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**Omics approaches to investigate
plant-fungus interactions
in orchid mycorrhizal symbioses**

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Preface

1 This thesis is aimed at increasing our understanding of the
2 molecular mechanisms that govern the symbiotic relationship
3 between orchids and their associated mycorrhizal fungi. In
4 particular, the main aim of the thesis was to deepen our knowledge
5 of i) the nutritional aspects of the symbiosis, with a focus on the
6 essential element sulfur, and ii) how orchids interact with their
7 associated fungi during the initial stages of seed germination and
8 protocorm development. Over the past few years, the advancement
9 of *-omics* tools has been pivotal in expanding knowledge of the
10 molecular processes occurring during the interaction between
11 orchid mycorrhizal (ORM) fungi and their host plants. In particular,
12 targeted gene expression approaches that made use of RT-qPCR
13 and laser microdissection, coupled with ultra-high spatial resolution
14 secondary ion mass spectrometry (SIMS), have been applied to
15 study plant and fungal sulfur metabolism during symbiosis. In
16 addition, untargeted transcriptomics and metabolomics have been
17 used to investigate the mechanisms involved in the early stages of
18 the symbiosis, before physical contact. As a model system, the
19 Mediterranean terrestrial orchid *Serapias vomeracea* and the ORM

20 fungus *Tulasnella* sp. were investigated because of the genomics or
21 transcriptomic databases already available.

22

CHAPTER 1

Introduction

1.1 The Orchidaceae: general aspects and diversity

The study of orchid biology is important and fascinating at the same time because of some key aspects that characterize this peculiar group of plants (Benzing, 1981; Waterman and Bidartondo, 2008). The Orchidaceae represent one of the most species-rich plant families, comprising over 27,000 species (WCSP, 2022) that have successfully adapted to the most diverse environments. Orchid seeds exhibit a tiny, dust-like nature, making them among the smallest of the whole plant kingdom (Arditti and Ghani, 2000). Orchids exhibit a remarkable degree of ecological diversity, forming intricate interactions with both animals and fungi (Perez-Escobar et al., 2024) and establishing complex relationships with other plant species. The association of orchids with mycorrhizal fungi is crucial for the plant because these symbiotic fungi promote seed germination and provide essential nutrients for seedlings growth (Smith and Read, 2008). While certain orchid species have achieved a widespread distribution, others remain rare and elusive, confined to specific regions or habitats (Benzing, 1981), and it has been suggested that the presence and distribution of suitable OM fungi may influence the host plant distribution (McCormick et al., 2018). Numerous orchid species possess highly specialized pollination

mechanisms, that requires specific pollinators to ensure successful sexual reproduction (Benzing, 1981; Waterman and Bidartondo, 2008). The diversity of plant-pollinator interactions observed in orchids surpasses that of many other plant families, suggesting that the effects of pollinators may represent an additional driving force behind the remarkable diversification of this group, acting in concert with the fungal symbiosis that has garnered considerable research interest (Otero and Flanagan, 2006; Waterman and Bidartondo, 2008).

As mentioned above, orchids produce very small seeds that lack stored energy reserves and contain an immature embryo; thus, they heavily depend on specific ORM fungi for organic carbon, needed to provide both energy and chemical elements for germination (Arditti and Ghani, 2000; Dearnaley et al., 2016). This symbiotic relationship extends beyond the initial germination stage, playing a pivotal role in promoting growth and adaptation of orchid seedlings to environmental conditions, thereby facilitating their successful establishment (Bustam et al., 2014; Reiter et al., 2016; Phillips et al., 2020; Yang et al., 2020; Wang et al., 2021). Furthermore, the ORM fungal partners involved in these symbiotic associations can exhibit a high degree of specificity, highlighting the intimate and specialized nature of this mutualistic interaction (Jacquemyn et al., 2017).

Orchids first diversified between 112 to 76 million years ago (Ma), according to evolutionary and molecular dating studies (Ramírez et al., 2007; Givnish et al., 2016). Over the past few decades, significant progress has been made in reconstructing the evolution of orchids, largely facilitated by the numerous molecular phylogenetic studies that have shed light on the intricate relationships between various orchid tribes, subtribes, and subfamilies (Cameron et al., 1999; van den Berg et al., 2005; Carlswald et al., 2006; Górniak et al., 2010; Givnish et al., 2015; Zou et al., 2015; Li et al., 2016; Pérez-Escobar et al., 2017, 2021; Y.X. Li et al., 2019; Kim et al., 2020; Eserman et al., 2021; Serna-Sánchez et al., 2021; Wong and Peakall, 2022). In 2015, Chase and co-authors proposed a “classification summary tree” to simplify the taxonomic assignment system, linking subfamilies, tribes and subtribes (Fig. 1).

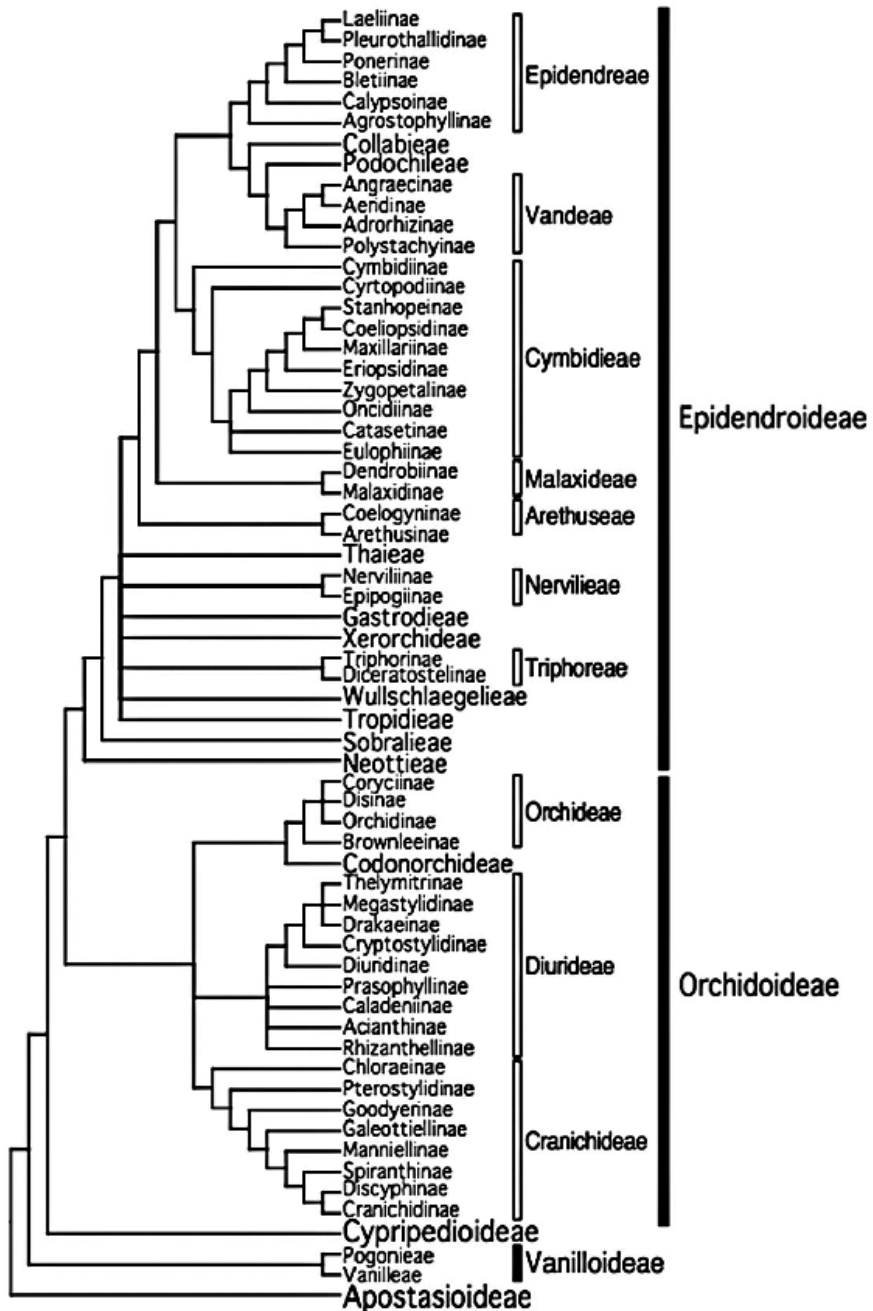


Figure 1. The ‘classification summary’ tree proposed by Chase et al. (2015). This tree summarizes the relationships among the subfamilies, tribes, and subtribes of Orchidaceae reported in the published literature.

In a recent study, Perez-Escobar et al. (2024) presented a new phylogeny of the Orchidaceae family integrating high-throughput and Sanger sequencing data, combining in particular target enrichment (low-copy nuclear loci) and Sanger sequences (matK and ITS). This study not only elucidated patterns of geographic range evolution and diversity within the orchid families but also shed light on the intricate processes of speciation, suggesting Central America as a significant hotspot for orchid diversification.

Based on their growth substrates, orchids can be broadly classified into three main lifestyles: terrestrial, lithophytic, and epiphytic, utilizing soil, rock surfaces, and other plants as their respective growth substrates (Dearnaley et al., 2012). The orchid used for the experiments described in this thesis is the Mediterranean terrestrial species *Serapias vomeracea* (Burm. f.) Briq., shown in Fig. 2, a member of the Subtribe Orchidinae (subfamily Orchidoideae). Based on molecular phylogenetics, Jin et al. (2017) investigated the phylogenetic relationships within this subtribe, that includes about 1800 species.



Figure 2. *Serapias vomeracea* grown in natural grassland in the locality of Cairo Montenotte (SV), Italy. a) a single individual, b) a small population of *S. vomeracea*.

Despite their evolutionary success and remarkable adaptability, orchids are among the most endangered plants globally (Fay, 2018). Numerous terrestrial orchid species are listed on the IUCN Red List, underscoring their vulnerable conservation status and the urgent need for effective conservation measures (Wraith et al., 2020; Bazzicalupo et al., 2023). Since the survival and growth of orchid species mainly depend on their complex relationships with mycorrhizal fungi and pollinators, they are particularly sensitive to human-related pressures like pesticide use, climate change, and harvesting (Bazzicalupo et al., 2023). For example, several orchids

depend on a single pollinator species for reproduction, thus being highly vulnerable to the rising global declines in pollinator populations (Chupp et al., 2015). The close ecological link with other organisms makes orchids useful indicators of ecosystem health (Lussu et al., 2024), leading to extensive reviews and analyses focused on their conservation efforts (e.g., Koopowitz et al., 2003; Dixon and Phillips, 2007; Fay, 2018), the development of intervention protocols (e.g., Swarts and Dixon, 2017) and analytical studies on the significance of the actions taken (e.g., Tsiftsis, 2021; Lussu et al., 2024).

1.2 The life cycle of terrestrial orchids

In Europe, members of the Orchidaceae family are mainly terrestrial and fall under the category of cryptophytes, specifically geophytes (Djordjević and Tsiftsis, 2022). According to Bartolucci et al. (2018), 237 species and subspecies of orchids, all terrestrial, can be found in Italy in diverse ecosystems such as forests, wetlands, or grasslands (Lussu et al., 2024).

According to Shefferson et al. (2020), the life cycle of terrestrial orchids comprises six distinct phases: seed, protocorm, juvenile, latent adult, vegetative adult, and flowering individual. In Figure 3 a

typical life cycle of the perennial terrestrial orchid *Orchis purpurea* is represented, with the possible transitions between the different growth stages.

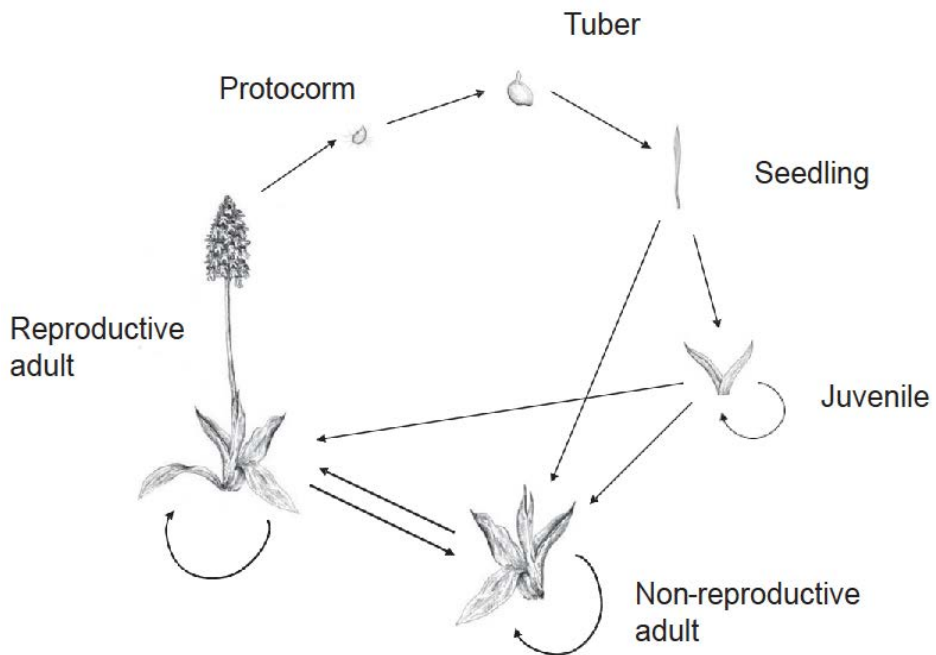


Figure 3. Life cycle of the terrestrial orchid *Orchis purpurea*. In this picture, the juvenile stage that follows the protocorm stage includes also a seedling and a tuber, which can be found only in some orchid species. Possible stage transitions are shown by arrows. Although not indicated in this picture, juveniles non-reproductive adults and reproductive adults may all go through vegetative dormancy (non-emergent stage) in a typical orchid population (modified from Shefferson et al., 2020).

One of the distinctive features of orchids is the production of copious dust-like seeds capable of dispersing over considerable distances (Arditti and Ghani, 2000). These minute seeds, lacking endosperm and showing a rudimentary embryo, are ill-equipped for independent growth and development (Arditti and Ghani, 2000). During the initial stages of their life cycle, orchids rely entirely on ORM fungi through a strategy known as mycoheterotrophy (Leake, 1994; Merckx, 2013). Orchid mycorrhizal fungi have been observed to infect seeds and to enter the embryo through the degenerated suspensor, the base of an epidermal hair or adjacent already colonized cells at a later stage of development (Peterson et al., 1998; Smith and Read, 2008). When it germinates, the embryo expands, causing the rupture of seed coat and forming a heterotrophic structure known as protocorm (Fig. 4) (Arditti, 1967; Leroux et al., 1997). This structure represents an intermediate stage between the embryo and the seedling, relying entirely on symbiotic fungi for sustenance (Leroux et al., 1997). Protocorms produce rhizoids and increase in size, and the parenchyma cells in the basal part of the protocorm are colonized by dense intracellular fungal coils, known as *pelotons* (Fig. 4 b), to allow for nutrient exchange (Rasmussen, 1995; Selosse, 2014; Dearnaley et al., 2016). The *pelotons* are the key structure of

the orchid mycorrhizae and represent the closest interaction between the fungus and the plant (Perotto and Balestrini, 2024).

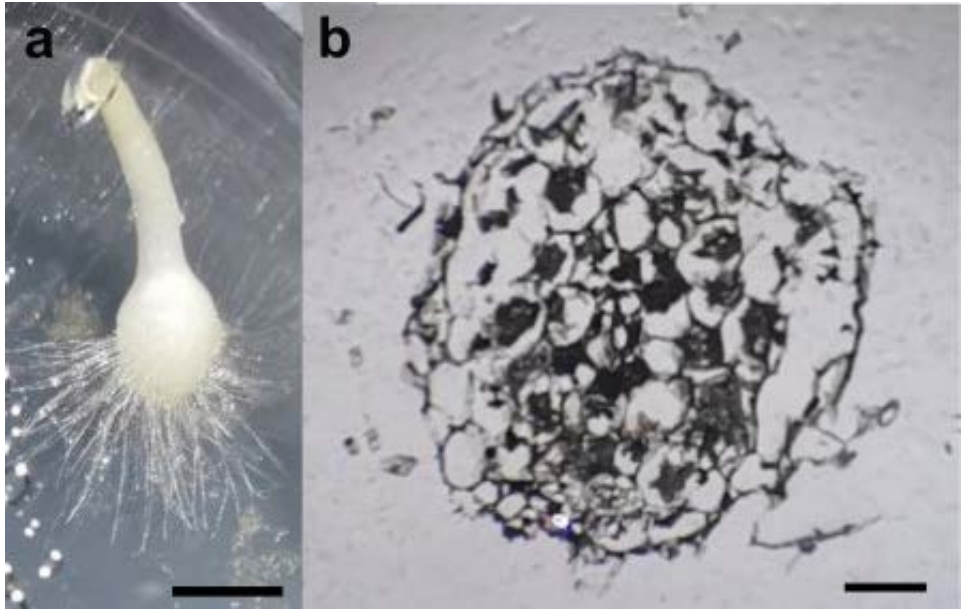


Figure 4. *S. vomeracea* protocorm obtained by *in vitro* symbiotic germination. **a)** A side view of a *S. vomeracea* protocorm with leaf primordia already forming in the apical part, and numerous rhizoids in the basal part. Near the protocorm are several ungerminated seeds. Bar is 1mm. **b)** Cross section of the basal part of a *S. vomeracea* protocorm showing parenchyma cells colonized with fungal pelotons at different developmental stages. Bar is 250 μm .

Protocorms establish an apical meristem that will eventually give rise to the first leaves, and will form adventitious roots (Arditti, 1967; Harrison, 1977). The juvenile phase follows, characterized by underground mycoheterotrophic structures (Fig. 3), more developed

than protocorms and seedlings, resembling adult plants but typically smaller and lacking resources for flowering (Shefferson et al., 2020). The cortical root cells of juvenile and adult plants are typically colonized by ORM fungal *pelotons*.

Only a small fraction of seeds successfully germinates and develops into adult plants. This suggests that orchid populations may be somewhat constrained by microsite availability, possibly due to spatially variable distributions of ORM fungi (Jacquemyn et al., 2011; McCormick et al., 2018). Indeed, the selection of suitable microsites for various orchid species is influenced by a combination of factors, including the requirement of specific ORM fungi by the orchids and the presence of suitable environmental conditions (biotic and abiotic) within a given area (Waud et al., 2016; McCormick et al., 2018). Dearnaley et al. (2012) emphasized the pivotal role of mycorrhizal associations for the survival and growth of terrestrial orchids. They treated how the protocorm and juvenile stages of orchids are particularly dependent on these symbiotic relationships, highlighting the need, for targeted conservation efforts, to protect the habitats and microbial communities that support these delicate orchid life stages. Furthermore, Djordjević and Tsiftsis (2022) reviewed the environmental factors that affect distribution, abundance, and richness of terrestrial orchids, and

discussed how the flowering and the reproductive success of terrestrial orchids are also influenced by various abiotic factors, such as soil pH, nutrient availability, and light conditions suggesting that a better understanding of these environmental requirements is crucial for the effective management and restoration of terrestrial orchid populations.

In summary, the life cycle of terrestrial orchids involves a complex interplay between biotic and abiotic factors, with the successful germination and maturation of these plants depending on the availability of suitable microsites, and on the presence of specific ORM fungi and environmental conditions.

1.3 Taxonomic diversity of ORM fungi

A diverse array of fungal taxa has been found in ORM tissues, usually reflecting the trophic strategy of the host plant (Wang et al., 2021). Fungi belonging to the phylum Basidiomycota, particularly the rhizoctonia-like group, are prevalent in these associations, along with fungi from the phylum Ascomycota (Smith and Read, 2008; Dearnaley et al., 2012). Like in many other plant species, a number of taxa of well-known endophytes, mostly Ascomycetes, have been also reported in orchid roots (Sisti et al., 2019). These fungi do not

form the intracellular fungal structure typical of ORM, and their role is unclear (Li et al., 2021).

Photosynthetic orchids are usually found to be associated with basidiomycetes in the rhizoctonia-like group, encompassing the genera *Tulasnella* and *Ceratobasidium* (both belonging to Cantharellales) and *Serendipita* (belonging to Sebaciniales) (McCormick et al., 2018; Li et al., 2021). These fungi exhibit a broad ecological spectrum, often displaying both saprotrophic behavior (i.e., they feed on decaying organic matter) and endophytism within non-orchid plants (Dearnaley et al., 2012; Selosse and Martos, 2014; Jacquemyn et al., 2017; McCormick et al., 2018; Selosse et al., 2018). This pattern is particularly evident in the Serendipitaceae family; for example, the widely studied endophytic model *Serendipita* (formerly *Piriformospora*) *indica* exhibits mycorrhizal associations with Brazilian orchids (Oliveira et al., 2014). Rhizoctonia-like ORM fungi have been identified both in terrestrial photosynthetic and epiphytic orchids, but the ORM fungi colonizing the aerial roots of epiphytic orchids and the roots of terrestrial orchids in the Reunion Island were found to belong to different guilds, possibly reflecting an ecological barrier between epiphytic and terrestrial subnetworks (Martos et al., 2012). In addition to rhizoctonia-like fungi, certain photosynthetic orchids have been

found to associate with saprotrophic fungi in the Auriculariales and Psathyrellaceae, as well as in the genera *Tricharina*, *Clavulina*, *Armillaria*, *Marasmius* (Waterman et al., 2011; Yagame et al., 2013; Jacquemyn et al., 2016; González-Chávez et al., 2018; Qin et al., 2019; May et al., 2020), but the actual role and mycorrhizal status of these fungi have not been demonstrated.

Achlorophyllous orchid species are found in forest habitats and are fully mycoheterotrophic, i.e. they rely on their ORM fungal partner throughout their life (Hynson et al., 2013). These orchids usually associate specifically with basidiomycetes also known to form ectomycorrhiza (ECM), like species in the Russulaceae and Telephoraceae (Roy et al., 2009; Lee et al., 2015). Additionally, Selosse et al. (2004) documented ECM Ascomycota from the genus *Tuber* in the mycoheterotrophic orchid *Epipactis microphylla* through molecular identification, transmission electron microscopy, and immunolabeling. Some orchid species growing in forest habitats, called mixotrophic, have retained most, or all, photosynthesis-related genes in their plastid genomes (Lallemand et al., 2019), but although they can be at least partially autotrophic, they have been found to rely on the ORM fungal partners for carbon (Selosse and Roy, 2009). These mixotrophic orchids typically have few or no rhizoctonia-like fungi in their roots, where ECM fungi predominate

(Paduano et al., 2011; Merckx, 2013; Gebauer et al., 2016; Schiebold et al., 2017; Li et al., 2021). Occurrences of Ascomycota have been sporadically noted in mixotrophic orchids like *Limodorum abortivum* and *Epipactis helleborine* (Girlanda et al., 2006; May et al., 2020), as well as in South African orchids (Waterman et al., 2011). Although it has been observed that mycoheterotrophic and myxotrophic orchids mainly associate with ECM fungi (Li et al., 2021), some partially and fully mycoheterotrophic plants may appear to be connected to rhizoctonias at first glance. For instance, *Ceratobasidium spp.* was identified in *Rhizanthella gardneri*, a mycoheterotrophic orchid (Bougoure et al., 2009), and in *Platanthera minor*, a mixotrophic orchid (Yagame et al., 2012). However, the *Ceratobasidium spp.* identified in these orchid species belong to a subclade that forms ECM, thus confirming the specific association of mycoheterotrophic/mixotrophic orchids with ECM fungi.

Finally, some mycoheterotrophic orchids have been found to associate not with ECM but with saprotrophic fungi, mostly wood decay fungi (Ogura-Tsujita et al., 2021). This fungal preference likely evolved independently at least nine times in the Orchidaceae, and involves well-established fungal genera such as *Mycena*, *Armillaria*, *Psathyrella*, *Coprinellus*.

In a recent preprint, Wang et al. (2022) provided a comprehensive summary of the ecological roles and phylogenetic distribution of fungi associated with orchid roots, combining into an integrated phylogenetic framework molecular data collected and produced on fungal taxa over the last years, mapping the diversity and distribution of the fungi at the order level (Wang et al., 2022). Using the DNA dataset of orchid symbionts obtained collecting in published literature fungal nuclear ribosomal internal transcribed spacer (ITS) accessions produced with Sanger or high-throughput sequencing (HTS) techniques, Wang et al. (2022) proposed a time-calibrated phylogenetic tree of the examined fungal taxa, linked to their ecological lifestyles (Fig. 5).

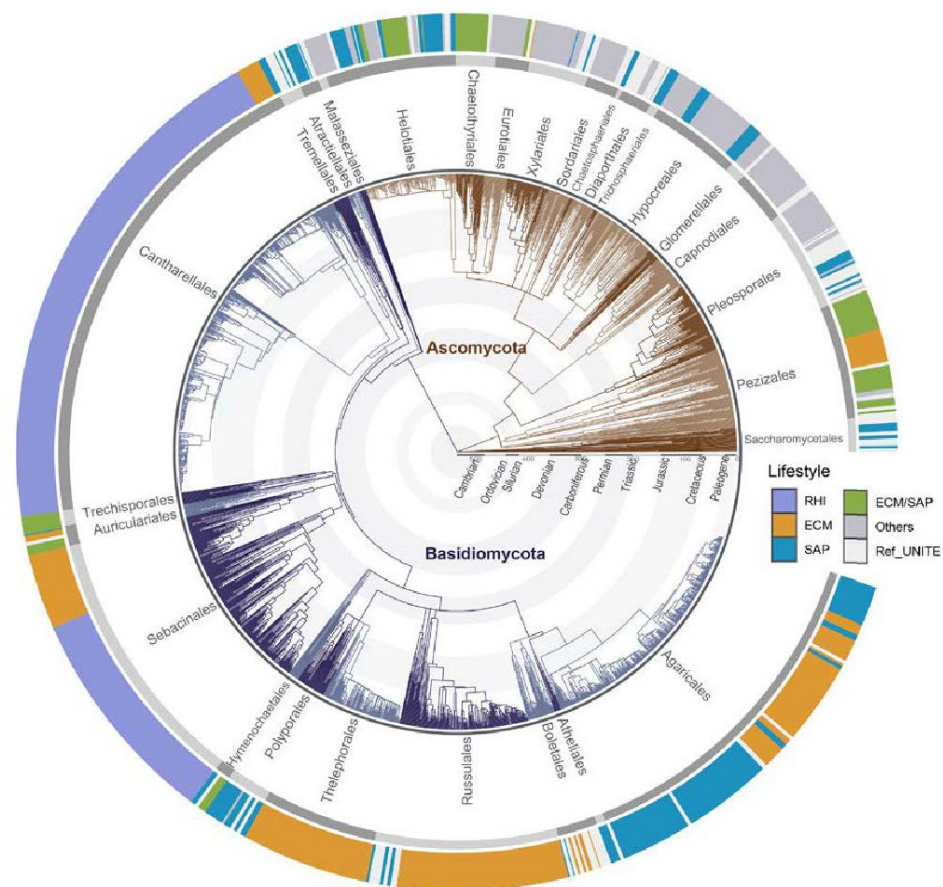


Figure 5. Phylogenetic tree of root-associated fungi from orchids, displaying lifestyle categories. The outer color ring indicates different fungal lifestyles: RHI (rhizoctonia-like) in purple, ECM (ectomycorrhizal) in orange, SAP (saprotrophic) in blue, ECM/SAP in green, and other lifestyles in gray. The reference sequences from the UNITE database were indicated in light gray. The proximity of various fungal orders is highlighted, with distinct branches representing Ascomycota (brown) and Basidiomycota (blue). The internal circle represents the geological time spans from the Cambrian to the present. This figure emphasizes the

evolutionary relationships and the functional diversity of fungal taxa involved in symbiotic and saprotrophic lifestyles (Wang et al., 2022).

Despite the diversity of ORM fungi, the formation of *pelotons*, the fungal structure typical of ORM, occurs independently of the fungal taxa involved, suggesting a primarily, if not entirely, plant-driven development of the microbial partner within the host plant cells (Perotto and Balestrini, 2024). For example, species in the genus *Russula* (Basidiomycetes) and *Tuber* (Ascomycetes) have been identified in ORM, where they form typical intracellular *pelotons*, despite also forming ectomycorrhizal symbioses with tree species (Selosse et al., 2004; Girlanda et al., 2006; Perotto and Balestrini, 2024).

1.4 Specificity and dynamics in orchid-fungus association

Typically, ORM fungi associate with their orchid hosts with a higher level of host specificity as compared to other mycorrhizal fungal guilds, like arbuscular mycorrhizal fungi (Polme et al., 2018). To assess this specificity can be measured by the breadth of the phylogenetic range of the ORM fungi associated with a given orchid

species, and represents a major challenge in research studies on ORM (Li et al., 2021).

According to Shefferson et al. (2020), host specificity in ORM can be classified into three types based on the level of specialization and ecological interactions: i) *assemblage specialization*, where ORM fungi are associated with specific orchid hosts and their interactions remain relatively unaffected by environmental factors; ii) *apparent generalism*, where ORM fungi are linked with a few orchid hosts and there is some correlation between their interactions and the environment; iii) *true generalism*, where ORM fungi are associated with multiple orchid hosts, and their interactions are significantly influenced by environmental factors (Shefferson et al., 2020).

Throughout the orchid life cycle, the communities of orchid associated ORM fungi can therefore display variability in their composition as a consequence of the dynamic co-regulation of environmental filtering, dispersal restrictions, spatiotemporal scales, biogeographic history, as well as the distribution, selection, and phylogenetic spectrum width of host orchids (Li et al., 2021).

Given the complexity of orchid biology and dependency of the plant on the ORM fungal partner, it is not surprising that the community of ORM fungi associated with orchid individuals can exhibit a strong

dynamic nature throughout all stages of the orchid life cycle (Cevallos et al., 2018).

The diversity of ORM fungi was found to decrease from early stages of mycoheterotrophic seeds and protocorms to seedlings, but then showed an increase during the adult stage, with an hourglass-like pattern (Li et al., 2021). ORM fungi identified in early germinating seedlings and adult plants typically originate from subsets of protocorms or seedlings (Bidartondo and Read, 2008; Waud et al., 2017). In *Dendrobium moniliforme*, Meng et al. (2019) observed a decrease in the diversity of cultivable *Tulasnella* species from early germination stages (six fungal species) to the appearance of the second leaf (two fungal species). By contrast, fully mycoheterotrophic orchids, which rely on ORM fungi as their sole source of carbon also at adult stages (Bidartondo, 2005; Jacquemyn and Merckx, 2019), tend to remain dependent on the same ORM fungi throughout their lives (Rasmussen, 2002; McCormick et al., 2012). An exception is *Gastrodia elata*, a mycoheterotrophic orchid associated with wood decay fungi, that requires symbiotic relationships with *Mycena* for seed germination and protocorm growth, and with *Armillaria* for plant growth and flowering (Liu et al., 2024)

Thus, the number and types of ORM fungi that associate with a particular orchid can vary widely, and this depends on several factors: the phylogenetic position of the orchid species (Shefferson et al. 2010; Jacquemyn et al. 2011; Martos et al. 2012), their growth stages, the environmental conditions and also their nutritional modes (Bidartondo and Read, 2008; Ogura-Tsujita et al., 2012; Jacquemyn et al., 2016). Orchid-fungus associations are therefore not static, but rather dynamic and influenced by various biotic and abiotic factors.

Partially and fully mycoheterotrophic orchids tend to primarily associate with fungi known to form ECM or with fungi that degrade wood and litter, whereas autotrophic orchids typically associate with rhizoctonia-like fungi (Wang et al., 2021). This preference for taxonomically and ecologically different ORM fungi suggests that shifts in trophic modes are likely linked to changes in fungal partners within the Orchidaceae family (Jacquemyn and Merckx, 2019). Detailed studies on Neottieae (Selosse et al., 2004; Selosse and Roy, 2009) and *Cymbidium* (Ogura-Tsujita et al., 2012) suggested an evolutionary relationship between orchid trophic modes and the type of ORM fungi they associate with. These studies indicate that transitions in orchid trophic modes often coincide with changes in the ORM fungal partners, particularly when orchids lose their ability

to photosynthesize. This flexibility in fungal partnerships may confer adaptive advantages, allowing orchids to exploit different nutrient sources and coping with shifts in the environmental conditions.

Thus, the remarkable diversity of OMF and the intricate dynamics of these symbiotic associations highlight the complexity of orchid biology. This underscores the importance of conserving not only the orchids themselves but also the intricate web of ecological interactions that sustain them (Jacquemyn et al., 2017).

