,

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Table 1 - List of fungal genes identified in mycorrhizal roots as being preferentially or exclusively expressed in arbuscule-containing cells by means of LMD technology (modified by Fiorilli et al., 2019).

Fungal species	Gene name	Putative function	Reference
Funneliformis mosseae	*GmosPT*	Phosphate transporter	Balestrini et al., 2007
	GmHA	H^+ATPase	Balestrini et al., 2007
	RiARF	ADP-ribosylation factor	Gomez et al., 2009
	RiAP	Probable autophagy protein	Gomez et al., 2009
	Rip24p	Related to p24 protein	Gomez et al., 2009
	RIGS	Glutamine synthetase	Gomez et al., 2009
	RiARG	Arginase	Gomez et al., 2009
	RiOATR	Probabile ornithine aminotrasferase	Gomez et al., 2009
	RiASS	Argininocusscinate syntase	Gomez et al., 2009
	Riacyl-CoA-dehy	Probable acyl-CoA dehydrogenase	Gomez et al., 2009
	RiDSR2	Phospholipid-transporting ATPase DRS2	Gomez et al., 2009
	RiFAD	Delta-9 fatty acid denatuase	Gomez et al., 2009
Rhizophagus irregularis	*RiPEIP1*	Preferentially Expressed in Planta	Fiorilli et al., 2016
	GintPT	Phosphate transporter	Fiorilli et al., 2013
	GintMST2	Monosaccaride transporter	Fiorilli et al., 2013
	RiAMT1	Ammonium transporter	Perez-Tienda et al., 2011
	RiAMT2	Ammonium transporter	Perez-Tienda et al., 2011
	EXTB106676_b0	No hit	Tisserant et al., 2012
	EXTA123933.b0	No hit	Tisserant et al., 2012
	RiABCT	ABC transporter	Tisserant et al., 2012
	RiATPase	ATPase	Hogekamp et al., 2011
	RiRB	RNA binding protein	Hogekamp et al., 2011
	RiZTF	C2H2 zinc finger transcription factor	Hogekamp et al., 2011
	RiPTR2	Dipeptide transporter	Belmondo et al., 2014
	-	Several secreted proteins*	Zeng et al., 2018

*Due to the large dataset, specific proteins have been not reported here.

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2012; Lin et al., 2014). Through an LMD approach, Zeng et al., (2018) revealed that different SP sets were expressed in the extraradical mycelium, the intraradical hyphae and the arbuscules. Interestingly, these authors also observed that the gene expression levels of 42 candidate effectors varied from host to host, suggesting that expression of secreted AM fungal proteins is stage and host dependent.

In addition to functional genes, LMD has been applied, in combination with sequence-based taxa identification, to study the AM fungal community inside mycorrhizal roots (Berruti et al., 2013). Comparison between the AM fungal taxa identified inside arbusculated cells by LMD and those identified in the DNA extracted from the whole root showed that the two AM fungal communities differed remarkably. In fact, whereas five AM fungal taxa were found to be involved in the production of arbuscules, two taxa were retrieved from the root DNA but were not found in the arbusculated cells. Thus, the LMD technique can be used to obtain more precise data on the symbiotically active intraradical AM fungal community. By focusing on the functional genes with a role in symbiosis, this technique could be also used to evaluate the efficiency of different AM fungal species/isolates.