1.5 The *in vitro* model system *Serapias vomeracea*-*Tulasnella*

The development of model systems to investigate the molecular bases of biological interactions between plants and fungi has proven to be instrumental to elucidate the mechanisms by which two partners recognize and respond to each other, as well as to identify key genes, proteins, and metabolites involved in the establishment and maintenance of the symbiotic interactions (Perotto et al., 2014; Valadares et al. 2014; Miura et al., 2018, Chen et al., 2020; Valadares et al. 2020; Miura et al., 2024). In orchids, the protocorm stage is of particular interest because all terrestrial orchid species are mycoheterotrophic during the early life stages, regardless of the

nutritional strategies they adopt as adults (Schiebold et al., 2017). The use of model system has greatly facilitated the exploration of the intricate molecular mechanisms that drive plant-microbe interactions, and lead to important discoveries on the signaling and nutritional exchanges occurring in other mycorrhizal symbiosis, such as arbuscular mycorrhiza (Harrison et al., 2002; Maillet et al., 2011; Lòpez-Ràez et al., 2011; Wang et al., 2017) and ECM (Plett et al., 2011; Casieri et al., 2013; Martin et al., 2016).

An *in vitro* model system was developed (Perotto et al., 2014; Ercole et al., 2015) to investigate plant and fungal gene expression in ORM, consisting of protocorms of the Mediterranean orchid *Serapias vomeracea* colonized by the mycorrhizal fungus *Tulasnella calospora* AL13 (Fig. 6). The sequencing of the complete genome of this fungal isolate (Kohler et al., 2015), together with post-genomics studies that included expression studies on target genes coupled with laser microdissection technology, transcriptomics and metabolomics, allowed a deeper understanding of these peculiar mycorrhizal interactions (Balestrini et al., 2014; Fochi et al., 2017a,b; Ghirardo et al., 2020).

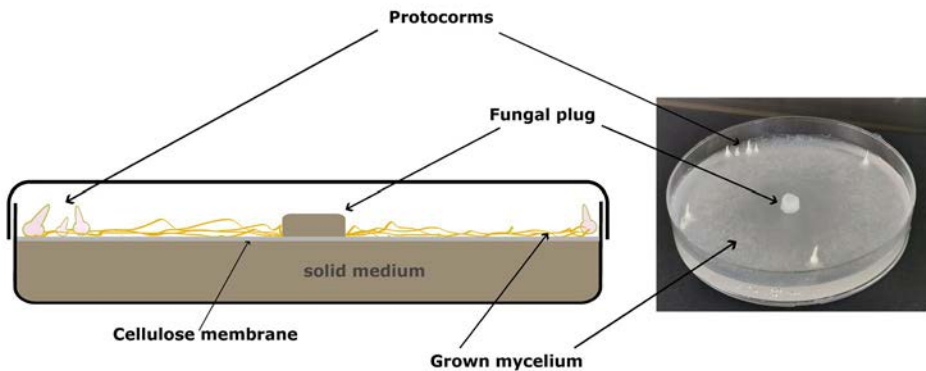


Figure 6. Representative scheme of *S. vomeracea* -*Tulasnella* sp. *in vitro* germination assay (modified from Ercole et al., 2015).

Due to a decreased symbiotic ability of the original *T. calospora* AL13 isolate, in the course of this thesis a new *Tulasnella* isolate (SV6) was used in combination with *S. vomeracea* as a model system. The *Tulasnella* sp. SV6 isolate is stored in the Mycotheca Universitatis Taurinensis (MUT) with the code MUT and proved to be effective in terms of *in vitro* mycorrhization and protocorm development.

1.6 Aims

In this thesis, I have addressed different aspects of the intricate interplay that occurs between the partners of ORM. In **Chapter 2**, I reviewed the recent advancement in the understanding of the

molecular mechanisms of plant-fungus interactions in the ORM symbiosis, as emerged from transcriptomics and metabolomics studies. **Chapter 3** provides novel insights into the nutritional framework in ORM, focusing on sulfur metabolism, through the use of ultra-high spatial resolution secondary ion mass spectrometry (SIMS), as well as a targeted gene expression approach (RT-qPCR) coupled with laser microdissection. **Chapter 4** is focused on the molecular responses of the ORM fungus in the early stages during the establishment of symbiotic interactions with *S. vomeracea* seeds *in vitro*, through transcriptomics and metabolomics. **Chapter 5** investigates the molecular changes in the fungal transcriptome and metabolome before physical contact with plantlets of *S. vomeracea* plants, in order to reveal possible signaling events. Finally, **the Appendix** describes the optimization of a DNA extraction protocol tailored for obtaining high-quality DNA to meet the requirements for Pac-Bio *de novo* sequencing, needed to sequence the genomes of *Tulasnella* sp. SV6 isolate, as well as new isolates of *T. helicospora* and *T. irregularis*.

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CHAPTER 2

Gene expression profiling in orchid mycorrhizae to decipher the molecular mechanisms of plant–fungus interactions

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1 Introduction

The Orchidaceae family comprises over 27,000 plant species (WFO 2022 <http://www.worldfloraonline.org/>) adapted to live in diverse environments, ranging from soil (terrestrial orchids), rock surfaces (lithophytic orchids) and on other plant species (epiphytic orchids) (Zhao et al. 2013).

Like 90% of plant species (Bonfante and Genre 2010), orchids form symbiotic associations with mycorrhizal fungi. From an ecological point of view, the study of orchid mycorrhizal (OM) symbiosis is pivotal since many orchid species are rare or at risk of extinction due to habitat destruction and over-harvesting. The presence of compatible OM fungi, necessary for seed development and plant growth, is therefore extremely crucial for the survival of plants in nature, as well as for ex situ horticultural growth. Furthermore, symbiotic germination from seeds may favor genetic variability compared to monocultures created by asexual propagation (Fig. 1) (Dearnaley et al. 2016).

In most mycorrhizal symbioses, the fungus provides inorganic nutrients in exchange for fixed carbon (C) from the photosynthetic host plant, which achieves several beneficial effects from this association (Genre et al. 2020). However, the OM symbiosis appears to be an unusual association because the usual mechanism of mycorrhizal nutrient exchange is reversed, at least during early development (Dearnaley and Cameron 2016); under natural conditions, orchids are entirely dependent on their associated symbiotic fungi for the supply of carbon and other

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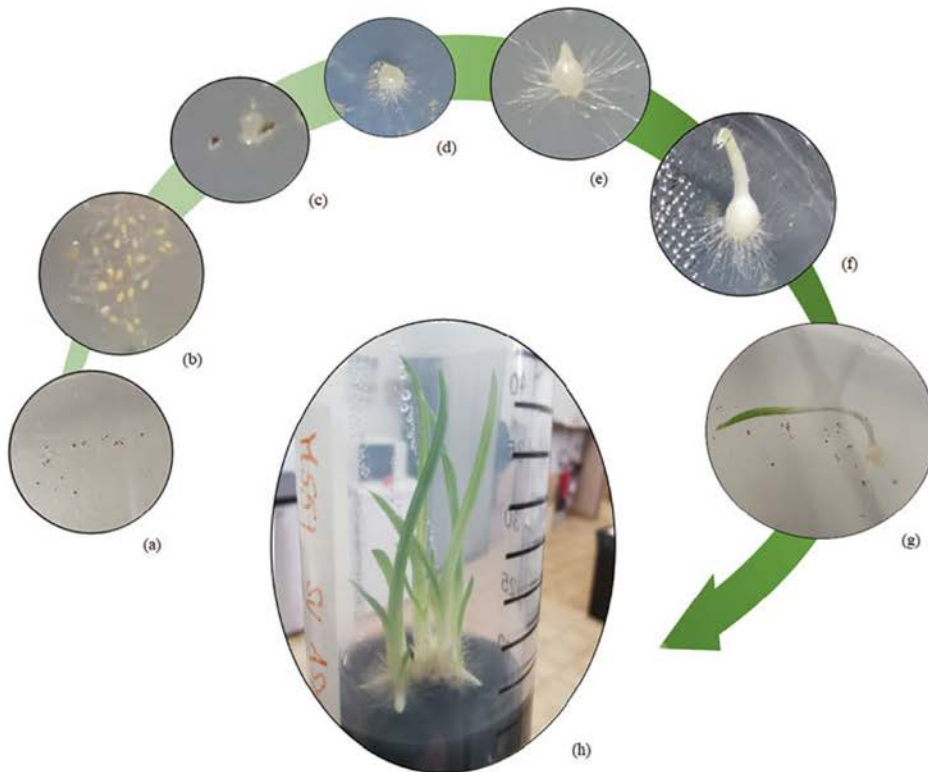


Fig. 1 In vitro development stages from seeds into the adult stage of *Serapias vomeracea*. (a, b) seeds seen under a stereomicroscope; (c) swollen seed; (d, e) protocorms; (f) protocorm with pre-leaf; (g) seedling; (h) adult plant

nutrients, particularly during seed germination and early plant development, apparently without getting anything in return (Selosse and Roy 2009). The plant dependency on the associated fungus is maintained throughout the orchid life cycle in achlorophyllous species. This peculiar trophic strategy, whereby orchids obtain carbon from their mycorrhizal fungi, instead of supplying carbon to their partner, is called mycoheterotrophy (Dearnaley et al. 2016).

The advent of *-omics* approaches, particularly transcriptomics, has contributed to elucidating the molecular mechanisms of this intriguing symbiosis and to providing insights into the nutrient exchanges between orchids and their associated fungi, thus helping to dissect this paradigm. By reflecting the gene expression changes during the development of organisms, as well as under the effects of biotic and abiotic factors, the transcriptome allows filling the gap between the genome and its phenotype at a particular time (Stark et al. 2019). Next-generation sequencing (NGS) approaches have now become widely available, thus providing the opportunity to explore differential gene expression at great resolution by the sequencing of whole transcriptomes, i.e., RNA-sequencing (RNA-seq; Marconi et al. 2014). RNA-seq approaches make it possible to investigate the extreme complexity of cellular life in

the round, redefining the fields of investigation that can be further explored by integrating with other *-omics* techniques (Lowe et al. 2017) or with target tools such as the use of laser microdissection (LM) technology (Balestrini et al. 2009).

In the next sections, the contributions that transcriptomics has made to the broad understanding of the mechanisms that drive this intriguing type of symbiosis, with reference to nutrient exchange, fungal genes and plant responses involved in the establishment of this association, are introduced and discussed.

2 Transcriptomics to Understand Nutrient Exchanges between Orchids and their Symbionts

As mentioned above, OM fungi play a key role during orchid seed germination and plant development during the early stages. Orchid seeds are often small (0.3–14 μg) and the embryos have limited nutrient reserves, which mainly consist of protein and lipids (Arditti and Ghani 2013; Zhao et al. 2013; Dearnaley et al. 2016). Germination of these tiny seeds requires the interaction with a compatible OM fungal species/isolate, which forms elaborate intracellular hyphal coils (Smith and Read 2008), called “*pelotons*,” responsible for nutrient exchanges between symbionts. The subsequent orchid developmental stages include the formation of tuber-like heterotrophic structures lacking chlorophyll, defined as protocorms (Smith and Read 2008). In the adult stage, orchids can develop different trophic strategies: most species become autotrophic, with green leaves and photosynthetic capacity (Dearnaley 2007), but around 100 species are reported as achlorophyllous and therefore completely dependent on the fungal symbiont for organic carbon (C), i.e., mycoheterotrophic (Selosse and Roy 2009; Hynson et al. 2013). Mixotrophy is an interesting evolutionary intermediate between the first two strategies, where the plant takes advantage of the fungal supply of organic C while retaining photosynthetic capacity (Julou et al. 2005; Gebauer et al. 2016). Mixotrophy allows strong adaptation under shady environments or in situations of reduced photosynthetic capacity (Girlanda et al. 2006; Lallemand et al. 2019).

Early studies on nutrient exchange based on experiments with isotope tracing showed that the OM fungus is responsible for the supply of C, phosphorus (P), and nitrogen (N) to protocorms and that the supply of P and N continues in seedlings and adulthood plants (Cameron et al. 2006, 2007, 2008). By performing a high-resolution secondary ion mass spectrometry, nutrient transfer through peloton lysis in the obligate mycoheterotrophic orchid *Rhizanthella gardneri* has been observed (Bougoure et al. 2014). In mycorrhizal protocorms of *Spiranthes sinensis*, the use of imaging of stable isotope tracers at the cellular level also demonstrated that C and N are translocated from the mycorrhizal fungus to the orchid cell either through intact pelotons or through the release of hyphal cytoplasm during peloton degradation (Kuga et al. 2014).

In the last years, several nutrient transporter genes have been detected and characterized in OM by transcriptomics (Perotto et al. 2014; Zhao et al. 2014; Fochi et al. 2017a, b), thus supporting the hypothesis that an active nutrient exchange takes place at the plant–fungal interface. Investigations have been mainly focused on symbiotic protocorms obtained *in vitro*, but recent work also considered the symbiosis in the roots of adult plants (Valadares et al. 2020, 2021). Before the advent of RNA-seq, methods relying on PCR-based amplification of cDNA fragments that differ from the control, like suppression subtractive hybridization (SSH), led to the identification of various genes involved in transport processes in the orchid *Dendrobium officinale* colonized by a *Sebacina* sp. fungus, including a cation transporter of the plant and an inorganic phosphate transporter of the fungus (Zhao et al. 2013).

By using RNA-seq technology, the transcriptomic responses of *Cymbidium hybridum* plantlets co-cultivated with two different beneficial fungi, one of them non-mycorrhizal, were investigated (Zhao et al. 2014). Among the different genes involved in nutrient transport, two plant phosphate transporters, co-regulated during interactions with both fungal species, were identified (Zhao et al. 2014). Two genes coding for phosphate transporters expressed in mycorrhizal roots of the adult green orchid *Oececlades maculata* collected in natural conditions were also recently identified (Valadares et al. 2020). These results provide evidence that the acquisition of inorganic phosphorus in adult plants is mediated by the associated fungus, as suggested for the terrestrial orchid *Goodyera repens* based on isotope studies (Cameron et al. 2007). In soils with limited P availability, naturally occurring orchids have been found to acquire significant amounts of inorganic P from the symbiotic fungal partners; plant–fungus combinations, which may be more or less efficient, strongly influence P acquisition, with plant-mediated niche differentiation (Davis et al. 2022).

Bidirectional transfer of C between a green adult orchid and its fungal symbiont has been demonstrated by isotope tracing experiments, thus allowing a more complete view of C fluxes in OM symbiosis (Cameron et al. 2006). Interestingly, an up-regulated putative bidirectional sugar plant transporter belonging to the SWEET family has been identified by high-throughput transcriptomics of a normalized cDNA library by 454 GS-FLX Titanium pyrosequencing in *Serapias vomeracea* protocorms colonized *in vitro* by the fungus *Tulasnella calospora* (Perotto et al. 2014). Pathogenic microorganisms and beneficial symbionts are both known to target plant SWEET transporters for nutritional gain (Chen et al. 2010). SWEET transporters were also identified in mycorrhizal roots of albino variants of *Epipactis helleborine*, a mixotrophic orchid (Suetsugu et al. 2017); in symbiotic protocorms of *Bletilla striata* (Miura et al. 2018) and mycorrhizal roots of adult orchids *O. maculata* and *Limodorum abortivum* (Valadares et al. 2020, 2021) both collected in nature. Intriguingly, a sucrose transporter that may allow sucrose import at the symbiotic interface in mycoheterotrophic *Gastrodia elata* associated with the fungus *Armillaria* has been found (Ho et al. 2021). Moreover, invertase, an enzyme cleaving sucrose into glucose and fructose, has been identified, through proteomic

approaches, in mycorrhizal protocorms of the orchid *Oncidium sphacelatum* (Valadares et al. 2014).

Most orchid tissues are highly N-enriched (Hynson et al. 2013,) and the fungus has been demonstrated to provide N to protocorms and adult green orchids by exploiting inorganic and organic N sources in the substrate (Kuga et al. 2014; Cameron et al. 2006). The exchange of N in OM has been clarified thanks to transcriptomics approaches. RNA-seq analysis of the plant and fungal N uptake pathways in the model system *S. vomeracea*-*T. calospora* identified several up-regulated plant and fungal genes associated with N metabolism (Fochi et al. 2017a). To understand the preferential N form taken up by the fungus and transferred to the orchid protocorm, the fungal mycelium was grown on two different N sources. Based on transcriptomic and genomic data, it has been hypothesized that the fungus can obtain N from organic and inorganic sources, excluding nitrate, and two ammonium fungal transporters were identified, one of which was up-regulated in symbiosis. Plant transporters for N compounds resulted to be up-regulated in symbiosis, such as ammonium and oligopeptide transporters, as well as amino acid transporters, including a plant lysine histidine transporter (LHT1). A homologous LHT1 gene was previously reported in mycorrhizal roots of *Cymbidium hybridum* (Zhao et al. 2014) and recently detected in symbiotic protocorms of *B. striata* (Miura et al. 2018) and symbiotic roots of adult orchid plants (Valadares et al. 2021). This repertoire of fungal and plant genes was further investigated through the use of laser microdissection (Balestrini et al. 2018; Fochi et al. 2017b). By combining a microscope and a computer-assisted laser beam to separate various cellular components from sections placed under a microscope slide, laser microdissection enables the quick separation of specific cells from a piece of heterogeneous tissue making it possible to extract a variety of cellular compounds, including RNA (Balestrini et al. 2009, Balestrini and Fiorilli 2020). The analysis of gene expression in RNA samples originating from orchid cells harboring fungal coils at diverse developmental stages, as well as cells non-colonized by the fungus, demonstrated that plant genes coding for transporters of N compounds are differentially expressed in symbiosis (Fochi et al. 2017b). Based on these findings, it has been hypothesized that N-rich amino acids may be transferred from the fungus to the host plant, also contributing to the C requirement. Moreover, in addition to active transport, recovery of organic N forms from peloton lysis may occur (Kuga et al. 2014). Recent transcriptomic and proteomic studies focused on orchid species collected in nature support this hypothesis (Valadares et al. 2021). For example, in the mycorrhizal roots of the orchid *L. abortivum*, genes encoding a lysine histidine transporter 1 (LHT1) and an amino acid permease, as well as several NRT1/PTR family members putatively associated with N transfer from the fungus to the host plant, were found to be up-regulated (Valadares et al. 2021). Similarly, it has been discovered that the mycorrhizal roots of the terrestrial orchid *O. maculata* regulates genes for LHT1 and NRT1/PTR family members, which are amino acid transporters (Valadares et al. 2021). Interestingly, members of this protein group have also been discovered to transport peptides, but also chloride, nitrite, glucosinolates, and several phytohormones such as auxin, abscisic acid, gibberellins, and jasmonate

(Corratgé-Faillie and Lacombe 2017). However, their roles in mycorrhizal symbiosis are still under debate (Corratgé-Faillie and Lacombe 2017).

A recent metabolomic study using the model system *S. vomeracea*—*T. calospora* integrated previous transcriptomic data (Fochi et al. 2017a, b) and showed that the external mycelium of the mycorrhizal fungus freely growing close to the host protocorms affected several metabolic pathways. The interaction between plant and fungus increased compounds associated with structural, signaling, and energy, mostly lipids, particularly glycerolipids (GP) and sphingolipids (SP) (Ghirardo et al. 2020). Lipids are known to be the main structural components of cell membranes but also provide other important biological functions, ranging from signaling, C storage, plant–microbe interactions, and even response to environmental stresses (Ghirardo et al. 2020). Notably, a percentage decrement of N- and S-containing compounds in the mycorrhizal fungus growing close to the host protocorms, led the authors to hypothesize that this depletion may mirror a transfer of N compounds to the host plant. However, among the identified S-containing compounds, the amount of S-adenosylmethionine (SAM) increased in the mycelium surrounding protocorms (Ghirardo et al. 2020). This molecule is used by methyltransferases as a methyl group donor for a variety of target substrates (Mato et al. 1997; Ghirardo et al. 2020). Currently, scarce information on S transfer from fungi to the host plant in OM is available. This nutrient is essential for plant growth and development, as a constituent of amino acids such as cysteine and methionine and sulfated peptides (i.e., glutathione or phytosulfokines) (Kopriva et al. 2019). Very recently, experiments with labeled S, N, and C showed that these elements could be translocated from the substrate to the protocorm cells via the fungal hyphae (De Rose et al., submitted), corroborated by target transcriptomic data which showed up-regulation of several plants and fungal transporter genes, as well as genes related to S assimilation enzymes involved in movement and redistribution of S in the cell. Overall, these findings support the hypothesis of transfer during symbiosis of S in a reduced organic form that also contains N, such as S-amino acids or small peptides, including glutathione (De Rose et al., submitted).

Based on the reports so far available, the model originally proposed for nutrient transport in OM (Dearnaley and Cameron 2016) can be strengthened and integrated with transcriptomic data. In non-photosynthetic stages, orchids may receive inorganic P, C, N, and S from the symbiotic fungal partner in the form of amino acids, and export ammonium in exchange (Cameron et al. 2006, 2007; Kuga et al. 2014; Fochi et al. 2017a, b). This exchange takes place across intact membranes (Kuga et al. 2014; Fochi et al. 2017b), but the lysis of senescent fungal pelotons may also release C, N, and P (Bougoure et al. 2014). Significant metabolic (particularly lipid-related) changes in the mycelium outside the plant could also participate in the nutrient supply to the host plant (Ghirardo et al. 2020). In photosynthetic orchids, C as sugars could be exported from the plant to the fungus, while inorganic P continues to be received by the mycorrhizal partner (Cameron et al. 2006; Valadares et al. 2020, 2021) in amounts that can vary, even greater, depending on plant-fungus compatibility (Davis et al. 2022). The flow of nutrients in the orchid mycorrhizal symbiosis may be even more complex than presented when considering the hyphal

interconnections that may exist in natural ecosystems, well investigated for mycoheterotrophic orchids, and the fungal diversity in orchid roots (extensively reviewed by Yeh et al. 2019). An example is the flow of C between the tree species *Salix repens* and *Betula pendula* with the orchid *Corallorhiza trifida* by hyphal network shared by the plants (McKendrick et al. 2000), or the coexistence of different endosymbionts in *Cymbidium hybridum* (Zhao et al. 2014).

3 Transcriptomics to Decipher the Mechanisms Involved in the Establishment of Symbiosis

Fungi that form mycorrhizal associations with orchids exhibit great phylogenetical and ecological diversity (McCormick et al. 2018). Orchids are known to associate with a plethora of fungi, including ectomycorrhizal basidiomycetes and ascomycetes wood degraders and other saprotrophic basidiomycetes (Bidartondo et al. 2004; Selosse et al. 2004; Dearnaley 2007; Ogura-Tsujita and Yukawa 2008; Martos et al. 2009; Kottke et al. 2010; Lee et al. 2015; Kinoshita et al. 2016). The most common taxa in photosynthetic orchid species include *Tulasnella*, *Ceratobasidium*, and *Serendipita* (Dearnaley et al. 2012).

The diversity of OM fungi in the soil may be a crucial element in determining the distribution and future of orchids (McCormick et al. 2018) because orchids in nature are entirely dependent on OM fungi at least for seed germination and early stages of development. After initial contact and seed germination, protocorms are colonized by OM fungi; the hyphae penetrate parenchyma cells, branch and fold to create thick hyphal coils (the pelotons) that at last degrade (Miura et al. 2018). The symbiotic germination of orchid seeds requires the coordinated expression of numerous functional genes as well as a crosstalk between genes associated with the mycorrhizal establishment and the germination process (Liu et al. 2015; Chen et al. 2020). According to Evangelisti et al. (2014), plant hormones may act as a node in the crosstalk between plant development and plant–microbe interactions. Strigolactones (SLs), for instance, are signaling molecules produced by plant roots either constitutively (Akiyama et al. 2005) or in response to low phosphorus levels (Kretzschmar et al. 2012) capable of recruiting arbuscular mycorrhizal (AM) fungi and promoting hyphal branching. In orchids, a carotenoid cleavage dioxygenase, involved in the strigolactones biosynthetic pathway, has been identified in a proteomic study in *O. sphecelatum* mycorrhizal protocorms by Valadares et al. (2014). Since the enzyme was more expressed in earlier stages of mycorrhizal protocorm development, it has been hypothesized that SLs may have essential early functions in luring compatible fungal symbionts to aid orchid seeds germination (Valadares et al. 2014).

The crosstalk of jasmonic acid (JA), abscisic acid (ABA), and SLs were investigated in *D. officinale* seeds colonized by *Tulasnella sp.* during the germination phase (Wang et al. 2018). The transcriptomic and RT-qPCR data, combined with the quantification of endogenous phytohormones, suggested that the OM fungus had a

role in hormone production (Wang et al. 2018). Additionally, endogenous JA, ABA, or SLs levels were maintained low to promote the formation of the *D. officinale*-*Tulasnella* protocorm-like structures (Wang et al. 2018). However, the great phylogenetic and ecological diversity of OM fungi might suggest that other signals are involved. A comparative analysis of gene expression in asymbiotic and symbiotic *Anoectochilus roxburghii* seeds through Illumina HiSeq 4000 transcriptome sequencing allowed focus on the regulatory module GA-GID1-DELLA (Liu et al. 2015). Gibberellins (GAs) are plant hormones that play key roles in growth and development (Wang and Deng 2014). The GID1 receptor and the DELLA repressor were found to be critical in the regulation of seed germination (Wang and Deng 2014). ABA, another important phytohormone, was found instead to inhibit the process through a finely tuned crosstalk (Liu et al. 2015). Among the differentially expressed plant transcripts identified in symbiotic and asymbiotic seeds, two transcripts coding for gibberellin 20 oxidase (GA20ox), two transcripts coding for gibberellin 2-oxidase (GA2ox) involved in GAs biosynthesis, and two transcripts coding for DELLA proteins, members of GRAS superfamily (Hernández-García et al. 2021), were established as common elements of the mycorrhizal signaling pathway (Jin et al. 2016). This study suggested that OM fungi could modulate the expression of these plant genes, possibly affecting the entire GA-GID1-DELLA regulatory module.

Investigations focused on the impact of GAs on symbiotic seed germination in the model system represented by *D. officinale* and *Tulasnella* sp. were also performed (Chen et al. 2020). Levels of endogenous gibberellic acid (GA3) using liquid chromatography-mass spectrometry (LC-MS/MS) were determined during symbiotic and asymbiotic germination of orchid seeds, and a significantly higher ratio between GA3 and ABA was found in symbiosis (Chen et al. 2020). Phenotypic and target gene expression investigations were conducted on the germination of seeds treated with various concentrations of exogenous GA3, showing a negative effect of high concentrations of GA3 on fungal colonization. These findings were combined with data obtained from RNA-seq and proteomic analyses that highlighted a significantly higher expression of an ABA receptor protein, PYR1, during the early stages of symbiotic germination in *D. officinale* (Chen et al. 2017). The expression profile of genes involved in GAs and ABA biosynthesis, identified in the transcriptomic data, and of genes reported to be part of the recognized common symbiotic pathway (including a calcium-binding protein and a calcium-dependent protein kinase), showed a fine-tuned regulation under the different GA treatments (Chen et al. 2020). These results suggest that an interplay between GAs metabolism and the establishment of symbiosis may occur in orchids (Chen et al. 2017, 2020).

A group of common symbiosis genes (CSG) have been identified in angiosperms forming arbuscular mycorrhizal (AM) symbiosis, reported to act in the signaling pathway for recognizing and transducing microbial signals that are diffuse throughout root colonization and nutrient exchange (Stougaard 2001; Kistner et al. 2005; Genre and Russo 2016). In contrast, angiosperms unable to form mycorrhizal associations with AM fungi, such as members of Brassicaceae, showed no functional or only a few members of CSG (Delaux et al. 2014). Members of the Pinaceae in the

gymnosperms, forming ectomycorrhiza, were also lacking CSG in their genomes (Garcia et al. 2015).

To explore the possible presence of CSG in orchids, protocorms of *B. striata* colonized by the mycorrhizal fungal taxa *Tulasnella* sp. were analyzed at different stages by transcriptomics (Miura et al. 2018). Notably, all of the CSG characterized in other plant species were found in the *B. striata* transcriptome (Miura et al. 2018). In vivo assay by functional complementation of *L. japonicus* CCaMK mutant through *B. striata* CCaMK gene showed that this gene complemented the function of LjCCaMK (Miura et al. 2018). Moreover, eight genes were strongly induced during symbiosis (Miura et al. 2018). The high similarity of these genes to the AM marker genes in rice (Gutjahr et al. 2008), as well as their strong induction during the plant–fungus interaction, allow considering this set of eight genes as marker genes also for OM (Miura et al. 2018).

A large-scale analysis of more than 250 transcriptomes and about 100 plant genomes, encompassing the whole land-plant diversity, demonstrated that a shared symbiosis signaling pathway occurred in all plants forming intracellular endosymbioses (Radhakrishnan et al. 2020). It is worth noting that co-evolution between plants and fungi began approximately 400 million years ago and that four mycorrhizal types evolved at different times: arbuscular mycorrhiza, orchid mycorrhiza, ericoid mycorrhiza, and ectomycorrhiza (Genre et al. 2020). The first three types are endosymbioses, i.e., fungal symbionts are harbored intracellularly inside plant cells. In particular, AM symbiosis is probably the most ancient plant–fungus symbiosis (Delaux et al. 2013) from which OM symbiosis appeared to be derived after plant diversification (Radhakrishnan et al. 2020). Comparative transcriptomic studies allowed us to identify six genes lost in non-mutualistic plant taxa: CcaMK, calcium- and calmodulin-dependent protein kinase, SymRK, a receptor-like kinase, CYCLOPS and RAD1, two transcription factors, and two transporters STR and STR2 showing a half-ATP-binding cassette (ABC). In particular, the first three genes were conserved in all plants forming intracellular endosymbiosis, while *STR*, *STR2*, and *RAD1* were supposed to be specific to AM (Radhakrishnan et al. 2020).

Starting from the earliest observations (Burgeff 1932; Burges 1939), OM has been often argued to represent a balanced antagonism between plant and fungus, since it has been documented that fungi occasionally might destroy the protocorm (Adamo et al. 2020) and because plant receives C from the fungus lacking a clear reward. However, large-scale transcriptomic data integrated with target gene expression analysis with RT-qPCR allowed to demonstrate the absence of a defense activation in *S. vomeracea* mycorrhizal protocorms (Perotto et al. 2014), as previously observed (Zhao et al. 2013). The OM symbiosis was suggested as “a friendly plant–fungus relationship” and the lack of a strong defense response was successfully confirmed by other transcriptomic studies in other orchid species (Suetsugu et al. 2017; Miura et al. 2018). Using 454 pyrosequencing, Perotto et al. (2014) proposed a nodulin-like gene called *SVNod1* as a marker of OM symbiosis (Perotto et al. 2014). In this transcriptomic study, a set of plant and fungal genes expressed in *S. vomeracea* protocorms colonized by OM fungus *T. calospora* was identified

(Perotto et al. 2014). In-depth gene expression analysis using an extremely efficient target approach, laser microdissection, tested the expression of several genes identified in the work by Perotto et al. (2014), confirming the results and suggesting *SvNod1* as a marker gene of orchid symbiosis, since its transcript was detected in the fungal colonized cells only (Perotto et al. 2014; Balestrini and Bonfante 2014).

An important process for endosymbiosis is clathrin-mediated endocytosis (Leborgne-Castel et al. 2010). The plant plasma membrane plays a pivotal role in the management of microbial interactions, as it senses and possibly allows the entry of endocellular symbionts or microbial substances, and endocytosis can regulate the entry of extracellular particles or cargoes into the cell (Zeng et al. 2017). The molecular components of this process have been investigated by transcriptomics in the peculiar OM system represented by the orchid *Gastrodia elata*, a fully mycoheterotrophic orchid able to establish a symbiosis with two genera of fungal partners, i.e., *Mycena* and *Armillaria*. *Mycena* species are known to interact with *Gastrodia* as symbionts during the early stages of plant development, including protocorm formation (Kim et al. 2006; Zeng et al. 2017). The transcriptomes of *G. elata* symbiotic seeds and protocorms were analyzed by RNA-seq and among the differentially expressed genes identified in the study, genes putatively linked to energy metabolism, plant defense, molecular signaling, and secondary metabolism were detected (Zeng et al. 2017). Genes coding for clathrin, an adaptor protein, dynamin, and HSC70, were found to be constitutively expressed in seeds but strongly expressed in protocorms, indicating that endocytosis mediated by clathrin may be crucial in *G. elata* during interactions with *Mycena* fungi (Zeng et al. 2017). Comparative transcriptome analysis was also used to unravel molecular mechanisms underlying gastrodin biosynthesis since this compound has been reported to have several positive effects on human health (Tsai et al. 2016). Two putative monooxygenases and one glycosyltransferase key enzymes involved in gastrodin biosynthesis were identified, which could be a target of genetic editing to improve gastrodin production (Tsai et al. 2016).

In OM symbiosis, the fungal hyphae must enter the cell walls of the epidermal cells to reach the internal parenchyma cells during the colonization of orchid seeds, protocorms, or roots (Chen et al. 2014; Favre-Godal et al. 2020). The plant cell wall is therefore the first physical structure where interactions between the host plants and the associated symbiotic partners take place (Balestrini and Bonfante 2014). Recently, Chen et al. (2022) compared transcriptomic profiles during seed germination, protocorm formation, and seedling development under symbiotic and asymbiotic conditions to further investigate changes in the expression of plant genes related to plant cell wall biosynthesis, structure modification, and the expression pattern of fungal genes related to plant/fungal cell wall degradation (i.e., CAZymes) (Chen et al. 2022). After being inoculated with two symbiotic fungi (*Tulasnella* sp. and *Serendipita* sp.), *D. officinale* seeds showed significantly increased expression of genes coding for secreted glycoproteins specifically associated with the epidermis, proline-rich receptor-like proteins, leucine-rich repeat (LRR) extensin-like proteins, and extensin-like proteins during the symbiotic stage. Extensins are a large class of hydroxyproline-rich glycoproteins that play a

variety of roles in plant defense, such as reinforcing the cell wall to preclude invasion by pathogens or to facilitate the interaction with symbiotic organisms (Chen et al. 2022). They were also probably essential for preventing fungal colonization of basal cells and spreading inside the whole protocorms (Li et al. 2018). The observed up-regulation of a microtubule-associated protein gene during the symbiotic stage suggested that cytoskeletal remodeling took place during the fungal colonization of orchid seeds (Chen et al. 2022). In addition, some genes involved in plant cell wall biosynthesis, including genes coding for cellulose synthase and pectin esterase, were significantly up-regulated in seeds of *D. officinale* inoculated with both fungal species, suggesting that the interaction between seeds and mycorrhizal fungi was particularly active in terms of modification in plant cell wall biosynthesis pathways (Chen et al. 2022). In the symbiotic fungal mycelium, several differentially expressed CAZymes were found, representing approximately 24.8% and 36.7% of the total number of CAZymes identified in *Serendipita* sp. and *Tulasnella* sp. genomes, respectively (Chen et al. 2022). These genes were hypothesized to play a role in the suppression of the plant defense responses, the identification of mycorrhizal fungi during the germination of orchid seeds, and also in the successful fungal colonization (Chen et al. 2022).

As mentioned earlier, OM fungi sometimes display a saprotrophic behavior that leads to disruption of the mycorrhizal association, and recent research by Adamo et al. (2020) focused on possible changes, during mycorrhizal symbiosis and saprotrophic growth, in the expression of fungal genes encoding CAZymes capable of breaking down the plant cell wall (PCW). They found that PCW-degrading enzymes are finely regulated in *T. calospora* during mycorrhizal and saprotrophic growth in the host and hypothesized that the expression of several important CAZymes was connected to OM fungal transitions from symbiotic to saprotrophic growth (Adamo et al. 2020).

Although most studies on OM have been focused on germinating seeds and protocorms, *omics* approaches are also revealing molecular mechanisms and actors involved in the adult stages of orchids. In comparison to protocorms, the interaction in adult plants is expected to be different because of it involves the root, an anatomically and metabolically complex plant organ (Valadares et al. 2020). Two recent studies have shed light on the mechanisms driving plant–fungus interactions in adult orchids in natural conditions (Valadares et al. 2020, 2021). The interaction between adult plants of the terrestrial orchid *O. maculata* and its mycorrhizal fungus *Psathyrella candolleana* (Basidiomycota) was analyzed using transcriptomic and proteomic analyses (Valadares et al. 2020).

In nature, the same individual of *O. maculata* can form older roots completely colonized by OM fungi as well as younger non-colonized roots (Valadares et al. 2020). For this reason, *O. maculata* is an excellent experimental system allowing the study of the molecular changes related to OM formation and functioning since it can be used to analyze both mycorrhizal and non-mycorrhizal roots from the same plant. Integration of transcriptomic and proteomic data showed that a chitinase and a mannose-binding lectin, two proteins involved in plant defense responses, decreased in mycorrhizal roots, suggesting that the proximity of the fungal symbiont might

cause a local decrease of plant defense responses in the orchid tissues (Valadares et al. 2020). Results also highlighted that allene oxide synthase transcripts, precursors involved in the biosynthesis of JA, were only found in non-mycorrhizal roots of *O. maculata*, while genes annotated as 9-lipoxygenase and allene oxide synthase were negatively regulated in mycorrhizal roots (Valadares et al. 2020). Based on these findings, the authors suggested that inhibition of JA production is probably needed to promote fungal colonization and OM formation (Valadares et al. 2020). In addition, the up-regulation of three ethylene-induced calmodulin genes and 15 ethylene-responsive transcription factors in the transcriptome of *O. maculata* mycorrhizal roots suggested that the activation of the ethylene pathways plays a role in OM (Valadares et al. 2020). Similar outcomes were also reported in *C. hybridum* mycorrhizal roots (Zhao et al. 2014).

To further explore the mycorrhizal interactions in adult orchids in natural conditions at the molecular level, a transcriptomic approach has been used to examine gene expression in roots of the mixotrophic orchid *Limodorum abortivum*, able to associate with ectomycorrhizal fungi of the taxon *Russula* (Valadares et al. 2021). This study, which focused on how the plant responds to the mycorrhizal symbiont(s) and used non-sterile non-mycorrhizal roots collected in nature as references, addressed for the first time OM interactions in an orchid species interacting with an ectomycorrhizal fungus. The comparison between non-sterile mycorrhizal and non-mycorrhizal roots made it simpler to distinguish between the general orchid responses to microbes and the mycorrhiza-specific plant responses. A shared core of plant genes engaged in endomycorrhizal symbioses already identified in arbuscular mycorrhiza was identified in *L. abortivum* and mirrored by the overexpression of several molecular marker genes for symbiosis in mycorrhizal roots. Further studies and gene characterization are needed to determine whether the unique characteristics of OM depend on the precise regulation of these elements, or if additional genes are involved in the process (Valadares et al. 2021). Among the genes differentially expressed *in planta*, pectin methyl esterase (PME) genes were detected to be significantly down-regulated in *L. abortivum*, while PME inhibitor genes were up-regulated in mycorrhizal roots, demonstrating that the main proportion of pectin is in a highly methylated state in the outer cell wall and/or symbiotic interface (Valadares et al. 2021). The high expression of two expansin coding genes in mycorrhizal roots further supports the hypothesis that a loosening of the cell wall during symbiosis may occur (Valadares et al. 2021).

The transcriptome of *L. abortivum* mycorrhizal roots also showed a significant up-regulation of subtilisin-like serine protease-coding genes. The major part of subtilisin-like serine proteases is primarily directed to the plant cell wall, in which they can play a role in the regulation of the structural remodeling of the cell wall (Schaller et al. 2018). Additionally, two genes in mycorrhizal *L. abortivum* roots coding for syntaxin-132 (SYP132) proteins were found to be up-regulated, and thus it has also been documented in mycorrhizal roots of other orchid species (Zhao et al. 2014; Valadares et al. 2020). Syntaxins have been characterized in the AM symbiosis and the SYP132 α isoform has been demonstrated by knockdown mutant experiments to be needed for arbuscule formation in the model plant *Medicago*

truncatula (Huisman et al. 2020). It has been hypothesized that SYP132, which is localized on the perisymbiotic plant membrane surrounding functional arbuscules, is essential for the development of a functional plant–fungus interface (Huisman et al. 2020). Even though it is currently unknown where the SYP132 proteins are located in OM roots, it is intriguing to hypothesize that the AM and OM symbioses share a similar exocytotic route (Valadares et al. 2021).

4 Conclusion

In the last decades, thanks to advances in technology applied to transcriptomics, the understanding of how orchids interact with their symbiotic partners has been strongly improved. The application of RNA-seq in several orchid species has allowed in-depth analyses of the molecular bases of nutrient transfer between OM fungi and their orchid hosts, as well as the identification of fungal and orchid genes involved in the establishment of the symbiotic association. The current availability of annotated orchid and fungal transcriptomes will help to fill the gap between the genomic data and the phenotypic observations, also in natural conditions. From the plant side, a broad transcriptomics resource for orchid species has been developed, named Orchidstra 2.0 database (<http://orchidstra2.abrc.sinica.edu.tw>), including data from EST libraries and RNA-seq of 18 species from the five major subfamilies of the Orchidaceae (Chao et al. 2017). This tool has already proven to be useful for the comparison of whole transcriptomes across different orchid species. In addition, international sequencing efforts, including the “1000 Fungal Genomes” project of the U.S. Department of Energy (DOE) Joint Genome Institute (JGI) (Grigoriev et al. 2014), boosted knowledge on the fungal genomes and transcriptomes of several fungal taxa, including OM fungi. The integration of this data with outcomes of other *-omics* approaches, such as metabolomics, will improve our knowledge of the orchids and their fungal “friends,” and allow a better understanding of this fascinating and complex symbiosis.

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CHAPTER 3

Plant and fungal gene expression coupled with stable isotope labelling provide novel information on sulfur uptake and metabolism in orchid mycorrhizal protocorms

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SUMMARY

Orchid mycorrhiza (OM) represents an unusual symbiosis between plants and fungi because in all orchid species carbon is provided to the host plant by the mycorrhizal fungus at least during the early stages of orchid development, named a protocorm. In addition to carbon, orchid mycorrhizal fungi provide the host plant with essential nutrients such as phosphorus and nitrogen. In mycorrhizal protocorms, nutrients transfer occurs in plant cells colonized by the intracellular fungal coils, or pelotons. Whereas the transfer of these vital nutrients to the orchid protocorm in the OM symbiosis has been already investigated, there is currently no information on the transfer of sulfur (S). Here, we used ultra-high spatial resolution secondary ion mass spectrometry (SIMS) as well as targeted gene expression studies and laser microdissection to decipher S metabolism and transfer in the model system formed by the Mediterranean orchid *Serapias vomeracea* and the mycorrhizal fungus *Tulasnella calospora*. We revealed that the fungal partner is actively involved in S supply to the host plant, and expression of plant and fungal genes involved in S uptake and metabolism, both in the symbiotic and asymbiotic partners, suggest that S transfer most likely occurs as reduced organic forms. Thus, this study provides original information about the regulation of S metabolism in OM protocorms, adding a piece of the puzzle on the nutritional framework in OM symbiosis.

Keywords: sulfur metabolism, orchid mycorrhiza, *Tulasnella*, *Serapias vomeracea*, gene expression, stable isotope tracer.

INTRODUCTION

Sulfur (S) is an essential macronutrient for all organisms, but conversion of inorganic to organic S compounds is primarily dependent on sulfate uptake and reduction in photosynthetic organisms and microorganisms (Takahashi et al., 2011). Sulfur is involved in multiple metabolic processes where it serves many functions as a structural constituent of amino acids (cysteine and methionine), coenzymes (S-adenosyl-methionine), prosthetic groups (iron-sulfur cluster), vitamins (biotin and thiamin), the anti-oxidant glutathione, sulfolipids, and secondary metabolites (Kopriva et al., 2019).

In green plants, S is the fourth most important nutrient element after nitrogen, phosphorus, and potassium, and S deficiency affects plant growth, plant performance, and yield (Hell, 1997; Zenda et al., 2021). For these reasons, S uptake and metabolism in plants have been extensively investigated (Kopriva et al., 2019; Li et al., 2020; Narayan et al., 2022).

Plants take up inorganic S from the soil mainly in the form of sulfate, which is then transferred to the shoot and reduced in the chloroplasts through the reductive sulfate assimilation pathway.

Uptake and internal transport of sulfate in plants, including translocation between different cell compartments, occurs

via sulfate transporters of the SULTR family (Li et al., 2020; Takahashi, 2019). SULTR is a multigene family of sulfate/H⁺ co-transporters, and different categories of SULTR genes have been proposed according to their coding sequence homology, biochemical properties, and functions (Takahashi et al., 2011). In *Arabidopsis*, the two high-affinity sulfate transporters SULTR1.1 and SULTR1.2 are major players in sulfate absorption from the soil and are mainly located in root hairs and epidermal root cells (Takahashi et al., 2000), whereas the SULTR2.1 isoform seems responsible of translocation from roots to shoots. An additional role as a S sensor has been proposed for the SULTR1.2 transporter (Takahashi, 2019; Zheng et al., 2014). Inside the plant cells, transporters of the SULTR3 family may directly mediate sulfate import into the chloroplasts, where sulfate is reduced (Cao et al., 2013; Chen et al., 2019). Sulfate can be stored in the vacuole, and sulfate transporters of the SULTR 4 group seem to be responsible for sulfate efflux from this organelle under S deficiency (Takahashi, 2019; Takahashi et al., 2011).

Sulfur incorporation into biomolecules requires reduction of inorganic sulfate to sulfide. In plants, the reductive sulfate assimilation pathway starts with the ATP-dependent activation of sulfate to adenosine 5'-phosphosulfate (APS), followed by reduction to sulfite via an APS reductase, and then to sulfide. Condensation of sulfide with O-acetylserine (OAS) leads to the formation of the amino acid cysteine, which is either used directly in protein biosynthesis or for the synthesis of the amino acid methionine, of the redox control molecule glutathione, and of the methyl group donor S-adenosylmethionine (SAM). Starting from APS, the plant sulfate assimilation pathway bifurcates into two pathways: one is the reductive pathway described above, whereas the other involves an APS kinase and leads to the biosynthesis of 3'-phosphoadenosine 5'-phosphosulfate (PAPS), which serves as sulfate donor for sulfation of peptides, hormones, and secondary metabolites crucial for oxidative stress responses (Chan et al., 2019; Kopriva et al., 2012).

In nature, most plants form symbioses with mycorrhizal fungi that assist them with mineral nutrition (Balestrini & Lumini, 2018; Bucher et al., 2014; Genre et al., 2020). Different types of mycorrhizal fungi can be distinguished (arbuscular, ecto, ericoid, and orchid mycorrhizal fungi), depending on the morphology of their association with the host plant (Smith & Read, 2008).

Thanks to their extraradical mycelium, mycorrhizal fungi play a pivotal role in the acquisition of mineral nutrients because their hyphae can explore large volumes of soil and extend into soil micropores that the plant roots cannot reach, thus gaining access to essential nutrients that may be unavailable to the plant (Smith & Read, 2008). Mineral nutrients absorbed by mycorrhizal fungal hyphae are translocated to the plant root, where they are released to the host cell across specialized interfaces (Balestrini &

Bonfante, 2005, 2014) and normally exchanged with photosynthesis-derived carbon. The uptake of phosphorus and nitrogen and their transfer to the mycorrhizal host plant have been extensively investigated (Bucher et al., 2014; Casieri et al., 2013), whereas much less is known about S uptake by mycorrhizal fungi and transfer to the host plant (Giovannetti et al., 2014).

Fungi can absorb inorganic S as sulfate through sulfate permeases belonging to the SLP family, although alternative S sources can be also exploited (Linder, 2018; Traynor et al., 2019). Mansouri-Bauly et al. (2006) identified two sulfate transporters in the ectomycorrhizal fungus *Laccaria bicolor*, but data on other mycorrhizal fungi are scanty and S metabolism in fungi in general has been investigated in a limited number of species (Pilsyk & Paszewski, 2009). Fungi can assimilate S through a reductive sulfate assimilation pathway slightly different from the one described in plants. After transport into the cell, sulfate is reduced to APS, which is then phosphorylated by an APS kinase to form PAPS. A PAPS reductase then releases sulfite from PAPS, which is reduced to sulfide by a sulfite reductase (Traynor et al., 2019). In fungi, the reductive sulfate assimilation pathway is therefore linear and includes PAPS as an intermediate.

Sulfur transfer to the host plant by mycorrhizal fungi has been investigated in arbuscular mycorrhiza, by far the most common and best studied mycorrhizal symbiosis. Arbuscular mycorrhizal colonization substantially improved the S status of the host in many plant species, especially under low-sulfate conditions (Allen & Shachar-Hill, 2009; Casieri et al., 2012; Sieh et al., 2013; Wu et al., 2018). By using ³⁵S-labeled sulfate, Allen and Shachar-Hill (2009) demonstrated sulfate uptake by the fungus and transfer to the mycorrhizal roots. Following mycorrhizal colonization, the SULTR1;1 and SULTR1;2 homologs from *Medicago truncatula* and *Lotus japonicus* were found to be expressed in cortical cells containing fungal arbuscules (Giovannetti et al., 2014), which are the site of nutrient exchange in arbuscular mycorrhiza. This finding suggests that, in arbuscular mycorrhiza, S is transferred to the plant cells mainly in the form of sulfate, although organic S forms may also be translocated (Allen & Shachar-Hill, 2009).

Information on S uptake and transfer to the host plant in other mycorrhizal types is very limited, and, to our knowledge, no previous studies have focused on S uptake and transfer in orchid mycorrhiza (OM). Plants in the Orchidaceae, one of the largest families of flowering plants, display minute seeds lacking storage reserves and an immature embryo, which need carbon and other nutrients for germination and development of a unique postembryonic structure called protocorm (Smith & Read, 2008). Orchid mycorrhiza represents therefore an unusual symbiosis because, at least in the early plant developmental stages, the mycorrhizal fungus transfers organic carbon to

the host plant in addition to other nutrients (see in Cameron et al., 2006; Kuga et al., 2014; Smith & Read, 2008). Nutrient transfer in OM has been mainly studied in mycorrhizal protocorms, where plant cells colonized by intracellular fungal coils, or pelotons, are considered the site of nutrients transfer. Kuga et al. (2014) demonstrated ^{13}C and ^{15}N transfer to *Spiranthes sinensis* protocorms colonized by the OM fungus *Ceratobasidium* sp. using secondary ion mass spectrometry (SIMS). Here, we used SIMS to investigate ^{34}S transfer from an isolate of the OM fungus *Tulasnella calospora* to mycorrhizal protocorms of its host plant *Serapias vomeracea*. ^{13}C and ^{15}N transfer were also measured for comparison. To elucidate the molecular bases of S uptake and transfer in OM, the expression profile of plant and fungal genes potentially involved in S metabolism was investigated in asymbiotic and symbiotic conditions.

RESULTS

Uptake of sulfate and other elements by *Tulasnella calospora* and transfer to mycorrhizal protocorms of the orchid host *Serapias vomeracea*

Stable isotope tracers above natural abundances were detected in the intracellular hyphae colonizing mycorrhizal protocorm cells, as shown in the ratio images of the tracers $r^{13}\text{C}$, $r^{15}\text{N}$, and $r^{34}\text{S}$ (Figure 1). In particular, the ratio images showed that the three stable isotopes highly labeled the peloton hyphae in the colonized protocorm cells, but not the fungal dead masses (Figure 1f–h). The average values of ^{13}C , ^{15}N , and ^{34}S tracer in the fungal hyphae are reported in Table 1. Because the natural abundance is about 1% for ^{13}C , 0.36% for ^{15}N , and 0.4% for ^{34}S , the expected values of $r^{13}\text{C} \times 100$, $r^{15}\text{N} \times 1000$, and $r^{34}\text{S} \times 100$ are about 1, 4, and 4, respectively. The significantly higher average values in the fungal pelotons for all isotopes (Table 1) indicate that the fungal hyphae absorbed the labeled tracers (as ^{34}S -labeled sulfate, ^{13}C -labeled glucose, and ^{15}N -labeled ammonium nitrate) from the agar medium and translocated them to the orchid protocorm.

In colonized protocorm cells, the plant nuclei showed contents above natural abundance for all isotopes, with ratio values that were significantly correlated with the ratio values of $r^{15}\text{N}$ and $r^{34}\text{S}$ measured, within the same cells, in the fungal hyphae (Figure 2). These results on the host nuclei indicate that tracers translocated by the fungal hyphae in the mycorrhizal protocorms were transferred to the host cell. The stable isotope imaging showed that $r^{34}\text{S}$, $r^{13}\text{C}$, and $r^{15}\text{N}$ measured in the plant nuclei were higher than the values of natural abundances not only in the colonized cells but also in uncolonized protocorm cells close to mycorrhizal cells or in the cortical and meristematic region (Table 1). In particular, the highest isotopes ratios were measured in the plant nuclei in the uncolonized cells next to the colonized nuclei (Figure 1b–d), with values

significantly higher when compared to those of the other cells, both uncolonized and colonized (Figure 3). Altogether, these results indicate that stable isotope-labeled compounds, once transferred from the fungal hypha to the host cell, were translocated in other protocorm regions.

A significant linear correlation was observed between different isotope tracers in both the intracellular fungal hyphae and the plant nuclei of colonized and uncolonized cells. The strongest correlation, both in the fungal and plant structures, was between $r^{15}\text{N}$ and $r^{34}\text{S}$ (Figure 4). These results would indicate that compounds containing N and S were translocated at the same rate, or that N and S were both present in the same translocated molecule.

Identification and expression of fungal genes involved in S uptake and assimilation

The complete genome sequence of *T. calospora* (Kohler et al., 2015), available on the MycoCosm portal (<http://genome.jgi.doe.gov/Tulca1/Tulca1.home.html>), was searched for fungal genes potentially involved in S uptake and metabolism. Specifically, a search using the keyword “sulfate” led to the identification of protein-coding genes potentially involved in the transport, transformation, or usage of this element (Table 2). Three genes coding for putative sulfate transporters of the SulP family (*TcSULP1*, *TcSULP2*, *TcSULP3*) were identified, showing homology with sequences of putative transporters and permeases of other basidiomycetes. The best BLASTX hits for all three genes were putative sulfate anion transporters (Table 2).

These *T. calospora* genes were found to be differentially expressed in the mycorrhizal symbiosis in a previous RNAseq experiment (Fochi, Chitarra, et al., 2017). Here, RT-qPCR were here used to investigate their expression in the free-living mycelium (FLM), in the fungal mycelium grown on the substrate next to mycorrhizal protocorms (MYC), and in the mycelium inside mycorrhizal protocorms (SYMB). Expression measured by RT-qPCR, with respect to expression in FLM, is reported in Table S4, and a graphical representation is provided in the heatmap in Figure 5. An opposite expression pattern was revealed for *TcSULP2*, whose expression was strongly down-regulated in MYC samples and up-regulated in SYMB samples, and the other two *TcSULP* genes, both up-regulated in MYC samples and down-regulated in SYMB samples (Figure 5). Five additional fungal genes known to be involved in the different steps of the S reductive pathway were selected in the genome of *T. calospora*, namely a gene coding for a putative ATP sulfurylase (*TcATPSul*) involved in the production of adenosine 5'-phosphosulfate (APS), two genes coding for putative APS kinases (*TcAphk1* and *TcAphk2*), that phosphorylate APS in 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a putative PAPS reductase (*TcPSRed*), that catalyzes the formation of sulfite ions, and a gene coding for a putative sulfite reductase involved in the last step of reduction in sulfite to sulfide

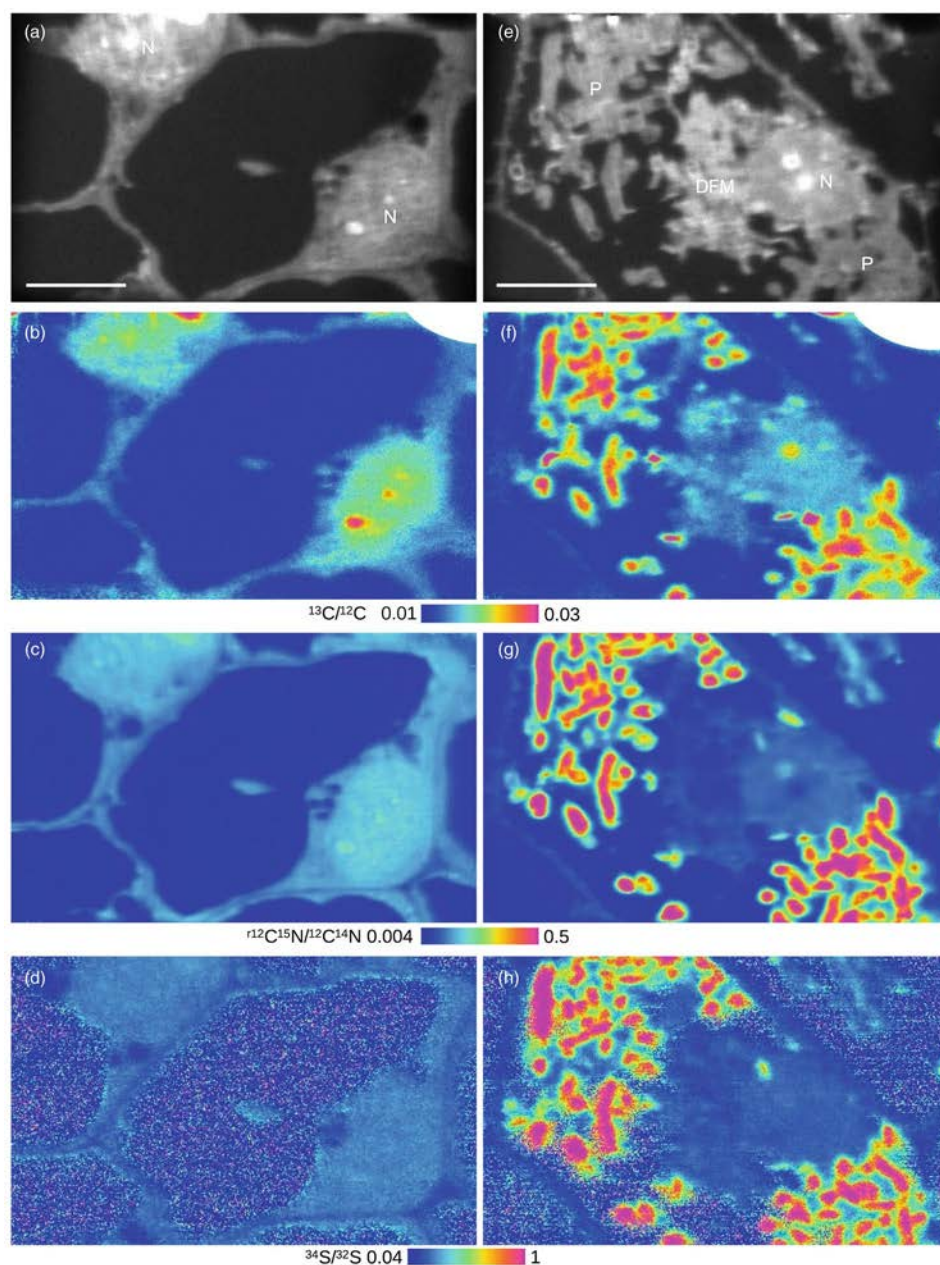


Figure 1. Secondary ion mass spectrometry imaging of a symbiotic protocorm of *Serapias vomeracea* associated with *Tulasnella calospora*. ^{13}C -glucose, $^{15}\text{NH}_4^{15}\text{NO}_3$, and $\text{Mg}^{34}\text{SO}_4$ were added only to extraradical hyphae and incubated for 6 days. Isotopograph of $^{12}\text{C}^{14}\text{N}$ (a,e), $^{13}\text{C}^{12}\text{C}$ (b,f), $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$ (c,g), and $^{34}\text{S}/^{32}\text{S}$ (d,h). (a-d) Uncolonized cortical cells next to colonized region. (e-h). Colonized cell with a fungal peloton and a dead fungal mass from previous colonization. DFM, dead fungal mass; N, plant nucleus; P, peloton. The lowest color indexes are the value of the natural abundance of each isotope. Bars, 20 μm .

Table 1 ^{13}C , ^{15}N , and ^{34}S tracer values of *Serapias vomeracea* protocorm symbiotically germinated with *Tulasnella calospora* (6 days labeling)

	Fungal pelotons	Plant nuclei			
		Colonized cells	Uncolonized cells		
			Cortical next to the colonized region	Cortical and meristem	All
Number of images	30	18	4	4	26
$^{13}\text{C}/^{12}\text{C} \times 100$	2.26 ± 0.50	1.48 ± 0.10	1.76 ± 0.12	1.48 ± 0.08	1.52 ± 0.14
$^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N} \times 1000$	322.35 ± 97.80	78.31 ± 12.03	148.63 ± 13.54	77.88 ± 10.25	89.06 ± 28.10
$^{34}\text{S}/^{32}\text{S} \times 100$	68.39 ± 16.18	18.33 ± 1.47	23.66 ± 1.54	16.40 ± 1.10	18.56 ± 2.59

An aqueous mixture of tracers (1% ^{13}C -glucose, 0.2% $^{15}\text{NH}_4^{15}\text{NO}_3$, 0.1% $\text{Mg}^{34}\text{SO}_4$) was added to the external hyphae and incubated for 6 days. $r^{13}\text{C}$, $^{13}\text{C}/^{12}\text{C}$; $r^{15}\text{N}$, $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$; $r^{34}\text{S}$, $^{34}\text{S}/^{32}\text{S}$. The expected values used in this study for the natural abundance of the isotopes (^{13}C , 1%; ^{15}N , 0.36%; ^{34}S , 4%): $r^{13}\text{C} \times 100 = c. 1$; $r^{15}\text{N} \times 1000 = c. 4$; $r^{34}\text{S} \times 100 = c. 4$. Average $\pm$ SD.

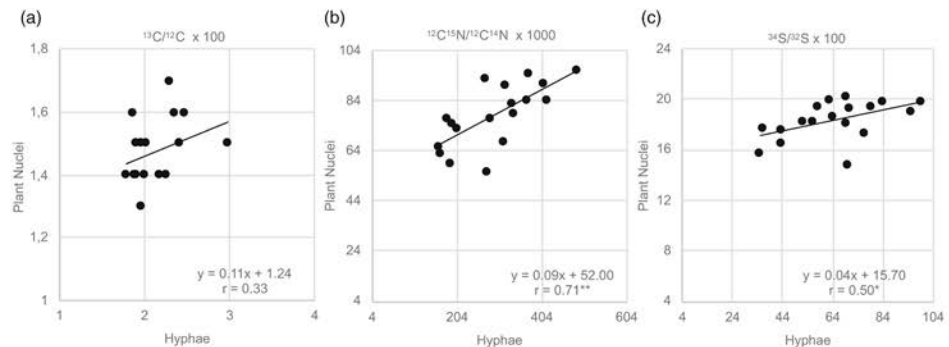


Figure 2. Scatter plots and Pearson's correlation coefficient showing the relationship between the isotope ratios measured in the fungal peloton hyphae and in the plant nuclei of the same colonized protocorm cell of *Serapias vomeracea*. (a) $^{13}\text{C}/^{12}\text{C} \times 100$. (b) $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N} \times 1000$. (c) $^{34}\text{S}/^{32}\text{S} \times 100$. $n = 18$. Pearson's coefficient correlation, both side: *, $P < 0.05$; **, $P < 0.01$.

ions. The transcripts of these genes all showed a similar expression pattern in the fungal mycelium, with a significant down-regulation in MYC samples, with respect to FLM, and a strong up-regulation in SYMB samples (Table S4 and Figure 5).

A gene encoding a putative sulfite efflux pump (*TcSul*), required for the secretion of sulfite as a reducing agent, was also selected in the genome of *T. calospora* as a gene putatively involved in S transport between partners.

This gene was significantly up-regulated in both the MYC and SYMB samples, as compared to FLM (Figure 5).

Selection and gene expression analysis of plant transcripts putatively involved in S uptake and assimilation

RT-qPCR was also performed on plant genes involved in S uptake, transport, and assimilation. The genome sequence of *S. vomeracea* is currently unavailable, and genes of interest were therefore selected from the RNA-Seq data

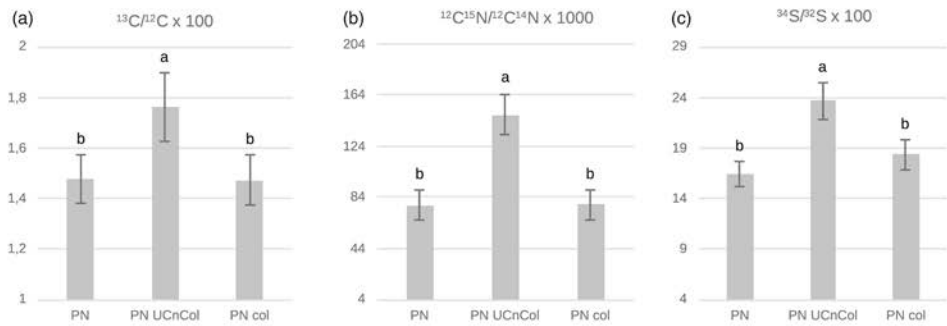


Figure 3. SIMS ROI analysis plant nuclei in a symbiotic protocorm of *Serapias vomeracea* associated with *Tulasnella calospora*. All isotope ratios of plant nuclei in meristematic cells (PN), colonized cells (PN col), and uncolonized cells next to colonized region (PN UCnCol) were higher than the natural abundances and reveal accumulation of elements of fungal origin. Among them, PN UCnCol shows highest elevations of all tracers. Tukey-Kramer multiple comparison, the different letters indicate statistical difference between the samples. (a) $^{13}\text{C}/^{12}\text{C} \times 100$. (b) $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N} \times 1000$. (c) $^{34}\text{S}/^{32}\text{S} \times 100$.