Application of LMD to study ORM fungal functions in symbiosis

All orchids depend on symbiotic mycorrhizal fungi for seed germination and produce a tuber-like structure, i.e. the protocorm, that will eventually give rise to the true seedling. Basidiomycetes in the genera *Tulasnella*, *Ceratobasidium* and *Serendipita* are the most common mycorrhizal symbionts in orchid species that develop into fully photosynthetic adult plants. As described above, ORM protocorms and roots are heterogeneous structures that comprise different plant cell-types, where cells colonized by intracellular fungal coils (the *pelotons*) at different developmental stages, due to the rapid coil turnover inside the colonized cells, are close to non-colonized plant cells in the same tissue section (Perotto et al., 2014). A protocol based on LMD was used to localize specific plant and fungal gene transcripts in different cell-type populations collected from mycorrhizal protocorms and roots of the Mediterranean orchid *Serapias vomeracea* (Burm.f.) Briq. colonized by *Tulasnella calospora* (Boud.) Juel (Fochi et al., 2017a, b; Balestrini et al., 2014). Total RNA was extracted from the different cell-type populations (i.e. cells fully colonized by large fungal coils, cells containing condensed coils and non-colonized plant cells). Transcripts corresponding to genes potentially involved in plant-fungus interactions were then investigated by RT-PCR, as well as genes related to nitrogen (N) uptake and transport previously identified as being up-regulated in symbiotic protocorms by transcriptomic experiments. The results showed that *SvNod1*, coding for a *S. vomeracea* nodulin-like protein containing a plastocyanin-like domain, is expressed only in protocorm cells containing intracellular fungal hyphae. Additionally, fungal and plant genes involved in the transport and metabolism of nitrogen compounds were found to be differentially expressed in cells containing fungal coils at different developmental stages, as well as in non-colonized cells. A list of the fungal genes identified in coil-containing cells can be found in Table 2.

Overall, the data derived from the *S. vomeracea* - *T. calospora* ORM model system support the hypothesis that ammonium is available in the apoplastic interface surrounding the pelotons and is actively taken up by the fungus, as suggested by the strong induction in colonized cells of a low-affinity ammonium transporter (*TcAMT2*). The induction, in the same colonized protocorm cells, of the ammonium scavenging enzyme glutamine synthetase (*TcGS1*) suggests that ammonium is rapidly assimilated once taken up by the fungal peloton (Fochi et al., 2017a). RNAs extracted from the same cell-type populations were used also to verify the expression of plant genes potentially involved in N uptake and transport, previously identified as being induced in mycorrhizal protocorms. A plant ammonium transporter gene (*SvAMT1*) was found to be expressed in the colonized protocorm cells, although it expression was not specific to this cell type (Fochi et al., 2017a, b). As hypothesized for the AM symbiosis (Guether et al., 2009; Calabrese et al., 2016), the presence of both fungal and plant

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AMTs in the same colonized orchid cells may lead to a competition between the plant and the fungus for nitrogen sources present in the interfacial apoplast. The LMD approach revealed that some plant amino acid transporters were differentially expressed in protocorm cells containing fungal coils at different developmental stages, adding an additional piece on the comprehension of the N metabolism in orchid symbiosis (Fochi et al., 2017b) and leading to the identification of new functional marker genes associated to coil-containing cells in ORM.

Table 2 - *Tulasnella calospora* genes found to be expressed in *Serapias vomeracea* protocorm cells containing fungal coils by means of LMD technology. Fungal transcripts code for transporters and enzymes involved in N uptake and metabolism (Fochi et al., 2017a).

Fungal species	Gene name	Putative function	References
Tulasnella calospora	*TcAMT1*	Ammonium transporter	Fochi et al., 2017a
	TcAMT2	Ammonium transporter	
	TcGS1	Glutamine synthetase	
	TcGS1	Glutamine synthetase	
	TcCAR	Arginase	
	TcURE	Urease	
	TcOAT	Ornithine aminotransferase	
	TcODC	Ornithine decarboxylase	
	TcAAT1	Amino acid transporter/permease	
	TcAAT2	Amino acid transporter/permease	
	TcAAT3	Amino acid transporter/permease	
	TcAAT4	Amino acid transporter/permease	
	TcAAT5	Amino acid transporter/permease	

Conclusions

LMD has proved to be a useful tool to study cell-type specific gene expression in symbiotic and pathogenic interactions. Overall, the results obtained so far in symbiotic mycorrhizal interactions demonstrate that LMD is a powerful technology to investigate the cellular complexity of both AM and ORM and can be applied to investigate both plant and fungal gene expression. The possibility to successfully apply the LMD technology to evaluate the expression level of both fungal and plant functional genes involved in the AM and ORM symbioses should help to dissect the functional diversity in different fungal - plant combinations and conditions. This knowledge may provide future guidelines to identify the best plant-fungus combinations to improve plant fitness under a changing ecological scenario.

Authors' contribution

RB, SP and VF equally contributed to writing this review.

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