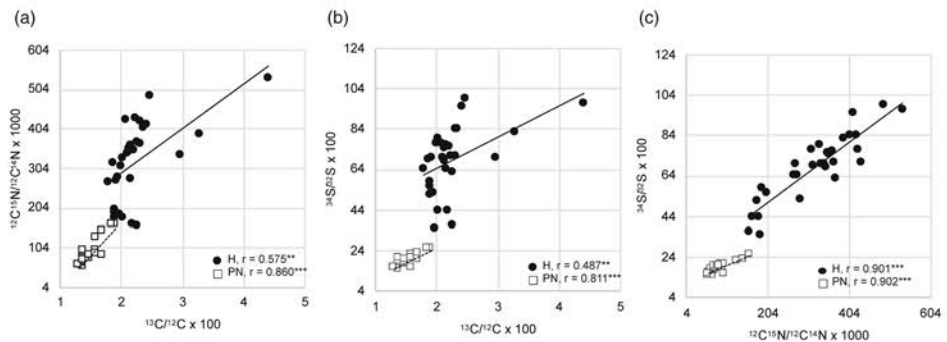


Figure 4. Scatter plots and Pearson's correlation coefficient showing the relationship between different isotope ratios measured in the same structure, either fungal peloton hyphae or plant nuclei, in *Serapias vomeracea* protocorm cells colonized by *Tulasnella calospora*.

(a) $^{13}\text{C}/^{12}\text{C} \times 100$ and $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N} \times 1000$.
(b) $^{13}\text{C}/^{12}\text{C} \times 100$ and $^{34}\text{S}/^{32}\text{S} \times 100$.
(c) $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N} \times 1000$ and $^{34}\text{S}/^{32}\text{S} \times 100$. ●, pelotons; □, plant nuclei. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

obtained by Fochi, Chitarra, et al. (2017) for this species and from a cDNA library obtained by 454 GS-FLX Titanium pyrosequencing (Perotto et al., 2014). Nine *S. vomeracea* sequences annotated as sulfate transporters (Table 3) were compared with sulfate transporters already described in the literature. Phylogenetic analysis (Figure 6) showed that six *S. vomeracea* sequences (SvST2, SvST4, SvST5, SvST6, SvST7, and SvST8) clustered with Group 3 plant sulfate transporters (SULTR 3) together with several sequences from other orchids, one sequence (SvST3) with Group 1 SULTRs and two sequences (SvST1, SvST9) with Group 4 SULTRs.

As compared to asymbiotic protocorms, analysis of the transcript expression profiles in the mycorrhizal

protocorms (Table S5 and Figure 6) showed strong up-regulation of the *S. vomeracea* sulfate transporters identified as Group 3 SULTRs by phylogenetic analysis (Figure 6a), except for SvST4 that was not significantly regulated. SvST3, a sulfate transporter clustering with Group 1 SULTRs, reported to be localized on the plasma membrane (Kopriva et al., 2016, 2019), showed no significant differences in gene expression between symbiotic and asymbiotic protocorms (Table S5 and Figure 6b). SvST9, one of the two sulfate transporters clustering in Group 4 SULTRs, reported to be efflux transporters on the tonoplast (Takahashi, 2019), was strongly up-regulated in symbiotic conditions, the other one (SvST1) being not differentially regulated (Table S5 and Figure 6b).

Table 2 List of the *T. calospora* sequences analyzed in this work

ID Sequence ^a	Sequence name	Best Blastx hit	Accession	% similarity	E-value
20 907	<i>TcSULP1</i>	Sulfate anion transporter [<i>Dichomitus squalens</i>]	XP_007370085.1	60.19%	0.0
20 008	<i>TcSULP2</i>	Sulfate anion transporter [<i>Rhizoctonia solani</i>]	XP_043176186.1	63.24%	0.0
215 409	<i>TcSULP3</i>	Sulfate anion transporter [<i>Rhizoctonia solani</i>]	XP_043183174.1	58.79%	0.0
14 111	<i>TcATPSUL</i>	ATP sulfurylase [<i>Fomitiporia mediterranea</i>]	XP_007266540.1	80.00%	0.0
75 899	<i>TcAphK1</i>	Adenylylsulfate kinase [<i>Dichomitus squalens</i>]	XP_007361681.1	78.89%	2e ⁻¹¹³
33 232	<i>TcAphK2</i>	Adenylylsulfate kinase [<i>Dichomitus squalens</i>]	XP_007361681.1	87.84%	1e ⁻⁴⁰
241 285	<i>TcPsRed</i>	Phosphoadenylyl-sulfate reductase [<i>Trametes versicolor</i>]	XP_008035525.1	64.88%	3e ⁻¹⁰⁸
65 027	<i>TcSR</i>	Assimilatory sulfite reductase [<i>Fomitiporia mediterranea</i>]	XP_007268242.1	52.88%	0.0
20 203	<i>TcSul</i>	Plasma membrane sulfite pump involved in sulfite metabolism [<i>Pleurotus ostreatus</i>]	XP_036630505.1	37.06%	4e ⁻⁹⁰

^aProtein ID from <https://mycocosm.jgi.doe.gov>.

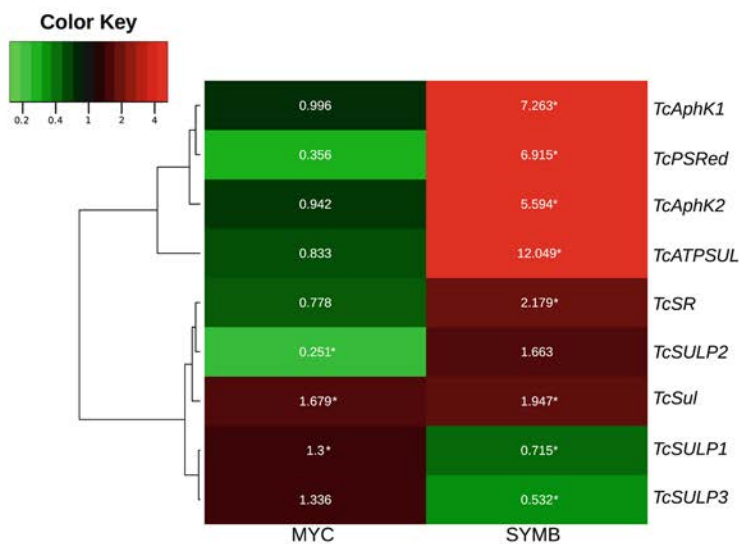


Figure 5. Heat map representation of the fungal transcript levels with a hierarchical clustering in MYC and SYMB samples. Expression levels are reported as fold change (FLM used as reference) and are colored green for low intensities (down-regulation) and red for high intensities (up-regulation). In order: adenylyl-sulfate kinase (*TcAphK1*), phosphoadenylyl-sulfate reductase (*TcPsRed*), adenylylsulfate kinase (*TcAphK2*), ATP sulfurylase (*TcATPSUL*), sulfite reductase (*TcSR*), sulfate transporter (*TcSULP2*), sulfite efflux pump (*TcSul*), and sulfate transporters (*TcSULP1* and *TcSULP3*). Asterisks indicate significant differences ($P < 0.05$) compared with the control (FLM) as inferred by the Pairwise Fixed Reallocation Randomization Test[©] performed by using REST[©].

Seven plant genes showing homology with enzymes involved in the reductive sulfate assimilation pathway or in the sulfation of secondary metabolites (Table 3) were further identified in the available *S. vomeracea* transcriptomes (Fochi, Chitarra, et al., 2017; Perotto et al., 2014). Gene expression analysis (Table S5 and Figure 6b) revealed that none of the plant genes involved in the sulfate reductive assimilation pathway was significantly regulated in symbiosis, whereas the sulfotransferase gene

SvSTRF, putatively involved in the sulfation of secondary metabolites, was strongly up-regulated in mycorrhizal protocorms (Table S5 and Figure 6b).

Laser microdissection (LMD) and gene expression in mycorrhizal protocorms

To investigate cell-type specific expression of plant and fungal genes potentially involved in sulfate uptake, transport, and assimilation, about 1300 protocorm cells either

Table 3 List of the *S. vomeracea* sequences analyzed in the current work

ID Sequence	Sequence name	Best Blastx hit	Accession	% similarity	E-Value
<i>R2_C16339^b</i>	<i>SvST1</i>	Probable sulfate transporter 4.2 [<i>Phalaenopsis equestris</i>]	XP_020585462.1	87.76%	4e ⁻⁵⁰
<i>R2_Rep_C202^b</i>	<i>SvST2</i>	Probable sulfate transporter 3.4 [<i>Dendrobium catenatum</i>]	XP_020699429.1	75.86%	4e ⁻³⁶
<i>R2_Rep_C5242^b</i>	<i>SvST3</i>	Sulfate transporter 1.3-like [<i>Phoenix dactylifera</i>]	XP_008780888.2	79.67%	2e ⁻⁹⁷
<i>R2_C13484^b</i>	<i>SvST4</i>	Sulfate transporter 3.1 [<i>Dendrobium catenatum</i>]	XP_020699282.1	69.47%	3e ⁻³⁷
<i>TRINITY_DN68835_c1_g2_i1^a</i>	<i>SvST5</i>	Probable sulfate transporter 3.4 [<i>Dendrobium catenatum</i>]	XP_020699429.1	82.89%	2e ⁻⁸⁰
<i>TRINITY_DN63650_c0_g1_i1^a</i>	<i>SvST6</i>	Probable sulfate transporter 3.4 [<i>Phoenix dactylifera</i>]	XP_008793448.1	71.23%	7e ⁻¹⁶⁴
<i>TRINITY_DN63650_c0_g2_i1^a</i>	<i>SvST7</i>	Probable sulfate transporter 3.4 [<i>Dendrobium catenatum</i>]	XP_020690822.1	77.39%	2e ⁻⁵³
<i>TRINITY_DN76349_c3_g11_i1^a</i>	<i>SvST8</i>	Probable sulfate transporter 3.4 [<i>Dendrobium catenatum</i>]	XP_020699429.1	72.22%	2e ⁻⁵²
<i>TRINITY_DN63650_c0_g4_i1^a</i>	<i>SvST9</i>	Probable sulfate transporter 3.3 [<i>Phalaenopsis equestris</i>]	XP_020585326.1	46.55%	1e ⁻²⁸
<i>TRINITY_DN69207_c1_g1_i1^a</i>	<i>SvATPSul</i>	ATP sulfurylase 1, chloroplastic [<i>Phalaenopsis equestris</i>]	XP_020586112.1	87.04%	0.0
<i>TRINITY_DN70992_c0_g1_i2</i>	<i>SvAPSKin2</i>	Adenylyl-sulfate kinase 3 [<i>Dendrobium catenatum</i>]	XP_020689777.1	73.61%	2e ⁻¹⁴²
<i>TRINITY_DN58139_c0_g1_i1</i>	<i>SvAPSRd1</i>	5'-adenylylsulfate reductase 3, chloroplastic [<i>Dendrobium catenatum</i>]	XP_020699460.1	77.51%	0.0
<i>TRINITY_DN76001_c0_g1_i1</i>	<i>SvSR2</i>	Sulfite reductase [ferredoxin], chloroplastic [<i>Dendrobium catenatum</i>]	XP_020698352.1	88.21%	0.0
<i>TRINITY_DN74740_c3_g1_i1</i>	<i>SvSTRF</i>	Flavonol 3-sulfotransferase-like [<i>Phalaenopsis equestris</i>]	XP_020575587.1	62.12%	1e ⁻¹¹⁰
<i>R2_C9449^b</i>	<i>SvAPSRd2</i>	5'-adenylylsulfate reductase-like 3 [<i>Phalaenopsis equestris</i>]	XP_020581002.1	61.62%	1e ⁻⁷⁷
<i>R2_C11840^b</i>	<i>SvAPSRd3</i>	5'-adenylylsulfate reductase-like 5 [<i>Phalaenopsis equestris</i>]	XP_020574787.1	63.33%	8e ⁻⁶⁰
<i>R2_Rep_C7777^b</i>	<i>SvSR1</i>	Sulfite reductase 1 [ferredoxin], chloroplastic [<i>Dendrobium catenatum</i>]	XP_028554576.1	80.26%	1e ⁻³⁴

^aFrom RNA-seq by Illumina HiSeq2000 (Fochi, Chitarra, et al., 2017).

^bFrom cDNA library by 454 GS-FLX Titanium pyrosequencing (Perotto et al., 2014).

with or without visible fungal pelotons were collected by LMD for each of the three biological replicates and used for RNA extraction and One-Step RT-PCR. RNA samples were amplified by RT-PCR using primers specific for the *T. calospora* elongation factor *TcEF-1α*, to detect the presence of the fungus in the LMD collected cells (Figure 7). Absence of an amplified product in the RT minus reactions excluded genomic DNA contamination (not shown). No differences in plant gene expression were observed between the two microdissected cell-type populations but, unfortunately, transcripts corresponding to the fungal housekeeping gene *TcEF-1α* could be amplified in samples of LMD plant cells that did not contain visible fungal coils. The amplification of fungal transcripts in apparently uncolonized cells may be due to the fact that small pelotons, either very young or already collapsed, may go undetected in some cortical cells during microdissection. This difficulty in the collection of specific cell types was already reported for orchid mycorrhizal roots by Fochi, Falla, et al. (2017). To avoid presenting a biased data, only results from colonized cells

have been presented here. The successful amplification of *TcSULP2*, *TcATPSul*, *TcAphK1*, *TcAphK2*, *TcSR*, and *TcSul* transcripts in at least two replicates of the colonized protocorm cells agreed with the expression in symbiosis revealed by the RT-qPCR analysis. The two sulfate permeases *TcSULP1* and *TcSULP3*, which appeared to be down-regulated in the whole protocorms by RT-qPCR, could not be amplified in any of the RNA samples from colonized LMD cells, thus suggesting a low level of transcripts in the symbiotic stage. The only discrepancy with the quantitative data on the protocorms (Figure 5 and Table S4) was for *TcPsRed*. This result could be mainly due to differences in the RNA extraction protocols and amplification method (One-Step RT-PCR vs RT-qPCR). Additionally, since RT-PCR has been performed on RNA extracted from subpopulations of intracellular fungal coils, we cannot exclude that *TcPsRed* might be exclusively expressed in intercellular hyphae inside the protocorms, or in a specific step of fungal coil development. The latter hypothesis would explain the absence of transcripts in some of the colonized cell

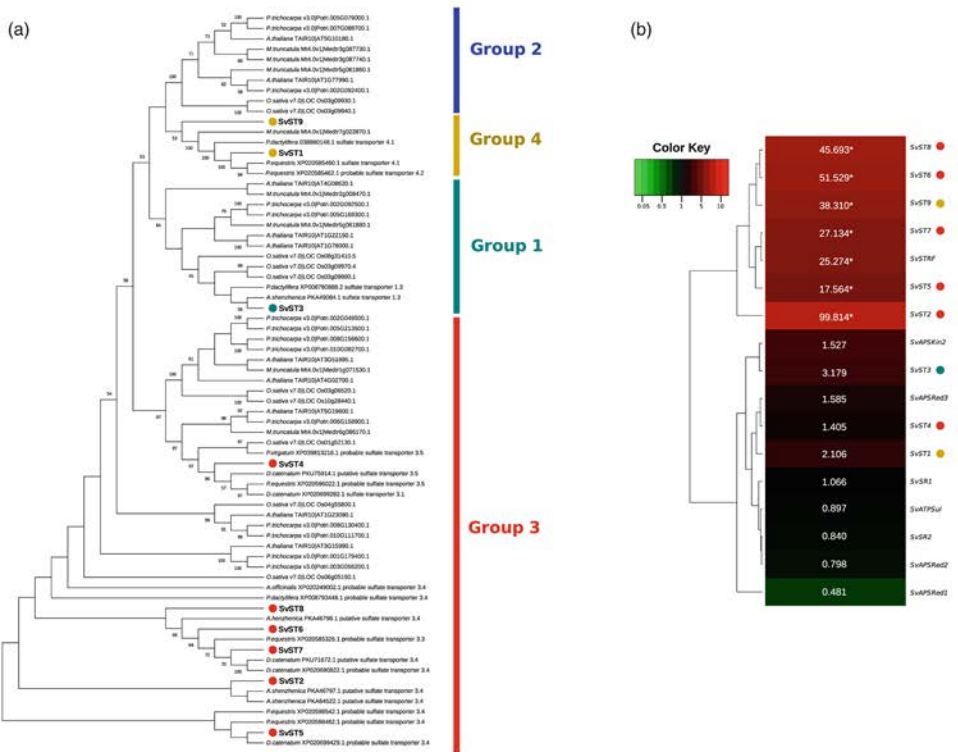


Figure 6. Phylogenetic analyses of plant sulfate transporters and gene expression on plant sulfur metabolism genes. (a) Phylogenetic analysis of sequences putatively coding for *S. vomeracea* sulfate transporters (SvST1, SvST2, SvST3, SvST4, SvST5, SvST6, SvST7, SvST8, and SvST9) as a bootstrap consensus tree. Only bootstrap values >50% are showed. (b) Heat map representation of the plant transcript levels with a hierarchical clustering in symbiotic protocorms. Expression levels are reported as Fold Change (asymbiotic protocorms used as reference) and are colored green for low intensities (down-regulation) and red for high intensities (up-regulation). SvST8, SvST6, SvST9, and SvST7 (sulfate transporters), SvSTR (sulfotransferase), SvST5 and SvST2 (sulfate transporters), SvAPSKin2 (adenylsulfate kinase), SvST3 (sulfate transporter), SvAPSRd3 (5'-adenylsulfate reductase), SvST4 and SvST1 (sulfate transporters), SvSR1 (sulfite reductase), SvATPSul (ATP sulfurylase), SvSR2 (sulfite reductase), SvAPSRd2 and SvAPSRd1 (5'-adenylsulfate reductases). Asterisks indicate significant differences ($P < 0.05$) compared with the control (ASYMB) as inferred by the Pairwise Fixed Reallocation Randomization Test© performed by using by REST©.

replicates also observed for other fungal transcripts. By using primer sets designed for plant transcripts, RT-PCR with specific primers for the *S. vomeracea* housekeeping gene (the elongation factor *SvEF-1 α*) yielded an amplified fragment of the expected size in all RNA samples (Figure 7). Sulfate transporters SvST2, SvST3, SvST5, SvST6, SvST7, and SvST9 showed amplification in at least two of the three biological replicates (Figure 7), while SvST1 did not show amplification in any of the samples (not shown). Most transcripts corresponding to plant genes involved in S assimilation (SvSR1, SvSR2, SvATPSul, SvAPSKin2, SvAPSRd1, SvSTR) appeared not significantly up-

regulated in RT-qPCR (Figure 6b and Table S5), but they could be detected in colonized cells.

DISCUSSION

Mycorrhizal fungi can assist plants in the uptake of macro-nutrients essential for their metabolism and growth, and a role in S uptake, especially under low-sulfate conditions, has been demonstrate in the ubiquitous arbuscular mycorrhizal fungi (Allen & Shachar-Hill, 2009; Giovannetti et al., 2014; Ma et al., 2022; Sieh et al., 2013; Wu et al. 2018). The role of ectomycorrhizal fungi in S nutrition has not been extensively investigated, but they are

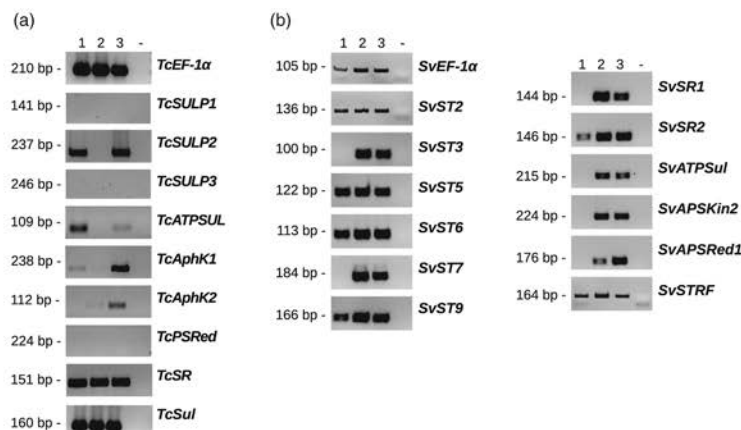


Figure 7. One-Step RT-PCR of laser microdissected mycorrhizal cells from *S. vomeracea* protocorms using fungal (a) and plant (b) primers. Numbers (1), (2), and (3) correspond to the three biological replicates, while (–) correspond to the negative control. In (a), tested genes: *TcEF-1α* (elongation factor), *TcSULP1* (sulfate transporter), *TcATPSUL* (ATP sulfurylase), *TcAphK1* and *TcAphK2* (adenylylsulfate kinases), *TcSR* (sulfite reductase), and *TcSul* (sulfite efflux pump). In (b), tested genes: *SvEF-1α* (elongation factor), sulfate transporters (*SvST2*, *SvST3*, *SvST5*, *SvST6*, *SvST7* and *SvST9*), *SvSR1* and *SvSR2* (sulfite reductase), *SvATPSul* (ATP sulfurylase), *SvAPSKin2* (adenylylsulfate kinase), *SvAPSRed1* (5'-adenylylsulfate reductase), and *SvSTRF* (sulfotransferase).

suggested to contribute to the regulation of sulfate uptake by the host (Mansouri-Bauly et al., 2006).

Here, we showed that an orchid mycorrhizal fungus in the genus *Tulasnella* can assist protocorms of the host plant *Serapias vomeracea* not only in C and N nutrition, as already demonstrated in other orchid species (see in Kuga et al., 2014; Smith & Read, 2008), but also in S nutrition. Compounds labeled with stable isotopes (^{34}S -sulfate, ^{13}C -glucose and ^{15}N - ammonium nitrate) were taken up by the external mycelium of *T. calospora* and translocated to the mycorrhizal protocorms via the fungal hyphae. Intracellular fungal hyphae accumulated high amounts of stable isotopes, whereas collapsed fungal masses were not labeled in our study. This is likely because they were already non-functional in nutrient transport when labeling was applied to the external medium, as fungal pelotons in mycorrhizal protocorm cells undergo a rapid turnover culminating in hyphal degeneration and collapse (Uetake et al., 1992). Labeling of plant nuclei indicate that, inside the mycorrhizal protocorms, labelled compounds were transferred from the intracellular hyphae to the host cells and further allocated to the uncolonized cells and the meristem. Interestingly, plant nuclei in uncolonized cells next to the colonized region accumulated more stable isotopes than nuclei in peloton harboring cells, suggesting a higher cellular activity than the colonization events, such as reported in pathogenic infections and arbuscular mycorrhizal colonization (Balestrini et al., 1992; Deslandes & Rivas, 2011). In which chemical form these stable isotopes reach the

neighboring cells once they are transferred from the fungal pelotons to the colonized cells, and by which route (symplastic or apoplastic), remains to be elucidated.

The *T. calospora* sulfate permease *TcSULP1* may be involved in the uptake of sulfate from the substrate, as it was significantly up-regulated in the fungal mycelium outside the mycorrhizal protocorms and down-regulated inside the orchid protocorms. A similar trend was also found for *TcSULP3*, although gene regulation was significant only inside the protocorm. In addition to mycorrhizal fungi, some fungal endophytes can improve the host plant nutritional status (Baron & Rigobelo, 2022). The genus *Serendipita* (Serendipitaceae, Basidiomycetes) is intriguing because it harbors well-established fungal endophytes but also species forming orchid mycorrhiza (Oktalira et al., 2021). *Serendipita indica* is a well-characterized endophyte that displays nutrient transportation abilities (Card et al., 2016). This fungal endophyte enhances S uptake in maize plants thanks to the activity of a high-affinity sulfate transporter *SiSulT* (Narayan et al., 2021). Knockdown strains lacking *SiSulT* failed to support plant growth under low-sulfate conditions, demonstrating the importance of this fungal transporter (Narayan et al., 2021). The importance of the two *T. calospora* sulfate transporters *TcSULP1* and *TcSULP3* in assisting the orchid host in S uptake remains to be established.

Plant roots acquire S directly from soil in the form of sulfate, thanks to sulfate transporters belonging to Group 1, represented in *Arabidopsis* by *SULTR1;1* and *SULTR 1;2*

(Takahashi et al., 2000). In symbiotic plant–fungus interactions, nutrients taken up from the substrate by the external fungal hyphae are translocated to the host, where they are released across specialized plant–fungus interfaces (Ivanov et al., 2019). In arbuscular mycorrhizal *Lotus japonicus*, the LjSULTR1;2 promoter was activated in cortical root cells containing arbuscules, the highly branched intracellular fungal structures typical of this symbiosis, suggesting that S is transferred as sulfate from the fungus to the host plant across the plant-derived periarbuscular membrane (Giovannetti et al., 2014). Translocation of S to the respective host plants in the form of sulfate has been also suggested for the ectomycorrhizal fungus *Laccaria laccata* (Mansouri-Baully et al., 2006) and the fungal endophyte *S. indica* (Narayan et al., 2021).

The form of S translocated to the plant in orchid mycorrhiza is currently unknown, but results of transcriptomic experiments in this study suggest that S is at least in part translocated as an organosulfur compound. In particular, the strong activation of the sulfate reductive assimilation pathway in the fungus *T. calospora* when inside the mycorrhizal protocorm, together with the lack of a significant induction of the reductive assimilation pathway in the orchid host when in symbiosis, suggest that at least part of S is taken up as sulfate from the substrate and reduced by the fungus before S translocation to the plant.

The genome sequence of *S. vomeracea* is not currently available and the set of plant genes tested in this study as being involved in sulfate uptake and metabolism likely provides an incomplete picture of S metabolism in this orchid species. However, transcripts were selected from transcriptomic experiments on mycorrhizal protocorms, ensuring that genes expressed in symbiosis should be represented in the tested gene set. This was confirmed by LMD experiments, where most of the plant genes analyzed could be revealed in at least two of the three biological replicates. It was therefore intriguing and consistent with the hypothesis of organosulfur compounds being transferred to the plant, that the only Group 1 sulfate transporter identified (SvST3) was slightly but not significantly induced in mycorrhizal protocorms, as tested by RT-qPCR.

Interestingly, SIMS data indicate a very strong linear correlation between ^{15}N and ^{34}S labeling in both the intracellular fungal hyphae and the plant nuclei. The most likely explanation of these results is that organic compound(s) containing both N and S, like the S-amino acids cysteine and methionine, are translocated from the fungus to the plant, although we cannot exclude the possibility that separate compounds containing either N or S are translocated to the host at the same rate and/or that separately transferred N and S are used inside the same molecule formed in the host cell. Although sulfate seems to be the main S compound translocated in arbuscular mycorrhiza, the S-amino acids cysteine and methionine were also suggested

to be released from arbuscular mycorrhizal fungi to plant roots (Allen & Shachar-Hill, 2009).

Specific cysteine (or methionine) membrane transporters have been described in fungi, such as the high-affinity cysteine importer Yct1 in yeast (Kaur & Bachhawat, 2007), but it is unclear whether similar specific transporters exist in plants or whether a less specific transport of S-amino acids occurs through general amino acid permeases (Gigolashvili & Kopriya, 2014). Plants possess a high number of amino acid transporters, many of them potentially capable of transporting cysteine, at least *in vitro* (Lee et al., 2014).

Previous work on orchid mycorrhiza showed that plant amino acid transporters are strongly up-regulated in mycorrhizal orchid protocorms, as compared to asymbiotic conditions (Fochi, Chitarra, et al., 2017; Miura et al., 2018). These transporters have been suggested to be responsible for the uptake of nitrogen released by the fungus in the form of amino acids (Dearnaley & Cameron, 2017; Fochi, Chitarra, et al., 2017). Notably, transcripts coding for two general amino acids permease (AAPs) and a Lysine Histidine Transporter 1 (LHT1) accumulated specifically in *S. vomeracea* cells containing viable fungal coils of *T. calospora* (Fochi, Falla, et al., 2017). The AAPs transport a broad spectrum of amino acids (Yang et al., 2020), and it is therefore possible that they may facilitate S-amino acids uptake across the plant–fungus intracellular interface. On the other hand, S-amino acids may also be metabolized in the fungal cells to form tripeptides that can be then transported by plant oligopeptides transporters. Cysteine is involved in the formation of the tripeptide glutathione, and some members of the oligopeptide transporter (OPT) family indeed transport this compound (Cagnac et al., 2004; Zhang et al., 2016). Interestingly, many plant OPTs were found to be very strongly induced in mycorrhizal orchid protocorms (Fochi, Chitarra, et al., 2017; Miura et al., 2018), but their specificity and role remain to be established.

Most *S. vomeracea* Group 3 sulfate transporters were found to be strongly up-regulated in mycorrhizal protocorms, as compared to asymbiotic protocorms. In *Arabidopsis*, all five members of Group 3 sulfate transporters have been suggested to be redundantly involved in sulfate uptake across the envelope membrane of chloroplasts, the main site for sulfate reduction in plants (Takahashi et al., 2011), based on the SULTR3 localization on the chloroplast membrane (Chen et al., 2019) and decreased chloroplast sulfate uptake in single knockout mutants (Cao et al., 2013). If orchids receive S from the symbiotic fungal partner in an already reduced organic form, as suggested by the results of SIMS and transcriptomic experiments, reduction of sulfate in the plastids would be unnecessary, and plastid transporters would be expected to be down-regulated. However, other reports on Group 3 transporters suggest that members of this large group of sulfate transporters have variable subcellular localization and

functional roles (Takahashi, 2019). For example, SULTR3;5 is a plasma membrane protein expressed in the root vasculature of *Arabidopsis*, where is thought to aid SULTR2;1 in root-to-shoot sulfate translocation (Kataoka et al., 2004). Members of the SULTR3 family are also expressed in *Arabidopsis* seeds, where they are suggested to be involved in the distribution of sulfate and S metabolites during seed development (Zuber et al., 2010). Intriguingly, the *Arabidopsis* SULTR3;3 ortholog in *Oryza sativa* did not show transporting abilities *in vitro*, and mutation of the corresponding gene altered the accumulation of phosphate and sulfate in rice seedlings (Zhao et al., 2016), thus revealing unexpected interactions between P and S assimilation pathways in plants (Sacchi & Nocito, 2019). Thus, the roles of SULTR3 subfamily members are variable and may depend on localization, plant species, and/or development stage. Their strong up-regulation in orchid mycorrhizal protocorms requires therefore further investigations.

To summarize, we have demonstrated that mycorrhizal protocorms of the terrestrial orchid *S. vomeracea* are assisted in S nutrition by their symbiotic fungal partner *T. calospora*, as labeled ^{34}S could be translocated from the substrate to the protocorm cells via the fungal hyphae. As previously suggested for N (Fochi, Chitarra, et al., 2017), we suggest that S may be also transferred to the plant in a reduced organic form, such as an amino acid or a small peptide such as glutathione. Reduction of sulfate and nitrate is an energy demanding process that normally occurs in the chloroplasts of photosynthetic plants. In this respect, translocation of molecules containing already reduced N and S could be an advantage for achlorophyllous orchid protocorms, that rely completely on the fungal symbiont for their energy supply in the form of organic carbon, a strategy termed mycoheterotrophy (Hynson et al., 2013). It would be therefore interesting to investigate S metabolism in fully photosynthetic adult orchid stages, where the role of the mycorrhizal fungus in nutrient uptake is largely unknown. Recent studies suggest that many photosynthetic meadow orchid species, like *S. vomeracea*, acquire organic compounds from their associated fungi throughout their entire life cycle, though being simultaneously photosynthetic (Gebauer et al., 2016; Schiebold et al., 2017; Stöckel et al., 2014). This observation, together with the finding that most adult orchid species, including *S. vomeracea*, acquire N from their fungal partner (Liebel et al., 2010), suggests that organic N forms are also taken up by adult orchids. Up-regulation of some amino acid transporters in mycorrhizal roots of adult *Oeceoclades maculata* (Valadares et al., 2020) and *Limodorum abortivum* (Valadares et al., 2021) provides further support to the hypothesis of translocation of organic N forms in adult orchids. We have currently no information of S translocation in adult *S. vomeracea* plants because our work focused on the early stages of this orchid species. However, a situation in adult plants like the one suggested

for N could be speculated also for S and deserves to be further investigated.

Research on S metabolism in plants has been mainly focused on S uptake and primary metabolism, whereas less attention has been paid to the PAPS branch of sulfate assimilation and the synthesis of sulfated compounds, despite their crucial roles in stress defense and signaling (Chan et al., 2019; Kopriva et al., 2012). Sulfotransferases (STRF) are involved in the transfer of the functional sulfo group to hydroxylated substrates using PAPS as the sulfate donor, and the *S. vomeracea* STRF significantly up-regulated in the mycorrhizal protocorms may be involved in the synthesis of S-containing secondary metabolites. We have currently no information on the nature and role of sulfated compounds in *S. vomeracea* and metabolomic investigations are ongoing, but it is worth noting that S compounds can play special roles in some orchid species. For example, the spider orchid *Caladenia crebra* produces sulfurous pheromone mimics to attract its male wasp pollinator (Bohman et al., 2017).

Our study provides a glimpse on S metabolism in mycorrhizal protocorms and poses intriguing questions on the strategies that orchids evolved to obtain reduced forms of essential nutrients from the fungus, at least during the achlorophyllous stages. Although the molecular mechanisms that regulate S sensing and homeostasis are far from being understood in plants (Li et al., 2020; Ristova & Kopriva, 2022), a further step in the understanding of the orchid-fungus interactions would be the identification of the mechanisms that regulate fungal S uptake and transfer to the plant in symbiosis.

MATERIALS AND METHODS

Gene expression analyses and laser microdissection

Growth of free-living mycelium

The fungal isolate used in this study was originally isolated from mycorrhizal roots of the terrestrial orchid *Serapias vomeracea* grown in meadows in Northern Italy (Girlanda et al., 2011) and deposited in the mycological collection of the University of Turin (MUT) with the accession number MUT 4178. To confirm the identity of this *Tulasnella* sp. isolate, genomic DNA was extracted for Internal transcribed spacer (ITS) sequencing. The mycelium was grown in 2% malt extract liquid medium at 25°C in the dark in 250 ml flasks under continuous stirring. Once the mycelium reached 2 cm in diameter, it was collected by using sterilized tweezers and stored at -20°C.

To obtain actively growing mycelium for symbiotic orchid seed germination, the fungal isolate was grown on 2% malt extract agar (MEA) at 25°C in the dark for 10 days.

For RNA extraction, a mycelial plug with a diameter of 6 mm was transferred onto a sterilized cellulose membrane placed in 9 cm Petri dishes on top of solid oat agar (OA) medium (0.3% oat flour, 1% agar), as described by Schumann et al. (2013). After 15 days of growth in the dark at 25°C, this free-living mycelium (FLM) was collected with a spatula, immediately frozen in liquid nitrogen, and stored at -80°C.

Tulasnella genomic DNA extraction and ITS region sequencing

Genomic DNA from fungal mycelium grown in liquid medium was extracted using DNeasy® Plant Mini Kit (Qiagen, Valencia, CA, USA) following the protocol with some modifications. Total genomic DNA was quantified using spectrophotometry (NanoDrop 1000; BioRad Laboratories, Hercules, CA, USA) and a PCR reaction with ITS1 and ITS4 primers (White et al., 1990) was carried out. The amplification reaction was performed with BIORAD thermocycler with the following protocol: incubation at 95°C for 3 min, 35 cycles at 95°C for 30 sec, 54°C for 30 sec, and 72°C for 45 sec, followed by extension at 72°C for 7 min. The resulting ITS amplicon was purified with The Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sent to the Sequencing Service of the Ludwig-Maximilians-Universität (LMU) in Munich (Germany) for sequencing. The ITS sequence was analyzed by BLASTn and identity of MUT 4178 isolate as *Tulasnella calospora* was confirmed.

Symbiotic and asymbiotic seed germination of *Serapias vomeracea*

Serapias vomeracea (BURM.) BRIQ. seeds were obtained from mature capsules collected from plants grown in the locality of Cairo Montenotte (SV) during spring 2019. To obtain symbiotic protocorms, the mycelium of the mycorrhizal fungus *T. calospora* (MUT 4178 isolate) and *S. vomeracea* seeds were co-inoculated in 9 cm Petri dishes as described in Perotto et al. (2014). Briefly, orchid seeds were surface sterilized in a solution of 1% sodium hypochlorite and 0.1% Tween-20 for 20 min under shaking, followed by three 5-minute rinses in sterile distilled water to remove residues of the sterilization solution. Seeds were re-suspended in sterile water and dropped on autoclaved cellulose membrane positioned on solid OA medium (0.3% oat flour, 1% agar) in 9 cm Petri dishes. In the center of each Petri dish, a plug (6 mm diameter) of actively growing *T. calospora* mycelium was placed, and plates were incubated at 23°C in full darkness for 45 days.

Asymbiotic protocorms were obtained by directly placing surface-sterilized *S. vomeracea* seeds on modified BM1 culture medium (Van Waes & Debergh, 1986) at 23°C in darkness for 90 days (Fochi, Chitarra, et al., 2017), with pH adjusted to 5.8. The fungal mycelium grown on the cellulose membrane next to the protocorms (MYC sample), symbiotic and asymbiotic protocorms were collected, immediately frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

Gene identification and primer design

Identification of fungal genes coding for proteins putatively involved in sulfur (S) uptake, transfer and assimilation was performed using the *T. calospora* genome database on the Joint Genome Institute (JGI) fungal genome portal MycoCosm (<http://genome.jgi.doe.gov/Tulca1/Tulca1.home.htm>).

Selection of plant sequences coding for proteins potentially involved in S uptake, transfer and assimilation was performed using the RNA-Seq database obtained in the study of Fochi, Chitarra, et al. (2017) and a cDNA library obtained with a 454 GS-FLX Titanium pyrosequencing described in Perotto et al. (2014). Phylogenetic analysis of protein sequences putatively encoding for S transporters was performed through MEGAX software v. 11 including 62 sequences retrieved in NCBI Protein database and described as plant sulfate transporters (Table S1). A tree was generated using maximum likelihood (ML) method, by setting JTT model as substitution model and 500 bootstrap iterations.

PRIMER3PLUS (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>) was used to design specific primers for fungal and

plant genes involved in S uptake and metabolism and their specificity was tested *in silico* using the tool PRIMER BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and through PCR experiments on DNA extracted from and *T. calospora* mycelium and *S. vomeracea* symbiotic protocorms.

RNA extraction and RT-qPCR

RNA extraction for RT-qPCR was conducted following the method of Chang et al. (1993) from symbiotic (SYMB) and asymbiotic (ASYMB) *S. vomeracea* protocorms as well as from *T. calospora* free-living mycelium (FLM) and mycelium grown on the cellulose membrane next to the mycorrhizal protocorms (MYC).

Before RNA quantification using the NanoDrop 1000, Turbo DNA-free TM reagent (Ambion, Austin, TX, USA) was used to remove genomic DNA according to the manufacturer's instructions. Absence of genomic DNA was checked through One-Step RT-PCR kit (Qiagen) using primers specific for fungal and plant housekeeping genes (see Table S2 and S3). cDNA synthesis was obtained using the SuperScript® Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), starting from approximately 500 ng of total RNA for each sample, following the manufacturer's instructions. At the end of the reaction, cDNA was diluted 1:10 for quantitative gene expression analysis, performed using a Rotor-Gene Q (Qiagen) instrument. Reactions were carried out in a final volume of 15 µl with 7.5 µl of Rotor-Gene SYBR® Green Master Mix, 5.5 µl of a mix of forward and reverse primers (prepared by adding 16 µl of each primer at 10 µM stock concentration to 168 µl of water) and 2 µl of cDNA. The RT-qPCR cycling program consisted of a 10 min/55°C RT step, 10 min/95°C holding step followed by 40 cycles of two steps (15 sec/95°C and 1 min/60°C).

Expression of target transcripts was quantified after normalization to the reference genes, that is, the fungal and plant elongation factor (EF) genes *TcEF-1α* and *SvEF-1α*, respectively, using the 2^{-ΔΔCT} method (Livak & Schmittgen, 2001). Gene expression data were calculated as expression ratios. All reactions were performed with three biological and two technical replicates.

Laser microdissection and RT-PCR

To prepare tissue sections for laser microdissection (LMD), symbiotic protocorms were treated with Farmer's fixative (absolute ethanol/glacial acetic acid 3:1) and maintained at 4°C overnight; samples were successively dehydrated and embedded in paraffin as described in Fochi, Falla, et al., 2017. 12-µm thick sections were prepared using a rotational microtome and placed on Leica RNase-free PEN foil slides (Leica Microsystems) with ddH₂O filtered with a 0.2 µm filter. Slides were dried on a 40°C warming plate, stored at 4°C and used within 1 day. Before LMD cutting, the slides with the sections were submerged in Neoclear for 8–10 min, rinsed in 100% ethanol for 1 min, and then air-dried. A Leica LMD 6500 LMD system (Leica Microsystems, Inc., Germany) was used, in which the slides without paraffin were placed face-down on the microscope and cells containing visible fungal coils and uncolonized cells were collected in 0.5 ml tubes. Three biological replicates were prepared, in each of which approximately 1200–1300 cells were pooled. Every pool was eluted to a final volume of 50 µl with Pico Pure extraction buffer and RNA was extracted with Pico Pure kit (Life Technologies, Carlsbad, CA, USA) following the protocol with some modifications (*i.e.*, DNase treatment was not done on column). RNA was treated with Turbo DNA free (Ambion, Austin, TX, USA), according to the manufacturer's instructions.

RT-PCR reactions were carried out using the One-Step RT-PCR kit (Qiagen) in a final volume of 10 µl containing 2 µl of 5× buffer, 0.5 µl of 10 mM dNTPs, 0.25 µl of each primer (10 mM),

0.25 µl of One-Step RT-PCR enzyme mix and 0.5 µl of total RNA diluted 1:2. Samples were incubated for 30 min at 50°C, followed by 15 min at 95°C. After this step, amplification reactions were run for 40 cycles at 94°C for 30 sec, 60°C for 30 sec, and 72°C for 40 sec using the same *T. calospora* and *S. vomeracea* specific primers used for RT-qPCR (Table S2 and S3). DNA contamination in the RNA samples were checked using primers for the plant (*SvEF-1α*) and the fungal (*TcEF-1α*) housekeeping genes, following the One-Step RT-PCR protocol. PCR products were visualized after electrophoresis on a 1.5% agarose gel.

Statistical analysis

For statistical assessment of significant differences (P -value ≤ 0.05) in gene expression among conditions, REST[®] Relative expression software tool (Pfaffl et al., 2002) was used. The mathematical model used in this software is based on the correction for exact PCR efficiencies and the mean crossing point deviation between sample groups and control groups. Subsequently the expression ratio results of the investigated transcripts were tested for significance by a pairwise fixed reallocation randomization test ©.

Stable isotope tracer and high spatial resolution secondary ion mass spectrometry (SIMS)

Sample preparation and labeling

Seeds of *S. vomeracea* were surface sterilized in a solution of a sodium hypochlorite (0.5% available hypochlorite) for 1 min followed by three 1-minute rinses in sterile distilled water and germinated symbiotically on 0.3% OA medium with the fungus *T. calospora* for 1 month. The stable isotope tracers were provided to the fungal hyphae by using the same setup described by Kuga et al. (2014). Briefly, six symbiotically grown protocorms were transferred on an OA medium disc (5 mm in diameter) placed on the cap of a 0.5-ml Eppendorf tube overlying OA medium on a glass slide and kept in a moist Petri dish. 40 days after transfer, the fungal hyphae had reached the OA medium layer on the glass slide and 40 µl of an aqueous mixture of D-glucose ($U\text{-}^{13}\text{C}_6$, 99%; Cambridge Isotope Laboratories Inc., Andover, MA, USA), $^{15}\text{NH}_4^{15}\text{NO}_3$ (98%; Cambridge Isotope Laboratories Inc.), and $\text{Mg}^{34}\text{SO}_4$ (98%, Shoko science Co., Ltd., Yokohama, Kanagawa, Japan), at final concentrations of 1, 0.2, and 0.1%, respectively, were added to the OA medium below the plastic cap, so that only the hyphae that had reached it could absorb the tracers. All incubation processes and labeling of the symbiotic protocorms were conducted in the dark at 25°C. 6 days after the isotope tracers had been added to the hyphae, protocorms were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 50 mM PIPES buffer (piperazine-1,4-bis (2-ethanesulfonic acid), pH 6.9) for 4 h at room temperature, cut in half with a sharp razor blade, and then post-fixed with 1% OsO_4 in the buffer for 2 h at room temperature. The fixed materials were then dehydrated with a graded ethanol series (50, 70, 80, 90, 95, and 100%). After infiltration with propylene oxide (50% and then 100%), samples were infiltrated and embedded in an epoxy resin (Embed-ITM; Polysciences Inc., Warrington, PA, USA), which was polymerized at 70°C for 5 days. Semithin longitudinal sections were cut using glass knives and stained with toluidine blue O (TBO) to obtain structural information by light microscopy.

Isotope imaging of ^{34}S - ^{13}C - ^{15}N -labeled protocorms by isotope microscopy

For isotope measurements, 1 µm-thick sections were cut, transferred on a drop of distilled water placed on a silicon (Si) wafer ($7 \times 7 \text{ mm}^2$) put on a glass slide. The glass slide was placed on a hot plate so that

the resin sections were adhered to the surface of the Si wafer, as described by Kuga et al. (2014). The sections on Si wafers were coated with a 70-nm layer of gold to prevent the accumulation of positive charge generated by the primary beam of the isotope microscope. The Hokudai isotope microscope system (Cameca IMS 1270 equipped with a stacked CMOS active pixel sensor, SCAPS) was used to visualize isotope distribution in cells, a technique known as isotopography (Hamasaki et al., 2013; Kuga et al., 2014; Takeda et al., 2019; Sakamoto et al., 2007; Yurimoto et al., 2003). A Cs^+ primary beam of 20 keV was homogeneously irradiated on the sample surface of c. $100 \times 100 \text{ µm}^2$ was homogeneously irradiated with a Cs^+ primary beam of 20 keV and a beam current of c. 0.8 nA. Secondary ion images of $^{12}\text{C}^{14}\text{N}^-$, $^{12}\text{C}^{15}\text{N}^-$, $^{12}\text{C}^-$, $^{13}\text{C}^-$, $^{32}\text{S}^-$, and $^{34}\text{S}^-$ scattered from the sample surface were separated with a large-radius magnetic prism and transferred onto the SCAPS ion imager using stigmatic ion optics. Exposure times for each isotopograph were 10, 100, 10, 100, 50, and 150 sec, respectively. The exit slit was narrowed to prevent $^{13}\text{C}^{13}\text{C}^-$ and $^{10}\text{B}^{16}\text{O}^-$ from interfering with $^{12}\text{C}^{14}\text{N}^-$.

Region of interest (ROI) analysis

ImageJ (Fiji, 1.53 j; <http://rsbweb.nih.gov/ij/>) was used for the primary image processing and analyses. A set of the isotope images taken from one image site were aligned and made a ratio image as follows, for example, two of the ^{12}C images were processed to make one averaged image, and then $^{13}\text{C}/^{12}\text{C}$ ratio images were obtained by dividing the ^{13}C image by the averaged ^{12}C image (abbreviated as r13C in this study). $^{12}\text{C}^{15}\text{N}/^{12}\text{C}^{14}\text{N}$ (abbreviated as r15N) and $^{34}\text{S}^{32}\text{S}$ (abbreviated as r34S) images were produced similarly. 30 colonized cortical cells and 8 uncolonized cortical and meristematic cells were subjected to the region of interest (ROI) analysis. Using $^{12}\text{C}^{14}\text{N}$ and a light microscope TBO image, outlines of fungal peloton and host nucleus were defined using a polygonal selection tool.

Within the exact ROI of correlated r13C, r15N, and r34S images, the mean intensity, the standard deviation (SD), and the number of pixels (area) were obtained. The natural abundance of ^{13}C is 1.10% (limestone from a fossil belemnite), with an expected r13C value of 0.0111. The natural abundance of ^{15}N is 0.366% (N_2 gas in the atmosphere), with an expected r15N value of 0.00367. The natural abundance of ^{34}S is 4.21% (the Vienna-Canyon Diablo Troilite), with an expected r32S value of 0.0437. Therefore, in this study, the values of r13C, r15N, and r32S were multiplied by 100, 1000, and 100, respectively, so that the tracer isotope presences were detected if the analyzed values were larger than those of the natural abundances: 1.10, 3.67, and 4.37, respectively.

The comparisons between host nuclei of colonized and uncolonized cells of two protocorm regions were conducted by Tukey–Kramer test and correlations between hyphae and nuclei within colonized cells and between isotope ratios were analyzed by Pearson's correlation coefficient.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Table S1. Accession numbers of protein sequences retrieved from NCBI Protein database and described as plant S transporters used to generate phylogenetic tree of *Serapias vomeracea* protein sequences putatively encoding for S transporters (Figure 6).

Table S2. List of primers to amplify the *Tulasnella calospora* genes

Table S3. List of primers to amplify the *Serapias vomeracea* genes

Table S4. Fold change, standard error values and p-values of statistical assessment of differences performed by Relative Expression Software Tool REST© 2009 v. 2.0.13 of *T. calospora* genes in MYC and SYMB conditions compared to FLM.

Table S5. Fold change, standard error values and p-values of statistical assessment of differences performed by Relative Expression Software Tool REST© 2009 v. 2.0.13 of *S. vomeracea* genes in symbiotic conditions compared to asymbiotic.

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3.1 Supporting Information

Table S1. Accession numbers of protein sequences keepen in NCBI Protein database and described as plant S transporters used to generate phylogenetic tree of *Serapias vomeracea* protein sequences putatively encoding for S transporters (figure 6).

Organism	Accession number	Secondary accession number
<i>Arabidopsis thaliana</i>	NP_001319061	TAIR10 AT1G22150.1
<i>Arabidopsis thaliana</i>	NP_001321366.1	TAIR10 AT1G78000.1
<i>Arabidopsis thaliana</i>	NP_192602.1	TAIR10 AT4G08620.1
<i>Arabidopsis thaliana</i>	NP_565165.2	TAIR10 AT1G77990.1
<i>Arabidopsis thaliana</i>	NP_196580.1	TAIR10 AT5G10180.1
<i>Arabidopsis thaliana</i>	NP_188220.1	TAIR10 AT3G15990.1
<i>Arabidopsis thaliana</i>	NP_173722.1	TAIR10 AT1G23090.1
<i>Arabidopsis thaliana</i>	NP_568377.1	TAIR10 AT5G19600.1
<i>Arabidopsis thaliana</i>	NP_192179.1	TAIR10 AT4G02700.1
<i>Arabidopsis thaliana</i>	NP_190758.2	TAIR10 AT3G51895.1
<i>Apostasia shenzhenica</i>	PKA49084.1	
<i>Apostasia shenzhenica</i>	PKA46798.1	
<i>Apostasia shenzhenica</i>	PKA46797.1	
<i>Apostasia shenzhenica</i>	PKA64522.1	
<i>Asparagus officinalis</i>	XP_020249002.1	
<i>Dendrobium catenatum</i>	PKU75914.1	
<i>Dendrobium catenatum</i>	XP_020699282.1	

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<i>Dendrobium catenatum</i>	PKU71672.1	
<i>Dendrobium catenatum</i>	XP_020690822.1	
<i>Dendrobium catenatum</i>	XP_020699429.1	
<i>Medicago truncatula</i>	AES97926	Mt4.0v1 Medtr5g061880.1
<i>Medicago truncatula</i>	AES63416	Mt4.0v1 Medtr2g008470.1
<i>Medicago truncatula</i>	AES72252	Mt4.0v1 Medtr3g087730.1
<i>Medicago truncatula</i>	AES72253	Mt4.0v1 Medtr3g087740.1
<i>Medicago truncatula</i>	AES76723	Mt4.0v1 Medtr6g086170.1
<i>Medicago truncatula</i>	AES60845	Mt4.0v1 Medtr1g071530.1
<i>Oryza sativa</i>	XP_015650733.1	v7.0 LOC_Os08g31410.5
<i>Oryza sativa</i>	XP_015631773	v7.0 LOC_Os03g09980.1
<i>Oryza sativa</i>	XP_015631772.1	v7.0 LOC_Os03g09970.4
<i>Oryza sativa</i>	XP_015630786.1	v7.0 LOC_Os03g09940.1
<i>Oryza sativa</i>	XP_015630778.1	v7.0 LOC_Os03g09930.1
<i>Oryza sativa</i>	XP_015643230.1	v7.0 LOC_Os06g05160.1
<i>Oryza sativa</i>	XP_015637166.1	v7.0 LOC_Os04g55800.1
<i>Oryza sativa</i>	XP_015625323.1	v7.0 LOC_Os01g52130.1
<i>Oryza sativa</i>	XP_015613399.1	v7.0 LOC_Os10g28440.1
<i>Oryza sativa</i>	XP_015633200.1	v7.0 LOC_Os03g06520.1
<i>Populus trichocarpa</i>	XP_024456321.1	v3.0 Potri.005G169300.1
<i>Populus trichocarpa</i>	XP_002301036.1	v3.0 Potri.002G092500.1
<i>Populus trichocarpa</i>	XP_002302276	v3.0 Potri.002G092400.1
<i>Populus trichocarpa</i>	XP_024461005.1	v3.0 Potri.007G088700.1
<i>Populus trichocarpa</i>	XP_024461005	v3.0 Potri.005G079000.1

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<i>Populus trichocarpa</i>	XP_002303279.3	v3.0 Potri.003G056200.1
<i>Populus trichocarpa</i>	XP_024445957.1	v3.0 Potri.001G179400.1
<i>Populus trichocarpa</i>	XP_002314803.3	v3.0 Potri.010G111700.1
<i>Populus trichocarpa</i>	XP_006379763.1	v3.0 Potri.008G130400.1
<i>Populus trichocarpa</i>	XP_024459242.1	v3.0 Potri.006G158900.1
<i>Populus trichocarpa</i>	XP_002314667.2	v3.0 Potri.010G082700.1
<i>Populus trichocarpa</i>	XP_024463147.1	v3.0 Potri.008G156600.1
<i>Populus trichocarpa</i>	XP_002300821.2	v3.0 Potri.002G049500.1
<i>Populus trichocarpa</i>	XP_002307605.1	v3.0 Potri.005G213500.1
<i>Panicum virgatum</i>	XP_039813216	
<i>Phalaenopsis equestris</i>	XP_020585460	
<i>Phalaenopsis equestris</i>	XP_020585462.1	
<i>Phalaenopsis equestris</i>	XP_020596022.1	
<i>Phalaenopsis equestris</i>	XP_020585326.1	
<i>Phalaenopsis equestris</i>	XP_020598542.1	
<i>Phalaenopsis equestris</i>	XP_020598462.1	
<i>Phoenix dactylifera</i>	XP_038980148.1	
<i>Phoenix dactylifera</i>	XP_008780888.2	
<i>Phoenix dactylifera</i>	XP_008793448.1	

Table S2. List of primers to amplify the *Tulasnella calospora* genes

Sequence ID	Putative Sequence name	Primer forward	Primer reverse	Mean efficiency	% efficiency	St. error
20907	<i>TcSULP1</i>	ACCTCAGCTACCTGACGGAT	CAAGCGCCACCAGTTCTCTA	1.77	77.17	0.005
20008	<i>TcSULP2</i>	CGCGAGGTTACCTTCCACTT	AACGGGTTGGATGGTGCTC	1.74	74.33	0.004
215409	<i>TcSULP3</i>	CCTATCATGCTTGTGGCGC	GCTCGACGCTCCGGTATAAA	1.73	72.67	0.003
241285	<i>TcPSRed</i>	ACAAGAGCATCGGTGACTGG	TTAAACTTCGACCCGCGTGA	1.78	78.33	0.004
14111	<i>TcATPSUL</i>	AGACAGACAGGAAAGGCGTG	TCCACTGTAATATCGGCCGC	1.79	79.17	0.006
75899	<i>TcAphk1</i>	CGTCGTATAGCCGAGGTGTC	CCGAGATGCCCGTGAAATTCT	1.74	73.56	0.006
33232	<i>TcAphk2</i>	ATCACTTTCCACCCCGGATC	CGATCGTCGACTTGCCCACTA	1.78	78.33	0.005
65027	<i>TcSR</i>	CTCGCTACCATGCTCGGGCGG	CCGCCGCACTTCTCTGGTG	1.59	59.17	0.019
20203	<i>TcSul</i>	GCTCAACGCCCGAACACCCA	GTACACCGGCTTGCCCCACC	1.75	74.83	0.008
15813	<i>TcEF-1α</i>	CAAGCCTATGTCGTTGAGA	ACGAGAATGCGAAGGAAGAA	1.76	76.06	0.005

Table S3. List of primers to amplify the *Serapias vomeracea* genes

Sequence ID	Putative Sequence name	Primer forward	Primer reverse	Mean efficiency	% efficiency	St. error
<i>R2_c16339</i>	<i>SvST1</i>	ACTCGAGTGCAGTTCAAGCT	CTATCAGGCCAGCCCTTGAG	1.78	77.83	0.01
<i>R2_rep_c202</i>	<i>SvST2</i>	AAAGGGCGGTCGACATGAAT	AGTGCTGTGATCTGCTGTT	1.78	77.75	0.01
<i>R2_rep_c5242</i>	<i>SvST3</i>	CTGTGATGAGTTGTGCCCT	TCCACGAATTTTCGGGTAGCC	1.86	85.67	0.042
<i>R2_c13484</i>	<i>SvST4</i>	CCAAAGGCCAGGATTTCAG	CTTGACCCCTTGTCTGTCAA	1.73	73.25	0.011
<i>TRINITY_DN68835_c1_g2_i1</i>	<i>SvST5</i>	TGACCCCATCACCAGAGAT	TCGTGCTCTGCCCATCATCTG	1.83	82.67	0.024
<i>TRINITY_DN63650_c0_g1_i1</i>	<i>SvST6</i>	AGAGACCTCCAGGCTCAACA	GCTTTGTGCCCCCACTGATA	1.78	77.92	0.005
<i>TRINITY_DN63650_c0_g2_i1</i>	<i>SvST7</i>	TGGGAATTCTGATGGCATCC	TCAGTGCTTTCCTTGGGGTC	1.78	78.42	0.007
<i>TRINITY_DN76349_c3_g11_i1</i>	<i>SvST8</i>	TTCTTCCCCGACGATCCTCT	TGCATAGCTTATCCCCGTGCG	1.82	82.17	0.035

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TRINITY_DN63650_c0_g4_i1	SvST9	GAGGTACCCTACGATGCAGC	ATCTGCAGCAGCTCTCGATC	1.78	77.5	0.013
TRINITY_DN69207_c1_g1_i1	SvATPSu1	AGAAAGCTTCCCTTCGCAGAC	CTTCCCTCCTCCGAAACCC	1.78	77.83	0.009
TRINITY_DN70992_c0_g1_i2	SvAPSKin2	CACCGGTCCCAACCTATCC	TCTCTCTCGCTTTCTCCCGA	1.79	78.92	0.015
TRINITY_DN58139_c0_g1_i1	SvAPSRed1	AGGCATCATACGTCGAGCTG	CTCGGACGGATACTTCACCG	1.74	73.83	0.015
R2_c9449	SvAPSRed2	TCAATGTTCCCGAGCATCCC	AAAGCGGAAAGGGAACCGAT	1.79	78.83	0.008
R2_c11840	SvAPSRed3	TATGAAAGCCAGCGGAAGCA	ATCAACAGCAGGAAGTCGCA	1.75	74.92	0.012
R2_rep_c7777	SvSR1	AATGTTGGACAAGTGGGACG	ATGACGTCCATGGCTAGCTG	1.79	78.92	0.01
TRINITY_DN76001_c0_g1_i1	SvSR2	GATCTTCGACTCCGCGCTCAG	TAGCCGCCGTTCCCTTTTCT	1.76	75.92	0.014
TRINITY_DN74740_c3_g1_i1	SvSTRF	TTCCGAAGTGATACGCGGAG	ACATGTGCCTAACCTGGACG	1.77	77.42	0.016

Table S4. Fold change, standard error values and p-values of statistical assessment of differences performed by Relative Expression Software Tool REST© 2009 v. 2.0.13 of *T. calospora* genes in MYC and SYMB conditions compared to FLM.

<i>Tulasnella calospora</i> gene	Putative Function	MYC			SYMB		
		Fold Change	Std. Error	p-value	Fold Change	Std. Error	p-value
<i>TcSULP1</i>	Sulfate permease	1.300	1.055 - 1.605	0.008	0.715	0.588 - 0.902	0.006
<i>TcSULP2</i>	Sulfate permease	0.251	0.139 - 0.575	0.000	1.663	0.795 - 2.912	0.129
<i>TcSULP3</i>	Sulfate permease	1.336	0.817 - 2.419	0.260	0.532	0.341 - 0.752	0.001
<i>TcATPSUL</i>	ATP sulfurylase	0.833	0.442 - 1.468	0.545	12.049	7.716 - 19.076	0.002
<i>TcAphK1</i>	APS kinase	0.996	0.682 - 1.338	0.975	7.263	5.453 - 10.044	0.001
<i>TcAphK2</i>	APS kinase	0.942	0.690 - 1.483	0.700	5.594	4.577 - 6.918	0.002
<i>TcPSRed</i>	PAPS reductase	0.356	0.111 - 0.906	0.036	6.915	4.097 - 10.974	0.002
<i>TcSR</i>	Sulfite reductase	0.778	0.604 - 1.034	0.066	2.179	1.505 - 3.153	0.001
<i>TcSul</i>	Sulfite efflux	1.679	1.416 - 1.963	0.001	1.947	1.146 - 2.899	0.003

Plant and fungal gene expression coupled with stable isotope labelling provide novel information on sulfur uptake and metabolism in orchid mycorrhizal protocorms

Table S5. Fold change, standard error values and p-values of statistical assessment of differences performed by Relative Expression Software Tool REST© 2009 v. 2.0.13 of *S. vomeracea* genes in symbiotic conditions compared to asymbiotic.

<i>Serapias vomeracea</i> gene	Putative Function	Fold Change	Std. Error	p-value
<i>SvST1</i>	Sulfate transporter (Group 4 SULTR)	2.106	± 1.82	0.274
<i>SvST2</i>	Sulfate transporter (Group 3 SULTR)	99.814	± 16.48	0.000
<i>SvST3</i>	Sulfate transporter (Group 1 SULTR)	3.179	± 4.96	0.189
<i>SvST4</i>	Sulfate transporter (Group 3 SULTR)	1.405	± 3.90	0.440
<i>SvST5</i>	Sulfate transporter (Group 3 SULTR)	17.564	± 5.13	0.006
<i>SvST6</i>	Sulfate transporter (Group 3 SULTR)	51.529	± 22.88	0.000
<i>SvST7</i>	Sulfate transporter (Group 3 SULTR)	27.134	± 15.01	0.000
<i>SvST8</i>	Sulfate transporter (Group 3 SULTR)	45.693	± 21.10	0.001
<i>SvST9</i>	Sulfate transporter (Group 4 SULTR)	38.310	± 26.66	0.002
<i>SvATPSul</i>	ATP sulfurylase	0.897	± 1.06	0.874
<i>SvAPSKin2</i>	APS kinase	1.527	± 8.03	0.560
<i>SvAPSRRed1</i>	APS reductase	0.481	± 0.93	0.284
<i>SvAPSRRed2</i>	APS reductase	0.798	± 0.82	0.684
<i>SvAPSRRed3</i>	APS reductase	1.585	± 3.51	0.516
<i>SvSR1</i>	Sulfite reductase	1.066	± 0.20	0.898
<i>SvSR2</i>	Sulfite reductase	0.840	± 1.90	0.751
<i>SvSTRF</i>	Sulfotransferase	25.274	± 0.93	0.002

CHAPTER 4

Integration of fungal transcriptomics and metabolomics provides insights into the early interaction between the ORM fungus *Tulasnella* sp. and the orchid *Serapias vomeracea* seeds

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Integration of fungal transcriptomics and metabolomics provides insights into the early interaction between the ORM fungus *Tulasnella* sp. and the orchid *Serapias vomeracea* seeds

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Abstract

In nature, germination of orchid seeds and early plant development rely on a symbiotic association with orchid mycorrhizal (ORM) fungi. These fungi provide the host with the necessary nutrients and facilitate the transition from embryos to protocorms. Despite recent advances in omics technologies, our understanding of this symbiosis remains limited, particularly during the initial stages of the interaction. To address this gap, we employed transcriptomics and metabolomics to investigate the early responses occurring in the mycorrhizal fungus *Tulasnella* sp. isolate SV6 when co-cultivated with orchid seeds of *Serapias vomeracea*. The integration of data from gene expression and metabolite profiling revealed the activation of some fungal signalling pathways before the establishment of the symbiosis. Prior to seed contact, an indole-related metabolite was produced by the fungus, and significant changes in the fungal lipid profile occurred throughout the symbiotic process. Additionally, the expression of plant cell wall-degrading enzymes (PCWDEs) was observed during the pre-symbiotic stage, as the fungus approached the seeds, along with changes in amino acid metabolism. Thus, the dual-omics approach employed in this study yielded novel insights into the symbiotic relationship between orchids and ORM fungi and suggest that the ORM fungus responds to the presence of the orchid seeds prior to contact.

Keywords Orchids, Symbiosis, Mycorrhizal fungi, Omics, CAZymes

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Introduction

Like most land plants, terrestrial orchids form symbiotic relationships with soil fungi (Genre et al. 2020; Selosse et al. 2022). In their natural habitat, the symbiotic relationships between orchids and their mycorrhizal fungi play a pivotal role during the initial phases of plant growth. Although orchid seeds store lipids within the embryo, which are used in the early stage of germination (Lee et al. 2007), they generally lack sufficient internal energy resources to support germination. Therefore, for several orchid species, the symbiotic fungal partner supplies the plant with organic carbon and other essential nutrients. This trophic strategy is known as initial myco-heterotrophy (Merckx 2012). The supply of nutrients by orchid mycorrhizal (ORM) fungi influences seed germination and the subsequent development of the immature embryo into a protocorm, a postembryonic structure typical of orchids (Smith and Read 2010; Dearnaley et al. 2016). The ORM fungi are a heterogeneous group of mostly basidiomycetes belonging to different families including the families Tulasnellaceae, Ceratobasidiaceae (in the Cantharellales) and Serendipitaceae (in the Sebaciniales) (Dearnaley et al. 2012). Experimental evidence from stable isotope and gene expression studies has demonstrated that, in addition to carbon (C), ORM fungi can provide the plant with phosphorus (P), nitrogen (N), and sulfur (S) (Cameron et al. 2006, 2007, 2008; Kuga et al. 2014; Fochi et al. 2017a,b; De Rose et al. 2023a). The contribution of ORM fungi to plant nutrition is observed throughout the orchid life cycle, from protocorm formation to the development of adult plants, where the fungi become mycorrhizal partners (Valadares et al. 2021). The transfer of nutrients primarily occurs across the extensive plant-fungus interface that surrounds the *pelotons*, the coiled intracellular fungal hyphae formed in ORM (Peterson and Farquhar 1994).

The advancement of *omics* technologies has provided researchers with powerful tools to investigate symbiotic mechanisms, including ORM. In the last decade, transcriptomics and metabolomics approaches have revealed intriguing molecular aspects of ORM interactions and identified potential molecular players in nutrient transfer (Perotto et al. 2014; Fochi et al. 2017a, b a,b; Ghirardo et al. 2020). In particular, RNA sequencing (RNA-seq) studies have enabled the identification of plant amino acid, oligopeptide, ammonium, and phosphate transporters, thereby providing insights into the mechanisms by which nutrients are acquired by the plant (Zhao et al. 2014; Fochi et al. 2017a,b; Miura et al. 2018; Ho et al. 2021; Kambara et al. 2023; De Rose et al. 2023b). However, while previous studies have primarily concentrated on the regulation of plant responses and transporters, there is a paucity of information on fungal

genes, metabolites and associated pathways involved in the symbiotic interaction with the orchid host (Favre-Godal et al. 2020; Li et al. 2021). A proteomic approach identified the accumulation of fungal proteins involved in stress response, protein–protein interactions, and protein biosynthesis in the tissues of *Oncidium sphacelatum* during symbiosis with a *Ceratobasidium* sp. isolate (Valadares et al. 2014). Transcriptomic profiles of free-living and symbiotic mycelium of *Tulasnella calospora* inside *Serapias vomeracea* protocorms led to the identification of fungal genes coding for high and low affinity ammonium transporters and for several amino acid transporters/permeases (Fochi et al. 2017a). The expression pattern of these fungal genes, in conjunction with the expression pattern of the host plant genes, provided compelling evidence that nitrogen is transferred to the host plant as amino acids, at least in the studied species (Fochi et al. 2017a). More recently, a metabolomic approach was used to decipher the fungal response during ORM, showing the induction of structural and signalling lipids in the external fungal mycelium that developed in close proximity to the symbiotic protocorms (Ghirardo et al. 2020). These data highlighted the need for a detailed analysis of fungal gene expression remodelling and metabolomic shifts during the symbiotic relationships.

In the process of plant colonization, the first physical structure encountered by ORM fungi is the cell wall of the orchid seeds (Balestrini and Bonfante 2014; Dearnaley et al. 2016; Martin et al. 2016; Favre-Godal et al. 2020). Comparative genomic and transcriptomic studies of mycorrhizal fungi, including ORM fungi (Kohler et al. 2015), revealed that the genomes of ORM fungi belonging to the Tulasnellaceae, Ceratobasidiaceae and Serendipitaceae are characterized by an extensive set of genes coding for carbohydrate-active enzymes (CAZymes) that act on pectins, cellulose and hemicellulose. When compared with the genomes of ectomycorrhizal and endophytic fungi within Agaromycotina, the *T. calospora* genome exhibited the highest number (176) of CAZymes-related genes (Kohler et al. 2015). A significant proportion of these plant cell wall-degrading enzymes (PCWDEs) were expressed during the symbiotic stages (Kohler et al. 2015; Chen et al. 2022), thereby supporting the hypothesis that PCWDEs are functionally activated to facilitate the penetration of host cells by the fungus and thus contribute to the establishment of ORM. These enzymes undergo dynamic regulation during the interaction with the plant, as Adamo et al. (2020) found changes in the expression of ORM fungal genes encoding PCWDEs during the transition from a symbiotic to a saprotrophic interaction.

In this study, we employed both transcriptomics and non-targeted metabolomics to investigate *in vitro* potential changes in fungal gene expression and metabolite

production during the initial stages of interactions with the host. In particular, we tested the hypotheses that (I) the ORM fungus senses and responds to the presence of *Serapias vomeracea* prior to contact with the orchid seeds; (II) the transcriptomic and metabolomic profiles of *Tulasnella* undergo significant changes during transition from pre-contact to post-contact stages; (III) the expression of fungal genes related to PCWDEs is finely tuned during the early stages of contact with orchid seeds, to favor penetration of host cell walls. To address these hypotheses, the mycelium of *Tulasnella* sp. isolate SV6 was collected at two different developmental stages prior to contact with *S. vomeracea* seeds, and as mycelium growing either in close proximity or inside symbiotic protocorms after symbiosis establishment.

Materials and methods

Plant and fungal materials

Seeds of *S. vomeracea* used in this study were collected during summer 2021 from mature capsules of wild plants grown in the locality of Cairo Montenotte (SV, Italy; Latitude 44.406944 N, Longitude 8.319795 E) and stored at 4 °C. The fungal isolate SV6 (MUT4178), belonging to the *Tulasnella* genus, was originally isolated from *Serapias vomeracea* roots grown in Northern Italy (Girlanda et al. 2011) and stored in the Mycotheca Universitatis Taurinensis (MUT) at the University of Turin. After thawing, vitality of the fungal isolate was verified by growing it in pure culture on solid 2% malt extract agar at 25 °C in the dark.

Experimental design and sample preparation for transcriptomic and metabolomic analyses

The regulation of fungal gene expression and the associated metabolomic remodelling during the initial stages of the interaction and the establishment of the symbiosis were investigated in a time-course experiment. The fungus was grown in co-culture with the plant and collected at three different time points (T1-T3) for transcriptomic (RNA-seq) and metabolomic analyses: early symbiotic stage (T1), *i.e.*, fungal mycelium before contact with the orchid seeds; pre-symbiotic stage (T2), *i.e.*, the mycelium closely reached the seeds but had no physical contact; and symbiotic stage (T3), *i.e.*, the mycelium was in the proximity of symbiotic protocorms. Additionally, we collected the symbiotic protocorms containing the fungus (namely, 'SYMB'). A schematic representation of the sampling design is provided in Fig. 1. Co-inoculation of *Tulasnella* sp. SV6 and *S. vomeracea* seeds was performed as described in Perotto et al. (2014). Seeds were surface sterilized with a solution of 1% sodium hypochlorite and 0.1% Tween-20 for 20 min, with stirring. This was followed by three 5-min rinses with sterile

distilled water. Seeds re-suspended in sterile water were then placed on dishes of autoclaved cellophane (polymeric cellulose film) membrane (Hutchinson Le Joint Français, France) previously positioned on the solid oat medium (0.3% milled oats, 1% agar). The membrane covered the surface of the 9 cm Petri dishes up to 3–4 mm from the edge, and was used to allow easy removal of the fungal material from the solid medium (Liu et al. 2010). No part of the membrane was used in the RNA extraction. A portion of actively growing mycelium was then placed in the center of each Petri dish and incubated at 20 °C in complete darkness. Samples of early-symbiotic stage (T1) were collected five days post-inoculation (dpi), when the mycelium reached approximately 5 cm in diameter, from the edge of the growing mycelium to harvest only the active growth zone; samples of the pre-symbiotic stage (T2) were collected 7 dpi (8 cm in diameter) from the edge of the growing mycelium, while samples of the symbiotic stage, consisting of mycelia grown in the proximity of (T3) and inside symbiotic protocorms (SYMB), were collected 35 dpi. Although T1 was employed as a control in the metabolomic and transcriptomic analyses, a free-living *Tulasnella* sp. SV6 mycelium (FLM) was also used for comparison. The FLM was obtained by growing the fungus on solid oat medium at 25 °C in the dark for 10 days. Samples were collected by carefully removing the mycelium (T1, T2, T3, FLM) and protocorms (SYMB) from the cellophane membrane, to ensure that no part of the membrane was used in the subsequent RNA or metabolites extraction. Three biological replicates for each condition (T1, T2, T3, SYMB, FLM) were prepared for transcriptomics and four biological replicates for metabolomics, respectively. All samples were immediately frozen in liquid nitrogen and stored at -80 °C for subsequent transcriptomic and metabolomic analyses. The SYMB sample was excluded from metabolomic analysis as it was not possible to distinguish among plant and fungal metabolites (Ghirardo et al. 2020).

Transcriptomic analysis

For the RNA-seq experiment, protocorms (SYMB) and fungal mycelia for each tested condition (T1, T2, T3, FLM) were homogenized with TissueLyser (25 Hz, 1 min, twice, Qiagen Diagnostic GmbH, Hilden, Germany) and RNA was extracted from approximately 80 mg of biological material from each biological replicate using the "pine tree" CTAB-based method (Chang et al. 1993). The RNA, eluted in RNA-seq free water, was then quantified using NanoDrop 2000 and Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The Genartis Functional Genomics Lab (Department of Biotechnology of the University of Verona), which was responsible for library preparation and RNA sequencing, evaluated

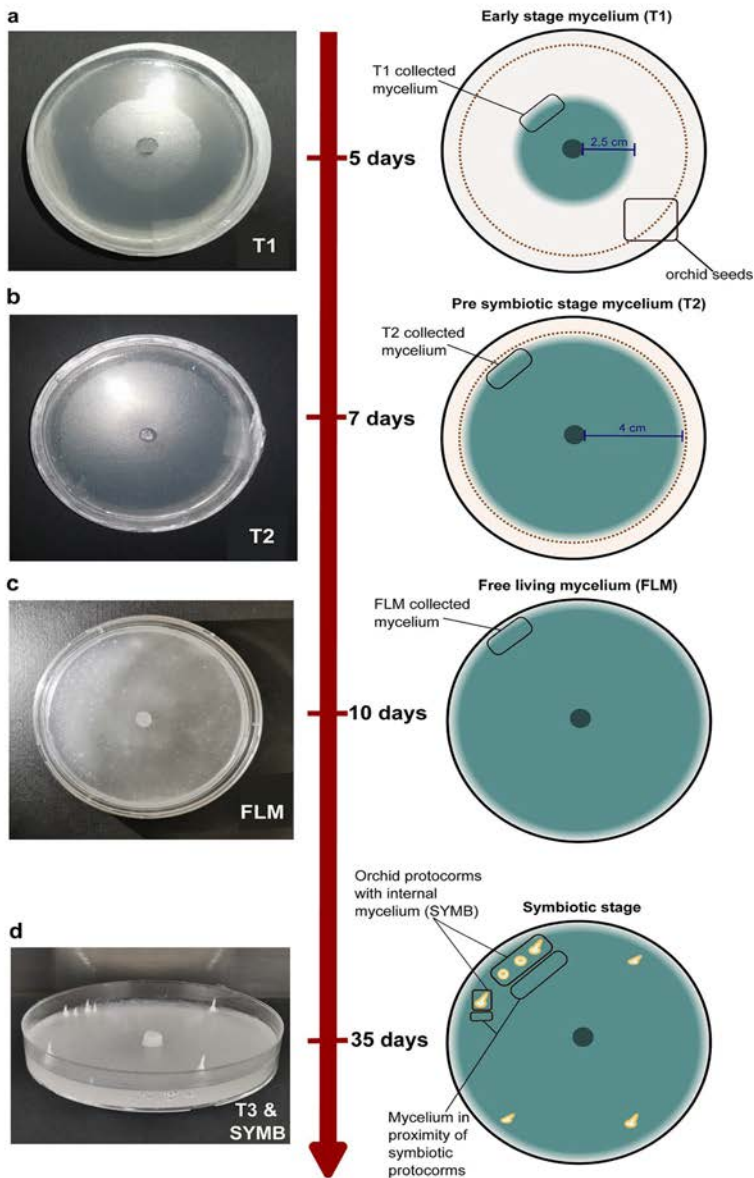


Fig. 1 Schematic representation of the sampling design for the time course experiment. Samples coded as T1 were collected at 5 days post inoculation (d.p.i) in plates (a), T2 at 7 d.p.i (b), FLM at 10 days after plug transfer in plates (c), T3 and SYMB at 35 d.p.i. (d)

the integrity and size of the RNA-seq libraries through capillary electrophoretic analysis using the Agilent 4150 Tape Station (Agilent Technologies). The generation of the library was unsuccessful for a single biological replicate of the T2 condition (replicate 1 of T2). After poly(A) capture and following the manufacturer's instructions, 14 Illumina RNA-seq libraries were created using the TruSeq stranded mRNA ligation kit (Illumina, San Diego, CA, USA) and were quantified by real-time PCR against a standard curve with the KAPA Library Quantification Kit (KAPA Biosystems, Wilmington, MA, USA). The sequencing was performed on a Novaseq 6000 sequencer with Illumina technology in 150PE mode. The reads were aligned to the *Tulasnella calospora* AL13/4D v1.0 genome available at <https://mycocosm.jgi.doe.gov/Tulca1/> (Kohler et al. 2015) using STAR v2.7.10a (Spliced Transcripts Alignment to a Reference; Dobin et al. 2013). Samtools 1.11v was used to convert SAM data into BAM files and index them (Li et al. 2009). Aligned reads on the reference genome were counted using HTseq-count 2.0.2v (Anders et al. 2014). Exon junctions and gene overlaps were mapped using the intersection method (strict).

Raw read counts were imported into the DESeq2 tool version 1.34.0 (Love et al. 2014) to identify differentially expressed genes (DEGs). Following default DESeq2 normalization of the count data (median of ratios method) and correction for multiple testing, DEG identification was carried out through Wald test and using T1 samples as controls. The three biological replicates per condition were employed to compute read count variation. To identify differentially expressed genes (DEGs), an adjusted p-value threshold of 0.05 was employed. Differentially expressed genes (DEGs) were functionally annotated in silico using Blast2GO v5.2.5 (Conesa et al. 2005), with their related Gene Ontology (GO) terms subsequently assigned. To perform a GO enrichment analysis and to provide a summary of the functions and pathways associated with the obtained sets of DEGs, Blast2GO v5.2.5 was employed. A specific database (<http://pcwde.riceb.last.snu.ac.kr/>) was used to identify genes related to PCWDEs among the detected DEGs. Furthermore, in order to identify DEGs potentially involved in transport, a search was conducted for DEGs that included the following terms: "transporter", "permease", "efflux", "carrier" and "facilitator".

Metabolomic analysis

A total of 16 frozen samples (four biological replicates for each condition; T1, T2, T3, and FLM) were freeze-dried at -50 °C under vacuum (Alpha 1–4 LDplus, Christ, Osterode, Germany) and finely ground with Tissue-Lyser (25 Hz, 1 min, Qiagen Diagnostic, Hilden, Germany). Approximately 10 mg of dried fine powder per

sample was extracted following the protocol used by Bertić et al. (2021). In short, 800 µL of methanol/2-propanol/water solution (1/1/1, v/v/v) containing 50 µL of an internal standard (IS) mixture were added to the sample (Table S2). The mixture was vortexed for 1 min, sonicated in an ultrasonic bath for 10 min at 5 °C and then centrifuged at 9300 g for 10 min at 5 °C, and 400 µL of supernatant was recovered. The centrifugation process was repeated, and the remaining 400 µL of supernatant was collected to obtain a total of 800 µL of the extracted metabolite solution. The supernatant was dried by Speed-Vac (Univapo 150H, Uniequip, Planegg, Germany), and re-dissolved in 350 µL of 50% (v/v) acetonitrile in water. After mixing, the solution was centrifuged at 9300 g for 10 min at 5 °C, and 300 µL of supernatant was transferred in amber glass vials for metabolomics analysis.

Non-targeted metabolomic analysis was performed as before (Bertić et al. 2021), using an ultra-performance liquid chromatography (UPLC) ultra-high resolution (UHR) tandem quadrupole/time-of-flight (QqToF) mass spectrometry (MS). The system consists of an Ultimate 3000RS UPLC (Thermo Fisher Scientific, Bremen, Germany), a Bruker Impact II (QqToF) and an Apollo II electrospray ionization source (Bruker Daltonics, Bremen, Germany). Metabolites were separated by reversed-phase liquid chromatography (RPLC) and by hydrophilic interaction liquid chromatography (HILIC), each run separately and eluted using H₂O + 0.1% (v/v) formic acid (solvent A) and acetonitrile + 0.1% (v/v) formic acid (solvent B) (see Bertić et al. 2021 for details). The spectra were acquired in both positive (+) and negative (–) ionization modes. The data were processed as outlined in Bertić et al. (2021), using the software Metaboscape v4.0 (Bruker Daltonics) and the parameters given in Table S1. Results of RP and HILIC analyses were merged manually and the zero values were replaced with random numbers below a threshold value of 300 area unit. Data were normalized for the IS mixture and dry mycelium weight. Annotation of compounds by matching MS/MS spectra with the libraries 'All Spectra' in MoNA (Mass Bank of North America, <https://mona.fiehnlab.ucdavis.edu/>), MS-DIAL Lipids (<http://prime.psc.riken.jp/>), MassBank (<https://massbank.eu/MassBank/>), Vaniya/Fiehn Natural Products Library (available through MoNA) and GNPS (<https://gnps.ucsd.edu/>). Mass features without MS/MS spectra were tentatively annotated on MS1 level for the precursor mass using 5.0 mDa tolerance using MetaboAnalyst 5.0 (www.metaboanalyst.ca, Pang et al. 2021). Furthermore, we used the multidimensional stoichiometric compound classification (MSCC) (Rivas-Ubach et al. 2018) approach to categorize the mass features that correspond to a molecular formula predicted by Metaboscape ('smart formula' tool). The MSCC algorithm

confidently classifies compounds into the chemical classes 'carbohydrates', 'nucleotides-related', 'lipids', 'secondary metabolites' and 'amino acids and protein-related' based on their elemental ratios of C/H, C/O, C/P, and C/S (Rivas-Ubach et al. 2018).

Pathway analysis was performed using MetaboAnalyst 5.0 (Pang et al. 2021). The Mummichog 3.0 algorithm (Li et al. 2013) coupled with Gene Set Enrichment Analysis (GSEA; Subramanian et al. 2005) was employed to perform an over-representation analysis of the entire dataset of mass features, including all ($\pm$) ionization measurements. This analysis was performed to aid in the functional interpretation of the data. Both algorithms were operated with a p -value cutoff of 0.05 and the *Saccharomyces cerevisiae* KEGG pathway library (<http://www.genome.ad.jp/kegg/>). Only pathways containing at least three entries were considered.

Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (PLS-DA) were calculated using the normalized peak areas, mean centered and pareto scaled in MetaboAnalyst 5.0. PCA was employed for analyzing sample similarities by examining their associated metabolomic patterns, whereas PLS-DA for deciphering metabolomic changes between sample groups. Mass features with a score of Variable Importance of Projection (VIP) > 1 in the first component were selected and resulting mass features were blank corrected and zero filtered. Log_2FC (using T1 as control) were calculated, applying Student t -test and Benjamini–Hochberg correction to discriminate significant values (adj. p value score < 0.05).

Integration between transcriptomic and metabolomic data

The online platform Omics Fusion (<https://fusion.cebit.ec.uni-bielefeld.de/>; Brink et al. 2016) was used to integrate data from RNA-seq and non-targeted metabolomics. The input data was the Log_2FC data of DEGs (adj. p value < 0.05) and metabolites (VIP ≥ 0.99 , FDR < 0.05 , and $\text{Log}_2\text{FC} \neq 1$). To identify co-regulated transcripts and metabolites, metabolomic and transcriptomic data were used as input for optimal cluster detection and grouping via neural gas cluster analysis (Qin and Suganthan 2004) embedded in Omics Fusion, setting 2 and 5 as minimum and maximum cluster to be computed. Furthermore, a correlation analysis was conducted to investigate the relationship between metabolomic and transcriptomic data across the two stages, T2 and T3. This was achieved by calculating the Pearson correlation coefficient. A scatter plot was generated for the purpose of data visualization. The dual-stage correlation analysis enabled the identification of biological patterns that were similarly or differentially regulated across the two time points. To show the dynamic shifts in transcript abundance and metabolite

contents in the different stages, alluvial plots using normalized read count and normalized mass feature peak areas, respectively, were generated. Both transcripts and metabolites were grouped based on GO terms and MSCC algorithm classification, respectively, to obtain five categories, *i.e.*, Carbohydrates, Lipids, Nucleotides, Proteins/peptides/amino acids, and Secondary metabolites.

Results

Distinct transcriptomic profiles of *Tulasnella* sp. SV6 during early stages of the interaction with *S. vomeracea*

Sequencing of RNA samples produced an average of 36 million raw reads per sample (ranging from 28,533,782 to 62,912,328; Table S3). Overall, reads mapped on the *Tulasnella calospora* AL13/4D v1.0 reference genome resulted in an average mapping rate of 72%. The mapping rate in fungus-containing protocorms (SYMB) was low (ranging from 51 to 58%) due to the presence of both fungal and plant transcripts, while samples T1, T2, T3 and FLM showed a mapping rate ranging from 75 to 80%. The total numbers of normalized read counts of *Tulasnella* sp. SV6 are reported in Table S4. The principle component analysis in Fig. 2a clearly shows that replicates of each RNA sample clustered into four distinct groups, with samples of early stage (T1), pre-symbiotic stage (T2) and mycelium grown in close proximity of symbiotic protocorms (T3) being well separate from samples of mycelium inside symbiotic protocorms (SYMB) by the first principal component (PC1), explaining 81% of the total variance. Differences between T1, T2 and T3 samples were less pronounced, as these samples were only separated in PC2, which accounted for 7% of the total variance (Fig. 2a). Fungal gene expression in T2, T3 and SYMB was compared to the early stage T1 in order to follow modulation of differentially expressed genes (DEGs) during the early events of symbiosis establishment. Sample T1 represents the earliest growth stage of the fungus in Petri dishes containing the orchids seeds, well before seed colonization, and it provided therefore a baseline condition. Additionally, gene expression in free-living mycelium (FLM) was compared with T1 samples to assess differences in gene expression between the mycelium grown in the presence (T1) and in the absence (FLM) of orchid seeds. A total of 8,153 genes (out of 19,659 *Tulasnella calospora* AL13/4D v.1 total genes) were found to be differentially regulated with adj. p value < 0.05 . The DEGs identified in the different stages of the interactions are listed in Tables S5–S8 for T2, T3, SYMB and FLM samples, respectively. In Fig. 2b, Volcano plots showed the distribution of DEGs detected in T2, T3, SYMB, as compared to T1.

A limited transcriptomic reprogramming was observed in T2 and T3 samples in comparison to T1 (Fig. 3a). In

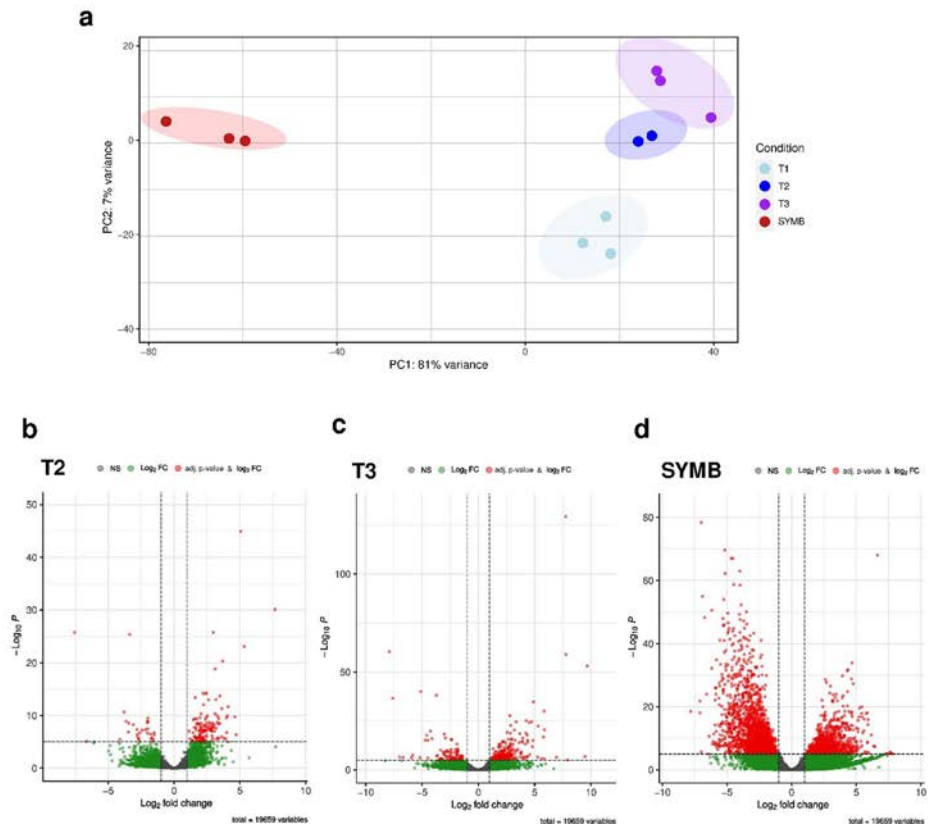


Fig. 2 RNA-seq results. In **a**, principal components analysis (PCA) of normalized read counts of all samples used in RNAseq experiment. In **b–d**, Volcano plots showing identified DEGs in T2, T3, SYMB, respectively, compared with T1 (control condition). Significant up- and down-regulated genes were represented by red dots ($\text{Log}_2\text{FC} > 1$ and < -1 , p adjusted value < 0.05). Green dots represented genes showing a $\text{Log}_2\text{FC} > 1$ and < -1 and a not-significant p adjusted value (p adjusted value > 0.05). Grey dots represented genes showing both a Log_2FC ranged from -1 and 1 , and a not-significant p -adjusted value (p adjusted value > 0.05)

the T2 samples, a total of 822 significantly up and 412 down-regulated fungal DEGs were found (Fig. 3b and Table S5), while T3 samples showed 678 significantly up- and 396 down-regulated DEGs (Fig. 3b and Table S6). As expected, a much more intense transcriptomic reprogramming was observed in SYMB samples (Fig. 3a). This stage showed the highest number of significantly up- and down-regulated fungal genes (2,813 up-regulated and 3,032 down-regulated DEGs; Fig. 3b and Table S7). As also expected, the FLM samples showed a very small number of DEGs when compared to the T1 samples (72

up- and 70 down-regulated genes; Table S8). A comparison of gene expression in the T2, T3 and SYMB samples revealed both shared and exclusive DEGs (Fig. 3b and Table S9). A core set of 182 genes were regulated across all conditions tested in the co-cultivation experiment. Among these DEGs, 44 transcripts increased in all three conditions and were primarily associated with fundamental cellular processes, including post-translational modifications. The other 142 DEGs were down-regulated in all three conditions and were mainly involved in protein post-translational modifications, but also in signal

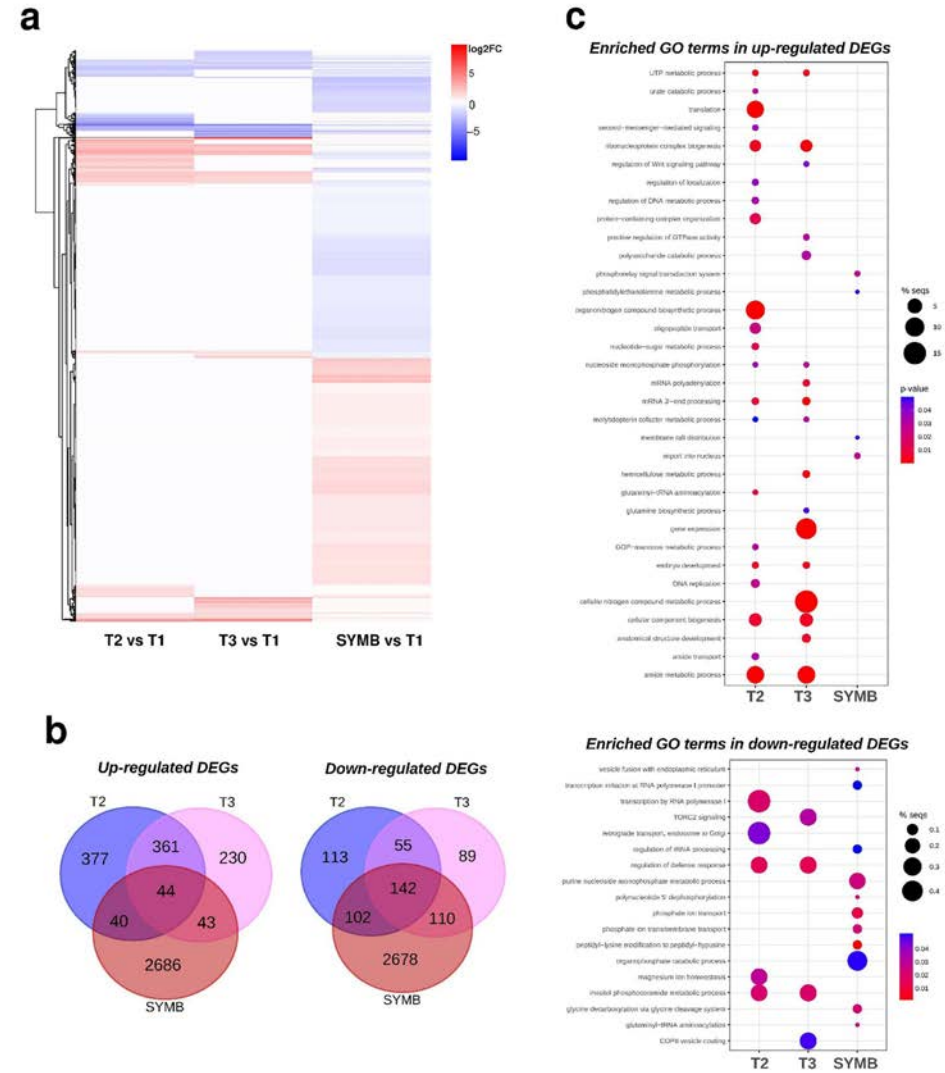


Fig. 3 Heatmap and Venn diagrams depicting DEGs in the conditions T2, T3 and SYMB versus T1, and GO analysis results. In **a**, heatmap and hierarchical clustering of DEGs using the McQuitty algorithm. The heatmap shows the expression patterns of DEGs across three conditions compared to T1, with red indicating up-regulated and blue representing down-regulated DEGs. Different color intensity indicates different levels of expression based on $\log_2FC$. In **b** Venn diagrams illustrate the overlap of up- and down-regulated DEGs, respectively, between pairwise comparisons of the conditions. In **c**, bubble plots showing GO-enriched terms classified as Biological Process (BP) in detected up and down regulated DEGs. The x-axis shows the fold enrichment values, i.e., the percentage of genes in the selected DEG list belonging to a pathway divided by the corresponding percentage in the all reference gene list, and the y-axis reports the GO terms. Sizes of bubbles are proportional to the number of genes assigned to the related GO term, while bubbles color indicates the significance of the enriched term (False Discovery Rate values) as calculated by the enrichment analysis by Blast2GO

transduction mechanisms (Table S9). It is noteworthy that 152 DEGs (Table S9) were shared among the three conditions tested yet exhibited different regulation in the mycelium outside or inside the host plant. In particular, they were up-regulated in T2 and T3, indicating induction in the fungal mycelium growing outside the host, either prior or after contact with the plant, but down-regulated in SYMB. The annotated biological gene functions associated to these 152 DEGs were mainly related to nucleic acid metabolism, oligopeptide transport and GTPase activity. Genes respectively coding for a protein annotated as a small secreted protein (transcript ID: 242048), a carbohydrate esterase family 15 protein (transcript ID: 18233), a $\text{Cu}^{2+}/\text{Zn}^{2+}$ superoxide dismutase SOD1 (transcript ID: 245590), and 5 proteases/peptidases (transcript IDs: 242177, 4507, 3720, 28523, 222978) were also identified as being up-regulated in T2 and T3 but down-regulated in SYMB samples (Table S9).

Some DEGs were identified exclusively in T2 (377 up-regulated and 112 down-regulated) or in T3 (230 up-regulated and 89 down-regulated) samples (Fig. 3b). In contrast, 416 DEGs were shared between T2 and T3 (Fig. 3b and Table S9) and were regulated with a similar transcriptional profile (*i.e.*, 361 up- and 55 down-regulated). Several of them encoded proteins involved in translation, ribosomal structure and biogenesis, signal transduction mechanisms, as well as genes putatively associated with transport and metabolism of lipids, amino acids, secondary metabolites, and carbohydrates (Table S9).

A total of 2686 up- and 2678 and down-regulated DEGs were found to be exclusive of the SYMB samples (Fig. 3b). This large DEGs dataset featured genes associated with transmembrane monodehydroascorbate reductase activity, intracellular trafficking, secretion and vesicular transport, cell wall/membrane/envelope biogenesis, transport and metabolism of macromolecules. Among the DEGs exclusive of SYMB samples, most genes putatively encoding glutathione S-transferases (9 out of 10) were down-regulated, whereas a glutathione synthase (transcript ID: 70710) was up-regulated (Table S9).

GO enrichment analysis suggests dynamic changes in metabolic processes before and after contact with the host

A GO enrichment analysis was conducted to identify the biological processes, molecular functions and signaling pathways that were involved in the early interactions between the fungus and the orchid seeds. The DEGs were grouped into functional classes, based on their putative involvement in specific biological processes (Fig. 3c). The GO terms in the T2 up-regulated transcriptome were mainly related to translation, organonitrogen compound

biosynthetic process, amide metabolic process and oligopeptide transport (Fig. 3c). In the T3 transcriptome, the most prevalent GO terms among the up-regulated genes were those involved in the cellular nitrogen compound metabolic process and gene expression (Fig. 3c). Among the down-regulated genes, the main enriched GO terms in T2 and T3 were involved in the regulation of defense response and inositol phosphoceramide metabolic process (Fig. 3c). Four different significant GO terms were identified for the up-regulated genes in the SYMB condition, although with a low percentage of sequences, and were related to the phosphorelay signal transduction system, import into nucleus, membrane raft distribution and phosphatidylethanolamine metabolic process (Fig. 3c). The GO terms enriched for the down-regulated genes in SYMB were mainly related to the organophosphate catabolic process (Fig. 3c).

Some fungal genes are up-regulated exclusively in the pre-symbiotic stage of the interaction (T2)

To investigate the influence of orchid seeds on the fungal transcriptome prior to contact with the plant, we focused on the top 20 DEGs up-regulated exclusively in the pre-symbiotic stage T2 (Table 1). The analysis revealed genes putatively encoding a PIH1/Nop17-domain-containing protein, a heat shock protein (HSP) family 20, a calmodulin, an oligopeptide transporter, an amphiphysin Hob1p, and a priA protein suggested to be involved in fungal fruiting body development (Yan and Ma 2024). A chitin synthase belonging to the Glycosyl Transferase (GH) Family 2 was also identified among the 20 most highly up-regulated genes in T2, in conjunction with an amino acid/polyamine transporter I, an ABC transporter, and two vegetative incompatibility proteins. In addition to the aforementioned top 20 up-regulated genes, other relevant genes within the entire dataset of T2 up-regulated DEGs (Table S5) included those coding for an additional HSP20-like chaperone (transcript ID: 241977, Log_2FC : 1.13), a myosin (transcript ID: 242888, Log_2FC : 1.55), and a calmodulin (transcript ID: 241437, Log_2FC : 1.68). Furthermore, putative kinase-like proteins, which are also involved in signal transduction mechanisms (Kosti et al. 2010), exhibited a slight increase in expression, as well as a C-type lectin (transcript ID: 17225, Log_2FC : 1.05). In the amino acid metabolism, two dehydrogenases were up-regulated, one NADP-specific for glutamate (transcript ID: 77118, Log_2FC : 2.07) and one homoserine specific (transcript ID: 79986, Log_2FC : 1.18). Additionally, genes coding for asparagine synthase (transcript ID: 66686, Log_2FC : 1.2), and a cobalamin-independent methionine synthase (transcript ID: 96062, Log_2FC : 1.49) were identified.

Table 1 List of top 20 DEGs up-regulated exclusively in T2 stage, as compared to T1

Transcript ID	Log ₂ FC	Log ₂ FCSE	Adj. p value	Blastx (best identified match) [species]	Accession	e-value	Identity (%)
19022	3.61	0.63	1.15E-06	Pre-RNA processing PIH1/Nop17-domain-containing protein [<i>Pisolithus orientalis</i>]	XP_051595886.1	1e-49	38.83
66522	3.43	1.16	3.73E-02	HSP20-like chaperone [<i>Suillus paluster</i>]	XP_041178010.1	2e-18	46.39
18523	3.35	0.60	2.56E-06	calmodulin [<i>Rhodofomes roseus</i>]	XP_047772407.1	6e-27	40.00
75873	3.29	0.77	7.46E-04	OPT oligopeptide transporter protein [<i>Rhizoctonia solani</i>]	XP_043179825.1	2e-67	76.06
246137	3.26	0.67	7.06E-05	Protein hob1 [<i>Psilocybe cubensis</i>]	XP_047754336.1	9e-109	62.11
27465	2.87	0.87	1.65E-02	Protein priA [<i>Psilocybe cubensis</i>]	XP_047743471.1	8e-12	30.14
58159	2.76	0.71	2.95E-03	Vegetative incompatibility protein [<i>Colletotrichum fructicola</i>]	XP_031875660.1	9e-10	33.33
67015	2.72	0.82	1.59E-02	SSU ribosomal protein S9P [<i>Lentinula edodes</i>]	XP_046085726.1	3e-48	70.37
247088	2.69	0.57	1.06E-04	40S ribosomal protein S17 [<i>Lentinula edodes</i>]	XP_046091369.1	4e-64	86.61
75118	2.66	0.55	8.57E-05	Amino acid/polyamine transporter I [<i>Filobasidium floriforme</i>]	XP_046040051.1	6e-70	59.47
22519	2.49	0.81	2.87E-02	Urate oxidase [<i>Kwoniella mangroviensis</i> CBS 8507]	XP_019001393.1	1e-39	66.67
79704	2.47	0.59	9.81E-04	Glycosyltransferase family 2 protein [<i>Suillus discolor</i>]	XP_041298901.1	0.0	68.27
22722	2.31	0.70	1.54E-02	Kinase-like domain-containing protein [<i>Suillus subalutaceus</i>]	XP_041251532.1	1e-04	32.06
74495	2.29	0.72	2.16E-02	60S ribosomal protein L30-1 [<i>Psilocybe cubensis</i>]	XP_047747268.1	1e-32	81.97
55002	2.25	0.74	3.09E-02	Sacsin [<i>Psilocybe cubensis</i>]	XP_047751045.1	2e-16	58.46
78780	2.25	0.74	3.15E-02	HAD-like protein [<i>Aureobasidium namibiae</i> CBS 147.97]	XP_013429611.1	2e-85	52.26
35692	2.14	0.64	1.45E-02	putative phosphatase HAD1 [<i>Psilocybe cubensis</i>]	XP_047750376.1	8e-30	59.76
19766	2.13	0.47	2.81E-04	ABC transporter [<i>Rhizoctonia solani</i>]	XP_043186107.1	8e-106	50.14
245006	2.12	0.51	1.26E-03	40S ribosomal protein S13 [<i>Laccaria bicolor</i> S238N-H82]	XP_001885801.1	2e-85	79.47
4711	2.11	0.67	2.33E-02	aldo/keto reductase [<i>Cryphonectria parasitica</i> EP155]	XP_040772933.1	4e-26	50.49

Genes exclusively up-regulated during symbiotic stages (T3 and SYMB) include several hydrolases as well as genes involved in primary metabolism

Specific metabolic changes have been previously identified in the mycelium growing in the close proximity of mycorrhizal orchid protocorms (T3; Ghirardo et al. 2020). However, gene expression in this stage of the symbiosis has never been investigated. Here, we compared gene expression in the external mycelium at stage T3 with the earliest symbiotic stage (T1), and focused on the top 20 DEGs exclusively up-regulated in T3 (Table 2; Fig. 1). Genes putatively coding for two α/β hydrolase proteins, an aminopeptidase puromycin sensitive, a serine/threonine-protein phosphatase ppe1, and a quinone oxidoreductase 15 were identified. Additionally, genes coding for a zinc metalloprotease, a Zn-dependent exopeptidase, a DsbA protein belonging to the Dsb (disulfide bond) enzymes family and three Carbohydrate Active enzymes (CAZymes) genes coding for Glycoside Hydrolases, belonging to Family 10, 11 and 16 were also strongly up-regulated in T3. Other relevant up-regulated genes (Table S6) code for proteins involved in lipid metabolism, including a sphingolipid C9-methyltransferase (transcript

ID: 85119, Log₂FC: 1.59) and a protein containing a beta subunit of fatty acid synthase (transcript ID: 216966, Log₂FC: 2.29). Together with proteins involved in sulfate assimilation and reduction such as phosphoadenosine phosphosulfate reductase (PAPS reductase, transcript ID: 241376, Log₂FC: 1.69), this gene is also up-regulated.

The top 20 DEGs up-regulated exclusively in the SYMB samples (Table 3) included genes coding for CAZymes belonging to Glycoside Hydrolase Family 30, 43 and 79, two CAZymes belonging to Polysaccharide Lyase Family 1, and a putative cellulose hydrolase harboring a Carbohydrate-Binding Module Family 1. In addition, other strongly up-regulated genes included an aquaporin-like, a putative DNA helicase ATP-dependent, a reductase belonging to SDR family and a galactose oxidase. Two putative kinase-like domain-containing proteins as well as a putative serine/threonine protein kinase and a dsk1 protein kinase were also detected (Table 3), and a putative GTPase Rab6 belonging to small G protein superfamily (transcript ID: 35046, Log₂FC: 6.09). Furthermore, several transcripts putatively involved in amino acid metabolism were identified among the DEGs up-regulated exclusively in SYMB condition (Table S9), from

Table 2 List of top 20 DEGs up-regulated exclusively in T3 stage, as compared to T1

Transcript ID	Log ₂ FC	Log ₂ FCSE	Adj. p value	Blastx (best identified match) [species]	Accession	e-value	Identity (%)
57403	5.86	1.08	2.90E-06	Epoxide hydrolase [Rhizoctonia solani]	XP_043182524.1	2e-131	51.54
34573	4.83	1.25	2.20E-03	Peptidase family M1 protein [Rhizoctonia solani]	XP_043187531.1	3e-26	72.06
21101	4.64	1.08	4.21E-04	Alkylated DNA repair protein alkB 6 [Malassezia restricta]	XP_027483908.1	5e-57	42.28
17709	4.58	1.02	1.87E-04	F-box-like domain-containing protein [Rhizoctonia solani]	XP_043180427.1	8e-09	33.67
241080	4.36	0.51	7.85E-15	Serine/threonine-protein phosphatase ppe1 [Sparassis crispa]	XP_027618106.1	0.0	79.50
23474	4.04	0.68	2.09E-07	Glycosyl hydrolases family 11-domain-containing protein [Lasiosphaeria miniovina]	XP_060296216.1	6e-49	64.39
242728	3.97	0.97	9.21E-04	Quinone oxidoreductase 15 [Heterobasidion irregulare TC 32-1]	XP_009552503.1	2e-50	40.18
18269	3.95	0.72	2.15E-06	TPR-like protein [Punctularia strigosozonata HHB-11173 S55]	XP_007389065.1	2e-90	37.20
16959	3.62	0.88	8.38E-04	Fungal-specific transcription factor domain-containing protein [Suillus subulatus]	XP_041249040.1	2e-22	70.37
226035	3.43	0.65	6.11E-06	Extracellular metalloprotease [Rhizoctonia solani]	XP_043176449.1	3e-131	65.96
24416	3.35	0.68	3.54E-05	Kinase-like domain-containing protein [Cantharellus anzutake]	XP_038918764.1	7e-32	30.72
24036	3.35	0.63	5.86E-06	Cytochrome P450 family protein [Rhizoctonia solani]	XP_043185935.1	3e-112	43.02
31503	3.27	0.71	1.19E-04	Glycoside hydrolase family 16 protein [Laccaria bicolor S238N-H82]	XP_001887475.1	6e-103	53.06
240495	3.09	0.79	1.73E-03	DSBA-like thioredoxin domain protein [Rhizoctonia solani]	XP_043179560.1	2e-48	44.88
28826	3.06	0.70	3.07E-04	Alpha/Betahydrolase YheT [Dacryopinax primogenitus]	XP_040623534.1	7e-07	26.81
225980	3.04	0.73	8.15E-04	Sodium/inorganic phosphate symporter [Rhizoctonia solani]	XP_043186227.1	0.0	55.29
231976	3.02	0.72	6.95E-04	Endo-beta-1,4-xylanase [Rhizoctonia solani]	XP_043180145.1	8e-111	68.01
22670	2.98	0.66	1.73E-04	Zn-dependent exopeptidase [Gloeophyllum trabeum ATCC 11539]	XP_007863390.1	4e-87	43.27
215906	2.97	0.70	5.00E-04	ATP-dependent RNA helicase DBP7 [Psilocybe cubensis]	XP_047752736.1	0.0	54.50
163733	2.91	0.40	9.24E-11	Glycerophosphocholine permease Git4 [Sparassis crispa]	XP_027618949.1	9e-167	59.10

biosynthesis (e.g., acetolactate synthase—transcript IDs 21435, Log₂FC: 2.23 -, prephenate dehydratase—12078, Log₂FC: 1.82 -, anthranilate synthase—71095, Log₂FC: 1.60 -, argininosuccinate lyase—245918, Log₂FC:1.91), to catabolism (e.g., ornithine cyclodeaminase – 8359, Log₂FC: 1.33 -, ornithine carbamoyltransferase—224256, Log₂FC: 1.92-, lysine-ketoglutarate reductase—215804, Log₂FC: 1.56). Genes coding for a putative PAPS reductase, which is involved in the sulfate reduction pathway (242229), and two putative enzymes involved in the metabolism of sulfur-containing amino acids (*i.e.*, a cystathionine gamma-synthase—214720 and a cysteine desulfurase NFS1—17165) were also found to be up-regulated (Log₂FC: 1.77, 2.80 and 4.63, respectively).

Gene expression possibly involved in fungal responses to external symbiotic signals

To identify fungal pathways potentially involved in the early response to plant signals (currently unknown in ORM), we selected those DEGs coding for fungal proteins putatively involved in signal transduction mechanisms, according to their KOG or description (Table S9). Differences in gene expression between FLM and T1 were minimal, indicating that the fungus at the initial stage of co-culture (T1) did not perceive (or respond to) the presence of the orchid seeds. By contrast, genes belonging to the KOG group “Signal transduction mechanisms” were up-regulated in later stages of co-culture (Table S9). A limited number of DEGs were shared by all different growth stages in co-culture, the majority of them being down-regulated in all samples (T2, T3 and SYMB). One exception was a gene coding for a divergent CRAL/TRIO domain-containing protein (223214),

Table 3 List of top 20 DEGs up-regulated exclusively in SYMB stage

Transcript ID	Log ₂ FC	Log ₂ FCSE	Adj. p value	Blastx (best identified match) [species]	Accession	e-value	Identity (%)
43357	7.81	1.61	8.61E-06	Uncharacterized protein LACBIDRAFT_395470 [Laccaria bicolor 5238N-H82]	XP_001888293.1	3e-43	47.25
26909	7.62	1.50	2.91E-06	Glycoside hydrolase family 30 protein [Trichoderma asperellum CBS 433.97]	XP_024760871.1	3e-84	74.58
53692	7.04	1.51	2.05E-05	Serine/threonine/tyrosine-protein kinase HT1 [Psilocybe cubensis]	XP_038918764.1	1e-28	31.70
247044	6.82	1.52	4.41E-05	cellulose hydrolase family 43 protein [Macroventuria anomochaeta]	XP_033558161.1	2e-42	59.42
35773	6.82	1.55	6.62E-05	ATP-dependent DNA helicase [Mycena indigotica]	XP_037223538.1	3e-32	42.62
27138	6.73	1.53	6.12E-05	cellulose hydrolase, putative [Aspergillus clavatus NRRL 1]	XP_001275546.1	1e-06	66.67
242138	6.64	1.56	0.000314	aquaporin-like protein [Dacryopinax primogenitus]	XP_040631163.1	3e-71	58.06
31239	6.24	1.55	0.000395	Pectate lyase [Rhizoctonia solani]	XP_043184226.1	8e-23	55.06
35046	6.09	1.52	0.000303	Ras-domain-containing protein [Coniophora puteana RWD-64-598 SS2]	XP_007772255.1	4e-35	35.53
32454	6.09	1.56	0.000642	Polyubiquitin [Schizophyllum commune H4-8]	XP_003030030.1	2e-54	42.40
247151	5.95	0.71	4.33E-15	Oxidoreductase [Gloeophyllum trabeum ATCC 11539]	XP_007864658.1	9e-61	44.67
246267	5.89	0.91	2.14E-09	Glycoside hydrolase family 79 protein [Desarmillaria tabescens]	XP_060333592.1	3e-15	48.08
25533	5.85	1.57	0.000843	Putative TIM8-translocase of the mitochondrial inner membrane [Jaminaea rosea]	XP_025361616.1	9e-15	55.56
28722	5.85	1.61	0.001277	Galactose oxidase [Stereum hirsutum FP-91666 SS1]	XP_007306347.1	3e-65	74.44
226373	5.81	1.44	0.000307	Kinase-like protein [Fomitiporia mediterranea MF3/22]	XP_007265203.1	2e-36	40.96
25837	5.76	1.58	0.001128	Pectin lyase-like protein [Stereum hirsutum FP-91666 SS1]	XP_007306015.1	4e-34	50.38
12135	5.74	1.54	0.000867	Nuclear protein 96-domain-containing protein [Suillus clintonianus]	XP_041208265.1	3e-40	38.16
84363	5.74	1.10	1.98E-06	Putative E3 ubiquitin-protein ligase rbrA [Colletotrichum siamense]	XP_036502071.1	2e-09	49.15
246249	5.67	1.58	0.001408	Phosphatidylserine decarboxylase [Kwoniella mangroviensis CBS 8507]	XP_019005511.1	2e-17	53.85
51640	5.64	1.47	0.000577	Kinase dsk1 [Fusarium graminearum PH-1]	XP_011323018.1	3e-14	44.30

that was up-regulated in all three conditions (T2, T3 and SYMB). The CRAL/TRIO motif is a common feature to several proteins that bind small lipophilic molecules (Panagabko et al. 2003; Gupta et al. 2012).

All DEGs shared by the external mycelium, either prior (T2) or after (T3) symbiosis establishment, showed the same regulation (i.e., up- or down-regulation), indicating a fungal response irrespective of the expression pattern inside the symbiotic protocorms (Table S9). Genes up-regulated in the T2 mycelium are of particular interest, as they suggest a fungal response to plant signals prior to contact. Among the genes exclusively up-regulated in T2 were two genes coding for calmodulin (transcript IDs: 18523 and 241437), with an average expression levels of Log₂FC ± SD: 2.5 ± 1.1 (Table 4). A single calmodulin gene was identified as DEG in T3 and SYMB conditions, but with lower average expression levels (Table 4). Two genes coding for kinase-like domain-containing proteins

(transcript IDs: 66501 and 72438, respectively) were exclusively up-regulated in T2, as well as a gene coding for a myosin regulatory light chain cdc4 (transcript ID: 242888), a gene coding for a small G protein (transcript ID: 30953) and two genes coding for ran-specific GTPase-activating proteins (transcript IDs: 12828 and 242041).

DEGs putatively coding for kinase-like proteins were found both in T2 and T3 samples (47 and 39 in T2 and T3, respectively), as well as GTPase-like proteins (4 and 6 in T2 and T3, respectively; Table 4). In particular, the most up-regulated gene in T2 was identified as a putative Ras guanine-nucleotide exchange factor, or RasGEF (transcript ID: 27558, Log₂FC: 3.69). This gene plays a role in the signalling cascade involving Ras and Ras-like proteins (Chen et al. 2020). The same gene was also found to be up-regulated in T3, although at a lesser extent (Log₂FC: 2.96), whereas it was not regulated in SYMB. Another RasGEF (transcript ID: 242442) was

Table 4 Main gene categories putatively involved in signalling processes in the different tested conditions

Category		T2	T3	SYMB	FLM
Kinase-like proteins	Number of DEGs	47	39	266	6
	average expression ($\log_2FC \pm SD$)	-0.109 ± 1.938	-0.179 ± 2.63	0.854 ± 1.498	-0.641 ± 2.42
Calmodulin-like proteins	number of DEGs	2	1	1	0
	average expression ($\log_2FC \pm SD$)	2.515 ± 1.181	2.591 ± 0.0	1.937 ± 0.0	NA
GTPase-like proteins	number of DEGs	4	6	29	2
	average expression ($\log_2FC \pm SD$)	-0.223 ± 2.060	1.177 ± 2.493	1.143 ± 2.314	-0.514 ± 2.907
C-type lectins	number of DEGs	1	0	4	0
	average expression ($\log_2FC \pm SD$)	1.05 ± 0.0	NA	-1.144 ± 1.612	NA

Number of DEGs detected, when compared to control condition (T1), and “function” expression values (as the expression average value of all the genes putatively annotated within the same category) are reported

up-regulated in both T2 and T3, but strongly down-regulated in SYMB samples. Another gene which may be involved in the downstream RAS signalling pathway (Mizunuma et al. 2013) was identified as a regulatory subunit of the cAMP-dependent protein kinase (transcript ID: 9609, $\log_2FC$: 1.25), and was specifically up-regulated in T2.

The largest number of regulated genes with a potential role in signal transduction mechanisms were identified in SYMB, with 20 of them exhibiting a $\log_2FC$ expression level greater than 4 (Table 4). The SYMB samples showed a considerable number of DEGs related to kinase-like proteins (a total of 266 up- and down-regulated DEGs) and GTPase-like proteins (a total of 29 up and down regulated DEGs). The average expression level of kinase-like proteins in the SYMB condition was $\log_2FC \pm SD$: 0.8 ± 1.5 (Table 4).

Expression of CAZymes and PCWDEs in *Tulasnella* sp. SV6 in co-culture with *S. vomeracea* suggests their involvement in symbiosis establishment

The expression of fungal genes putatively coding for CAZymes was found to be subject to different regulatory mechanisms during the different stages of the symbiosis establishment. In T2, 25 genes associated with CAZymes were identified as being up-regulated (Fig. 4), in addition to a Glycosyl Transferase Family 2-related gene that was among the top 20 most up-regulated genes (Table 1). In T3 and SYMB, the number of up-regulated CAZymes-related genes increased to 36 and 83, respectively (Fig. 4). Of the 176 CAZymes-related genes annotated in the *T. calospora* AL-13/4D v1.0 genome, 100 were classified as coding for PCWDEs based on the PCWDE database. Among the up-regulated genes in the three conditions tested, those belonging to the Glycoside Hydrolase (GH), Polysaccharide Lyase (PL), and Carbohydrate Esterase (CE) families of enzymes were identified as PCWDEs. In particular, the gene set included GH5 and former GH61

(now AA9), which are known to act on cellulose; GH10 and GH11, which are known to act on xylans (Puchart et al. 2021; Gavande et al. 2023); GH47 and GH2, which mainly target α and β -mannosides and β -D-galactosides (Herscovics 2001; Talens-Perales et al. 2016); GH28, PL4, PL3, and PL1, which target pectins (Bonnin and Pelloux 2020); and CE8 and CE12, which act on hemicelluloses and xylans through deacetylation (Gavande et al. 2023); GH28, PL4, PL3, and PL1, which target pectins (Bonnin and Pelloux 2020); CE8 and CE12, which act on hemicelluloses and xylans through deacetylation (Gavande et al. 2023). Seven of the genes (four belonging to the GH5 family, a GH47, a GH11 and a PL1) were found to be upregulated in T2, 13 (three belonging to the GH2 family, two belonging to the GH6 family, a GH47, three belonging to the GH11 family, a PL1 and two belonging to the PL2 family) in T3, and 30 in SYMB (Fig. 4). In contrast, eight PCWDE-related genes were only detected in the SYMB condition. It is noteworthy that certain genes encoding CAZymes in families GH5 (transcript IDs: 67006 and 20384), GH7 (transcript ID: 24394) and AA9 (transcript IDs: 218573 and 6298) showed increased expression in T2 and T3 yet exhibited decreased or no significant regulation in SYMB samples (Table S9). A single DEG corresponding to a GH5 was identified in the FLM when compared to T1 (Fig. 4).

Expression of membrane transporters in *Tulasnella* sp. SV6 in co-culture with *S. vomeracea*

Several DEGs were putatively identified as transporters. None of the transporters identified as DEGs in T2 and T3 were found to be regulated in the FLM condition, thereby confirming a similar gene expression during saprotrophic growth (FLM) and the early T1 stage. This also suggests that DEGs encoding transporters in T2 and T3 were somehow related to the presence of the host rather than by exploitation of the substrate. A total of 198 DEGs encoding putative transporters were found

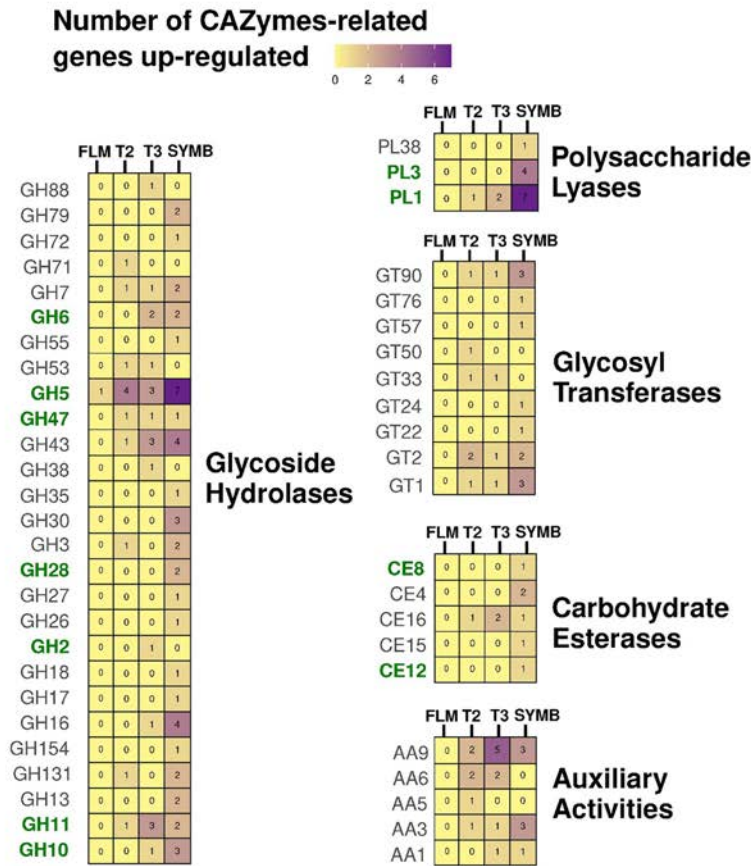


Fig. 4 Number of CAZymes-related genes up-regulated in the three conditions. Color intensity is related to number of up-regulated genes corresponding to the five CAZymes categories identified. Enzymes corresponding to PCWDEs are in bold and green colored

in our dataset (Table S10). Of the 19 DEGs putatively involved in transport exclusively detected in T2, all were up-regulated except for one gene associated with copper movement, which was down-regulated (transcript ID: 245837, Log₂FC: -2.29). In addition to the strongly up-regulated transporters already mentioned, different genes coding for major facilitator superfamily (MFS) proteins were identified, together with a putative urea transporter (transcript ID: 243251, Log₂FC: 1.57), an inorganic phosphate permease (transcript ID: 15435, Log₂FC: 1.42), three ABC transporters, one of which belongs to

the Pleiotropic Drug Resistance (PDR) proteins (transcript ID: 19766, Log₂FC: 2.13) and one protein possibly involved in iron export (transcript ID: 240789, Log₂FC: 1.22). Exclusively up-regulated in T2 were also a member of Yellow stripe like 5 (YSL5) metal-nicotianamine transporter (transcript ID: 228210, Log₂FC: 1.97) and two thiamine transporters thi9 (transcript IDs: 33469, Log₂FC: 1.73 and 29197, Log₂FC: 1.43).

Only six genes encoding transporters were differentially expressed exclusively in T3. Among them, a putative UDP-xylose and UDP-N-acetylglucosamine transporter

(transcript ID: 33298, Log₂FC: -3.47) was strongly down-regulated, while a phospholipid transporter (transcript ID: 163733) showed up-regulation (see Table 2).

As expected, a considerably greater number of DEGs encoding putative transporters was identified in SYMB. Among the 142 up- and down-regulated fungal transporters exclusively found in symbiotic protocorms, several were general substrate transporters belonging to the major facilitator superfamily (MFS) and the ATP-binding cassette (ABC) superfamilies, as well as different amino acid and oligopeptide transporters/permeases (Table S9). Highly up-regulated genes related to lipid and carbohydrate transport in SYMB included a phospholipid transporter (transcript ID: 211343; Log₂FC: 3.17) and a protein containing a triose-phosphate transporter family domain (transcript ID: 247150; Log₂FC: 4.41). In addition, genes coding for a phospholipid-translocating P-type ATPase (transcript ID: 25698) and a GDP-mannose 1 (transcript ID: 69923) transporter (Log₂FC: 1.65 and 1.30, respectively) were identified. Only an UDP-galactose transporter (transcript ID: 160960), was identified in the FLM. This transporter was shared with the symbiotic condition and was down-regulated in both the SYMB and FLM stages, with respective Log₂FC values of -2.40 and -1.43.

Metabolomic profiling of pre-symbiotic and symbiotic mycelial growth highlights changes mainly in lipid metabolism

The metabolomic profiles of the mycelium at the pre-symbiotic stage T2 and at the symbiotic stage T3 were compared with the profile at the early stage T1. The mycelium metabolome showed a significant divergence between the early (T1), the pre-symbiotic (T2) and the symbiotic (T3) stages of growth, as shown by the segregation in the first two components of the PCA, which collectively explained 63% of the total metabolome variance (Fig. 5a). The metabolome of the SYMB sample was not considered because it contains both fungal and plant metabolites that cannot be distinguished, unlike in the gene expression analysis. In total, the metabolome analysis could detect 12,648 metabolite-related mass features among the different stages. Of the total number of metabolite-related mass features, 3,971 were shared between T1, T2 and T3 (Fig. 5b), and 2,508 additional mass features were differentially accumulated (VIP > 1; $p < 0.05$, CV-ANOVA; PLS-DA Fig. S1). A considerable number of mass features were shared between T1 and T2 (2,693), which may be indicative of metabolites specifically involved in the active growth phase of the mycelium. In contrast, the 420 mass features shared between T2 and T3 may be indicative of the influence of the plant on the fungal metabolome. Among the 2,508 differentially accumulated mass features, 88 could be matched by MS/MS

data to chemical structures available in databases (Fig. 5c; Table S11). Furthermore, 281 mass features could be classified as 'lipids' (114, 40.6%), as 'amino acids (AA) and protein-related' (98, 35%), as 'secondary metabolites' (33, 12%), as 'carbohydrates' (20, 7%), or as 'nucleotides' (16, 6%) using the MSCC approach (Fig. 5c; Table S11). The heatmap (Fig. 5c) depicts the significant relative changes in the metabolome of T2 and T3 samples, in comparison to T1. Whereas minimal changes in the fungal metabolome were observed in T2, mainly a down-regulation, profound alterations were revealed in T3, with a specific and strong up-regulation (Log₂FC > 4) of several metabolites involved in the metabolism of amino acid, lipids, and secondary compounds (Fig. 5c). Among the putatively annotated compounds that exhibited a significant change (adj. $p < 0.05$; Table S11), several glycerophospholipids were identified in T3, including three glycerophosphocholines PC 36:3 (Log₂FC: 7.34), PC 34:2 (Log₂FC: 6.86) and PC 32:2 (Log₂FC: 2.77). Among the secondary metabolites, the levels of indole-3-carboxylic acid (ICA; Log₂FC: 4.88), the phenolic acid 3,4-dihydroxyphenylacetate (3,4-DHPA; Log₂FC: 3.82), the alkyl-phenylketone 4-O-methylphloracetophenone (Log₂FC: 3.77), and the pyranone 4-hydroxy-6-methyl-2-pyrone (Log₂FC: 2.42) were significantly increased in T3. A slightly higher level of ICA was observed in T2, although this was not significant. Among the putatively annotated compounds that decreased significantly in both the pre-symbiotic T2 and the symbiotic T3 stages, in comparison to T1, were three lyso-phosphatidylcholines (LysoPC 14:0, LysoPC 18:3, LysoPC 16:0) and a phosphatidylcholine (PC 18:0). In addition, three glycerophosphoglycerols (LPG 18:2, LPG 16:0, LPG 18:1), a glycerophosphoinositol (PI 36:4), two glycerophosphates (PA 36:3 and LPA 16:0) and two medium-chain fatty acids (MCFAs), the 10-hydroxydecanoic acid and the sebatic acid, were also decreased. Other metabolites that exhibited reduced abundance during the later (T3) stages of fungal development included the modified amino acid 3-methyl-L-histidine (Log₂FC T2: -1.50 and Log₂FC T3: -4.59), which is known to be a structural component of actin and myosin (Webb et al. 2010), the amino acid arginine (Log₂FC T2: -2.2 and Log₂FC T3: -2.8) and sclareol, a labdane diterpenoid (Log₂FC T2: -1.75 and Log₂FC T3: -1.66). Finally, compounds exhibiting a slight decrease in T2 and a pronounced decrease in T3 included the oxidized form of glutathione (GSSG), the pyrimidine ribonucleoside diphosphate cytidine 5'-diphosphocholine (CDP), the tetra saccharide stachyose, and the fatty aldehyde succinic acid (Table S11). The remaining annotated compounds, which included several amino acids such as serine, lysine, histidine, arginine, isoleucine, taurine or phenylalanine, as well as carbohydrates such as xylose, gluconic acid or

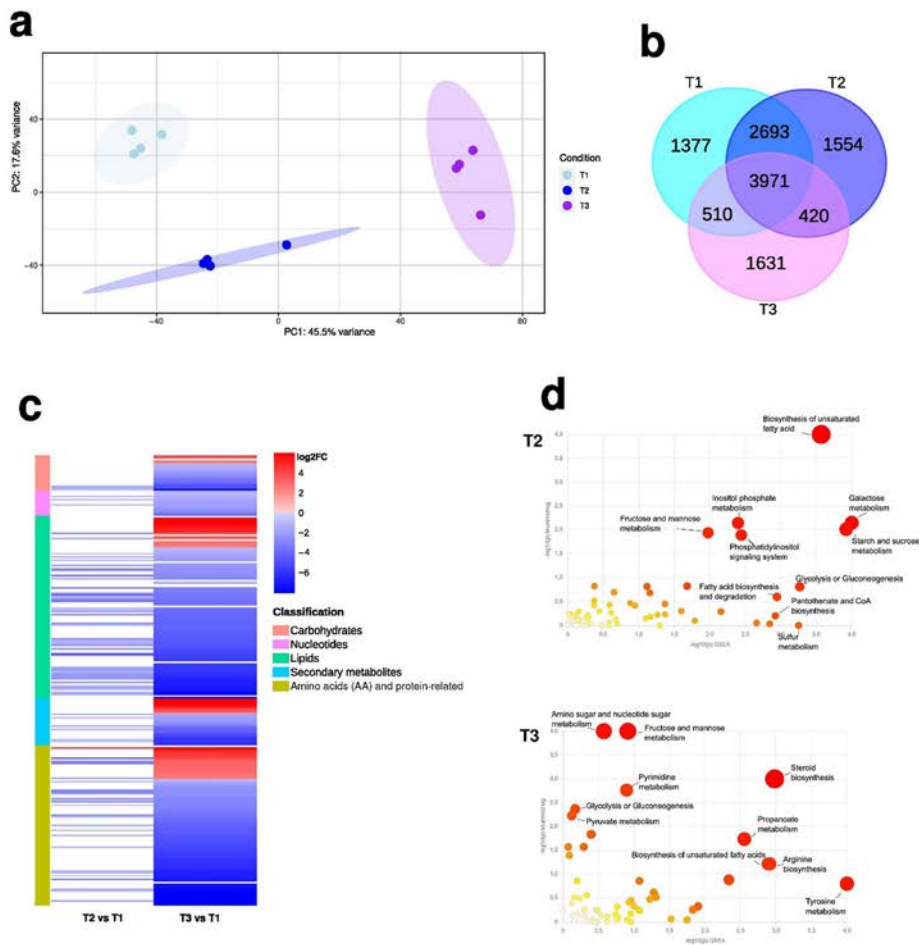


Fig. 5 PCA, heatmap, and Venn diagrams depicting metabolites in the conditions T1, T2, T3, and functional analysis results. In **a**, PCA on detected metabolites in T1, T2, and T3. In **b**, the heatmap shows the expression patterns of metabolites in the two conditions T2 and T3 compared to T1, with red indicating up-regulated and blue representing down-regulated metabolites. Different color intensity indicates different relative amount based on $\log_2FC$. In **c** Venn diagrams illustrate the overlap of detected mass features between pairwise comparisons of the conditions. In **d**, over-representation analysis shows enriched pathways (bubbles) in T2 and T3. $\log_{10}p$ -value of Mummichog and GSEA algorithms are represented by x and y axes, respectively

trehalose, showed significantly decreased levels in T3 in comparison to T1 (Table S11). According to our RNA-seq data (Table S9), a putative fungal trehalase (transcript ID: 20428) was significantly up-regulated in T2 ($\log_2FC=1.68$) and T3 ($\log_2FC=1.62$), suggesting a possible degradation of trehalose in the fungus.

Changes in the metabolome suggest activation of energy, carbon and nitrogen metabolic pathways in the different stages

The over-representation analysis of significant metabolomic changes revealed that the fructose / mannose metabolisms and the biosynthesis of unsaturated fatty acids were significantly enriched in both stages T2 and T3 (combined p values < 0.05 ; Fig. 5d and Tables S12–S13). Nevertheless, some metabolic pathways were specific to each condition. At the pre-symbiotic stage T2, five pathways (combined p values < 0.05) were affected, the majority of these pathways being related to energy and carbon metabolism. This included galactose metabolism, starch and sucrose metabolism, inositol phosphate metabolism, phosphatidylinositol signalling, and glycolysis or gluconeogenesis (Fig. 5d and Table S12). In T3, six metabolic pathways were identified as being affected, mainly related to nitrogen metabolism. These pathways included steroid biosynthesis, amino sugar and nucleotide sugar metabolism, tyrosine metabolism, propanoate metabolism, arginine biosynthesis and pyrimidine metabolism (Fig. 5d and Tables S12–S13). When the results of the Mummichog 3.0 and the GSEA algorithms were considered separately, the T2 samples were found to be enriched in sulfur metabolism, fatty acid biosynthesis and degradation, and pantothenate and CoA biosynthesis (GSEA p values: 0.0339, 0.04651, 0.04651, 0.04762, respectively; Fig. 5d). In T3, glycolysis or gluconeogenesis, and pyruvate metabolism (Mummichog 3.0 p values: 0.03 and 0.04, respectively; Fig. 5d) were found to be enriched.

Integration between transcriptomic and metabolomic data

A scatterplot of integrated transcriptomic and metabolomic data from T2 and T3 conditions demonstrated a strong correlation between gene expression and metabolomic data ($R^2 = 0.819$, p -value = 6.06×10^{-12} ; Pearson correlation). This indicates co-regulation of several genes and metabolites in the two conditions, particularly for down-regulated genes and metabolites (Fig. 6a). Notwithstanding the positive correlation, a group of metabolites showed down-regulation in T2 and up-regulation in T3 (Fig. 6a). Furthermore, neural gas cluster analysis facilitated the integration of transcriptomic and metabolomic datasets, resulting in the generation of 4 clusters based on the putative co-regulation of genes and metabolites (Fig. 6b). Among the identified clusters, cluster 3, which encompasses transcripts and metabolites showing a declining trend from T2 to T3 (Fig. 6b), is composed of a substantial number of mass features annotated as lipids, in addition to a DEG encoding a phospholipase A2. A list of metabolites and transcripts within the detected clusters is provided in Table S14. Alluvial plots

of transcriptomic data (Fig. 6c) showed shifts in transcript abundance across the five categories over distinct time points. In particular, transcripts related to biosynthesis/catabolism of proteins, lipids and carbohydrates showed a consistent increase in abundance from FLM to T1, T2 and T3. A notable increase in secondary metabolite-related transcripts was found in SYMB, and low levels of transcripts related to this category was highlighted in FLM. On the other hand, concerning the metabolomic data, lipids and carbohydrates exhibited the highest levels in T1, while a decreasing trend was observed in T2 and T3 (Fig. 6d).

Discussion

The application of *omics* techniques to investigate fungal-plant interactions has gained widespread recognition within the ORM community (De Rose et al. 2023b). In this study, we focused on the transcriptomic and metabolomic reprogramming of the ORM fungus *Tulasnella* sp. V6 during the early initial stages of its interaction with the host *Serapias vomeracea*, with the aim to elucidate the temporal molecular changes that occur in the fungus prior to and after contact with the plant. Thus, we have provided a wider picture of the ORM fungal behaviour in symbiosis, as previous investigations on ORM have only considered fungal gene expression and metabolite profiles in fully established symbiotic protocorms, as compared to FLM (Fochi et al. 2017a; Ghirardo et al. 2020; Chen et al. 2022).

Our integrated transcriptomic and metabolomic study indicates that the fungal mycelium initiates recognition of the host plant before physical contact, thereby preparing the fungal cells for establishment of the symbiosis. In particular, our results demonstrate the gradual activation of cell signalling pathways, the enhancement of the amino acid metabolism and nitrogen transport, as well as lipid remodelling and the expression of genes encoding plant cell wall-degrading enzymes (PCWDEs), which are responsible for the degradation of the constituents of the plant cell wall. It has been previously reported that nitrogen is transferred from *Tulasnella* sp. to *S. vomeracea* as amino acids (Fochi et al. 2017a, b). These processes are of great importance for the establishment and functioning of the symbiosis.

Tulasnella sp. SV6 activates calcium-related signalling pathways during the pre-symbiotic stage

Genes involved in cell signalling pathways were expressed in *Tulasnella* sp. prior to the symbiotic stage. In T2, the up-regulation of two calmodulin-coding genes is noteworthy, particularly considering their role in intracellular calcium signalling (Yang and Poovaiah 2003). Calcium ions play a crucial role in cell signalling, specifically in the

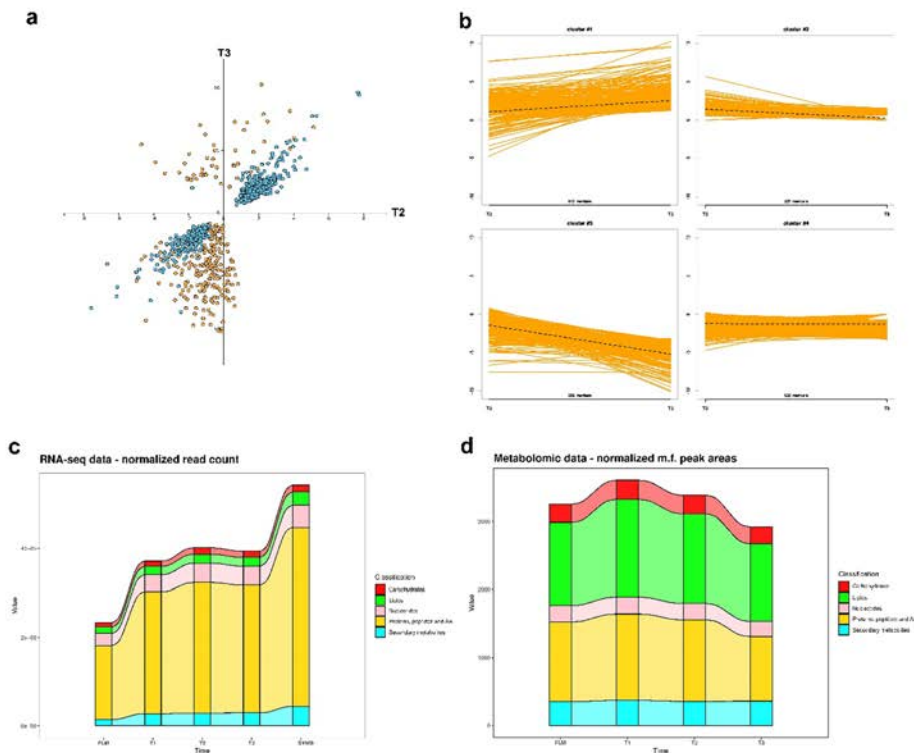


Fig. 6 Integration of transcriptomic and metabolomic data. In **a**, scatterplot depicting the correlation of DEGs and metabolites (Log-FC) between T2 (x-axis) and T3 (y-axis). Blue dots represent DEGs, while yellow dots represent metabolites. In **b**, detected clusters through neural gas cluster analysis. In **c** and **d**, alluvial plots of normalized transcriptomic and metabolomic data across the different tested stages, respectively

transduction of signals from the extracellular environment and in the activation of enzymes involved in gene expression (Roy et al. 2021). Calcium signalling response typically occurs at the initial stage of reaction to a stimulus, thus modulating fungal growth and development in response to the surrounding environment (Lengeler et al. 2000). In arbuscular mycorrhizal (AM) fungi, fungal genes related to calcium homeostasis and signalling are induced during symbiotic interactions with host roots, thus suggesting a role of Ca^{2+} -based signalling pathways during the differentiation of the hyphopodium, a specialized specialized hypha of an arbuscular mycorrhizal fungus, often branched and swollen, which adheres to the root epidermis (Bonfante and Genre, 2010). In our data, up-regulation of genes coding for calmodulins and

several kinase proteins in T2 would indicate that the fungus perceives the presence of the orchid seeds and activates an intracellular Ca^{2+} -signalling pathway prior to contact, possibly through recognition of chemical signals released from the imbibed seeds (Ortega-Cuadro et al. 2022). However, the actual existence and chemical structure of these plant signals remain unknown and warrant further dedicated studies.

Among the most up-regulated genes involved in signal transduction in T2, a putative Ras guanine-nucleotide exchange protein was also identified. This protein is responsible for the activation of Ras proteins by promoting the binding of GTP to the GDP-free Ras forms (Chen et al. 2020). The Ras superfamily, which includes monomeric GTPases, regulates a multitude of biological

processes in fungi, including morphogenesis, secondary metabolism, vesicle trafficking and virulence (Dautt-Castro et al. 2021; Pinar and Peñalva 2021; Yuan et al. 2022). The most common members of the Ras superfamily are Ras, Rho, Rab, Ran, Arf, and Miro (Cao et al. 2024; Xu et al. 2024). The activity of these GTPases within the cell is regulated by guanine-nucleotide exchange factors (GEFs) and by GTPase activating proteins (GAPs), which respectively catalyze binding of GTPase with either GTP (active form) or GDP (inactive form). The Ras signalling pathway, in particular, is responsible for orchestrating a range of cellular processes that are essential for hyphal growth in fungi (Martin-Vicente et al. 2019; Chen et al. 2020).

Intriguingly, Breuninger and Requena (2004) observed the up-regulation of genes involved in Ca^{2+} signalling and in the Ras signalling pathway in the fungal partner of AM symbioses during the early stages of appressorium development. Arbuscular mycorrhiza is the most common endomycorrhizal type (Genre et al. 2020), and notable similarities with ORM have been outlined, despite the phylogenetic distance of the plants and fungi involved. For example, orchids feature in their genome genes of the Common Symbiotic Signalling Pathway, a conserved signal transduction pathway found in all endosymbioses (Delaux et al. 2014; Miura et al. 2018). Furthermore, similarities among the intracellular structures formed by AM and ORM fungi in their respective hosts have been recently discussed (Perotto and Balestrini 2024). In AM, two main types of root colonization patterns were generally observed, the *Arum*-type and the *Paris*-type. Whereas the *Arum*-type AM are characterized by highly branched intracellular fungal structures called arbuscules, intracellular fungal structures formed in *Paris*-type AM are simpler and more similar to ORM pelotons. In addition, intracellular fungal structures formed in both AM and ORM show a similar life cycle, including a collapse phase (Perotto et al. 2014).

Beside physical contact, chemical signalling between the plant and the fungus likely plays a role in activating signal transduction pathways. In AM, it has been documented that plants exude strigolactones, which are sensed by AM fungi and promote spore germination, hyphal branching, and other biological processes (Genre et al. 2020). During the establishment of the symbiosis, AM fungi release chitin-derived molecules, called Myc factors, that trigger plant responses preparing them for colonization, including the fine tuning of expression of symbiosis-supporting genes as well as changes at cellular and physiological levels (Genre et al. 2020). Although presymbiotic chemical signalling is well studied in AM, in ORM the specific signalling molecules exchanged between host plants and their fungal partners are still

largely unidentified (Genre et al. 2020; Perotto et al. 2014). The activation of a similar signal transduction pathways between AM and ORM is intriguing, and further analyses are needed to elucidate this poorly explored aspect. In our work, it is noteworthy that metabolomic data indicate two unique enriched pathways at the pre-symbiotic stage (T2), namely inositol phosphate metabolism and the phosphatidylinositol signalling system. These pathways are putatively involved in signal transduction and cellular communication and may also be indicative of the activation of signalling processes during the pre-symbiotic stage.

Mycorrhizal fungi, including ORM fungi, feature in their genomes a large set of mycorrhiza-induced small secreted proteins (MiSSPs), which can be highly expressed in symbiosis (Kohler et al. 2015; Kang et al. 2020; Miyauchi et al. 2020). Some MiSSPs have been characterized in AM, ecto- and ericoid mycorrhizal fungi and have been demonstrated to be involved in the establishment of the symbiosis and/or in the inactivation of the plant defense mechanisms (Kloppholz et al. 2011; Plett et al. 2011; Casarrubia et al. 2018). In *T. calospora*, several genes strongly upregulated during the symbiosis with orchids encoded putative MiSSPs (Kohler et al. 2015). Here, we identified two DEGs up-regulated in the mycelium in the proximity of symbiotic protocorms (T3), one of which (transcript ID 242048) was also up-regulated in pre-symbiotic condition (T2) but down-regulated in the protocorms (SYMB). The relatively low number of up-regulated genes coding for MiSSP in our model system may be attributed to the fact that MiSSP tend to be highly species-specific (Pellegrin et al. 2015; Kim et al. 2016). Thus, we may have missed the annotation of species-specific MiSSP-related genes when we compared the *Tulasnella* sp. isolate SV6 (used in this study) with the genome of the sequenced *T. calospora* isolate available in MycoCosm. Anyhow, the two genes identified in this study warrant further investigation, as they may represent good candidates for the study of symbiosis effectors in ORM fungi.

Response to the host plant involves increase protein synthesis and transport in the pre-symbiotic stage

Among the fungal genes regulated prior to physical contact with the orchid seeds, we identified genes involved in amino acid metabolism, including an asparagine and a methionine synthase. Together with the specific enrichment of the GO term "Translation" in T2 and the up-regulation of several genes involved in ribosome biosynthesis, this suggests an increased primary metabolic activity in the pre-symbiotic stage, possibly associated with changes in fungal development. Not surprisingly, given the intense metabolic changes triggered by the

ORM symbiosis, sustained translation activity continued in the fungal mycelium after physical contact both outside and inside mycorrhizal protocorms.

Some fungal genes coding for membrane transporters or related to transport were also significantly up-regulated in T2, before physical contact with the host. In particular, 19 DEGs related to transporters were identified in the pre-symbiotic stage, suggesting transport activity for amino acids and oligopeptides. Membrane transporters in fungi are essential for the uptake of nutrients from the substrate and their subsequent redistribution within cellular compartments. In mycorrhizal fungi, they also play a pivotal role in nutrient exchanges with the host during symbiosis (Garcia et al. 2016; Wipf et al. 2019), and several transporters were identified in symbiosis in this and in previous studies (Zhao et al. 2014; Fochi et al. 2017a; De Rose et al. 2023a, b). Although it cannot be ruled out that, in the pre-symbiotic stage T2, up-regulation of fungal genes involved in translation and transport may simply reflect changes in substrate composition during fungal growth, it should be noted that expression of these genes was similar in T1, where the mycelium just started to colonize the substrate, and in FLM, where the fully grown mycelium covered the whole plate. Thus, if modulation of these fungal activities in the pre-symbiotic condition is related to changes in fungal growth, these changes appear to be caused by the proximity of orchid seeds. Of course, this hypothesis would suggest the release of so far unknown diffusible signals from the seeds.

Several CAZymes and PCWDEs are expressed in pre-symbiotic stages

CAZymes, and plant cell wall-degrading enzymes (PCWDEs) in particular, are instrumental for the degradation of the polymeric litter components by saprotrophic fungi. However, they also play a significant role during symbiosis (Kohler et al. 2015; Martin et al. 2016; Almario et al. 2017; Genre et al. 2020). A distinctive feature of ORM fungi such as *Tulasnella calospora* is their larger set of PCWDEs (Kohler et al. 2015) in comparison to other mycorrhizal fungi. It has been proposed that, thanks to these degrading enzymes, ORM fungi may maintain a double ecological niche, with saprotrophic and symbiotic lifestyles (Martino et al. 2018; Miyauchi et al. 2020). In addition, ORM fungi may also live as endophytes in the roots of non-orchid plants (Selosse et al. 2022).

In our experiment, comparison of gene expression with T1 samples (i.e., the early *Tulasnella* growth stage on fresh medium in the presence of orchid seeds) reduced the possibility that the DEGs identified were simply related to the exploitation of carbohydrates available

in the substrate. In fact, whereas only one DEG corresponding to a Glycoside Hydrolase (GH5) was identified in FLM when compared to T1, several CAZyme-related genes were up-regulated in the pre-symbiotic stage (T2) and in the external mycelium in the proximity of symbiotic protocorms (T3). In comparison to T1, 14.7% and 21% of the total number of CAZymes found in the *T. calospora* AL-13/4D v1.0 genome were induced in T2 and T3, respectively. The percentage of up-regulated CAZymes related fungal genes was considerably higher (47% of the total number of CAZymes in the *T. calospora* AL-13/4D v1.0 genome) in symbiotic protocorms (SYMB).

The up-regulation of a chitin synthase (GT2, transcript ID: 79704) unique to the pre-symbiotic stage is of particular interest, as it suggests an active remodelling of the fungal cell wall, possibly related to changes in the growth of fungal mycelium upon perception of the orchid seeds. It is noteworthy, that chitin synthase is also involved in the synthesis of lipochitooligosaccharides and short chitin oligomers, which are molecular signals released by AM fungi in the pre-symbiotic stages of the interactions (Maillet et al. 2010; Genre and Russo 2016). In addition, other genes related to glycoside hydrolases and glycosyl transferases and possibly involved in fungal cell wall remodelling (Bowman and Free 2006) were up-regulated in the pre-symbiotic stage.

The role of fungal CAZymes in the ORM symbiosis remains unclear. Several glycosyl hydrolases in the GH5 family and polysaccharide lyases in the PL1 family were induced at an early stage before symbiosis, and their number increased as the fungus established ORM symbiosis (T3 and SYMB). A set of enzymes belonging to the GH5 family was identified in the genome of the ectomycorrhizal fungus *Laccaria bicolor*, and were shown to be active on both fungal and plant cell walls (Veneault-Fourrey et al. 2014). Enzymes in the PL1 family include pectin and pectate lyases and are known to play a crucial role in plant cell wall degradation (Atasanova et al. 2018). Our RNAseq data thus suggest that GH5 and PL1 enzymes may be involved in modifying the orchid cell wall, potentially facilitating the symbiotic interaction. In SYMB condition, within the 83 CAZymes-related genes resulted as DEGs, 30 PCWDE-related genes were identified as up-regulated, suggesting that they might be involved in the fungal colonization and establishment of the symbiotic interface. This outcome also suggests that the plant cell wall degradative potential of *Tulasnella* is finely tuned during symbiosis, as previously reported (Adamo et al. 2020).

Remodelling of the fungal lipidic profiles during the pre-symbiotic and symbiotic stages

The enrichment analysis of metabolomic data revealed that biosynthesis of unsaturated fatty acids was induced in the *Tulasnella* mycelium before the fungus came in physical contact with the seeds (T2), and persisted when symbiosis was established (T3). Increased abundance of structural membrane lipids may reflect the stimulation of hyphal growth and a need for membrane biogenesis in the mycelium during both the T2 and T3 stages. However, the increase in potential membrane signalling molecules is intriguing. Phospholipids such as phosphatidylcholines (PC) are the dominant membrane lipids in fungi (Rella et al. 2016). In plants, a derivative of phosphatidylcholine, lyso-phosphatidylcholine (Lyso-PC) is known to act as a signal in the AM symbiosis (Drissner et al. 2007; Vijayakumar et al. 2015). In our experimental system, the amounts of metabolites putatively related to Lyso-PCs (Lyso-PC 14:0, Lyso-PC 18:3, Lyso-PC 16:0) were found to be higher in the early stages of co-cultivation, as compared to both FLM and T3. Lyso-PC content particularly decreased in T3, and was mirrored by an increase in the amount of PC. Intriguingly, the transcriptomic-metabolomic cluster analysis revealed that the decline of Lyso-PC levels in T3 correlated with the down-regulation of a gene encoding a phospholipase A2 (with Log_2FC of -4.73). Cleavage of PC by phospholipase A2 enzyme is the main process for the generation of Lyso-PC (Liu et al. 2020). Overall, these results indicate that Lyso-PCs may be involved during the pre-symbiotic stages of the *Tulasnella*-*Serapias* symbiosis, similar to what has been observed in the AM symbiosis (Vijayakumar et al. 2015). Once the symbiosis is established, Lyso-PCs appear to be depleted in the mycelium collected in close proximity to symbiotic protocorms (T3). Ghirardo et al. (2020) reported a significant up-regulation of the fungal phospholipase A2 gene in symbiotic protocorms, in comparison to FLM, but the substrate of this enzyme is unknown.

An indole related metabolite is produced before fungal contact with orchid seeds

In comparison to the very early T1 stage, stages T2 and T3 of the *Tulasnella*-*Serapias* interaction were characterised by a substantial increase (Log_2FC of 1.70 and 4.88, respectively) of a compound putatively annotated as indole-3-carboxylic acid (ICA). Indole-related compounds released from mycorrhizal fungi have been reported to regulate establishment of the symbiosis through lateral root formation (Ludwig-Müller 2010; Hanlon and Coenen 2011; Sukumar et al. 2013; Liao et al.

2018). Among indole compounds, ICA can be produced by ectomycorrhizal fungi, including *Paxillus involutus* and *Suillus luteus* (Rudawska and Kieliszewska-Rokicka 1997; Pan et al. 2022), and may promote mycorrhizal symbiosis (Wu et al. 2021). To the best of our knowledge, our data represents the first report of ICA in the genus *Tulasnella*, and its increased production prior to and during the establishment of the ORM symbiosis suggests similarities to its role in ectomycorrhizal fungi (Wu et al. 2021). However, manipulation of the endogenous levels of fungal ICA would be essential to explore its role in the ORM symbiosis. This could be achieved in future studies through the application of exogenous ICA, of specific enzyme inhibitors, or by genetic manipulation of its biosynthesis.

Conclusions

Our integration of transcriptomic and metabolomic data revealed the molecular changes that occur in *Tulasnella* sp. SV6 before and after physical contact with the orchid host *S. vomeracea*. In particular, the significant up-regulation of genes and metabolites potentially involved in early signalling suggests that the ORM fungus senses and responds to the presence of the host before physical contact with the orchid seeds. Early perception of orchid seeds by the fungus was further suggested by the increased production of specific fungal compounds such as an indole-related metabolite, putatively involved in the early stimulation of symbiosis.

Transcriptomics and metabolomics showed the up-regulation of some primary and secondary metabolic pathways during the initial stages of the symbiotic interaction. These pathways likely play a role in modulating fungal growth in response to the presence of orchid seeds. Furthermore, the up-regulation of *Tulasnella* genes related to PCWDEs during early contact with the *Serapias vomeracea* seeds supported the hypothesis that these enzymes play a role in fungal penetration and host cell colonization. Overall, our integrated approach enabled the identification of the dynamic nature of the ORM symbiosis, and the generation of useful data on the fungal side, which remains a relatively understudied topic (Favre-Godal et al. 2020).

Abbreviations

AM fungi	Arbuscular mycorrhizal fungi
CAZymes	Carbohydrate-active enzymes
DEGs	Differentially expressed genes
GO	Gene ontology
GH	Glycoside hydrolase
GSEA	Gene set enrichment analysis
HILIC	Hydrophilic interaction liquid chromatography
ICA	Indole-3-carboxylic acid
KEGG	Kyoto encyclopedia of genes and genomes
KOG	Eukaryotic orthologous groups

Lyso-PC	Lyso-phosphatidylcholine
MUT	Mycotheca Universitatis Taurinensis
MSCC	Multidimensional stoichiometric compound classification
ORM fungi	Orchid mycorrhizal fungi
PCA	Principal component analysis
PCWDE	Plant cell wall-degrading enzyme
PL	Polysaccharide lyase
PLS-DA	Partial least squares discriminant analysis
RPLC	Reversed-phase liquid chromatography
VIP	Variable importance of projection

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43008-024-00165-6>.

Additional file 1.

Additional file 2.

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Author contributions

RB and SP conceived and designed the experiments. AG, FS and SDR performed the experiments, contributed to the acquisition of the data and performed data analysis. FS, SDR and AG drafted the manuscript. RB, SP and JPS reviewed and improved the manuscript. All authors read and approved the manuscript.

Data availability

Transcriptomic raw data were submitted to Sequence Read Archive (SRA) of NCBI, under the BioProject ID: PRJNA979980.

Declarations

Ethics approval and consent to participate

Not applicable.

Adherence to national and international regulations

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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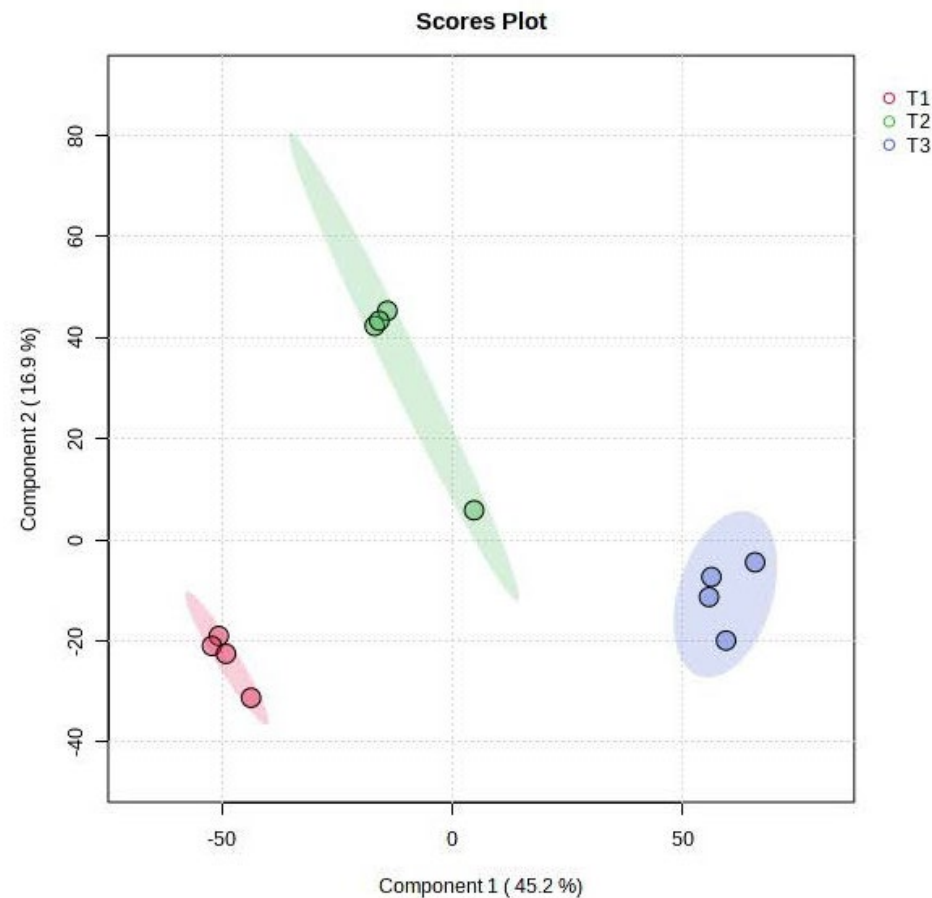
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4.1 Supplementary figure



Supplementary Figure 1: 2-D scores plot illustrating the distribution of detected metabolites across the different stages T1, T2 and T3 using PLS-DA.

CHAPTER 5

Omics approaches to investigate pre-symbiotic responses of the mycorrhizal fungus *Tulasnella sp. SV6* to the orchid host *Serapias vomeracea*

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Abstract

Like other plant-microbe symbioses, the establishment of orchid mycorrhiza (ORM) is likely to require specific communication and metabolic adjustments between the two partners. However, while modulation of plant and fungal metabolism has been investigated in fully established mycorrhizal tissues, the molecular changes occurring during the pre-symbiotic stages of the interaction remain largely unexplored in ORM. In this study, we investigated the pre-symbiotic responses of the ORM fungus *Tulasnella* sp. SV6 to plantlets of the orchid host *Serapias vomeracea* in a dual *in vitro* cultivation system. The fungal mycelium was harvested prior to physical contact with the orchid roots and the fungal transcriptome and metabolome were analyzed using RNA-seq and untargeted metabolomics approaches. The results revealed distinct transcriptomic and metabolomic remodelling of the ORM fungus in the presence of orchid plantlets, as compared to the free-living condition. The ORM fungus responds to the presence of the host plant with a significant up-regulation of genes associated with protein synthesis, amino acid and lipid biosynthesis, indicating increased metabolic activity. Metabolomic analysis supported the RNA-seq data, showing increased levels of amino acids and phospholipids, suggesting a remodelling of cell structure and

signalling during the pre-symbiotic interaction. In addition, we identified an increase of transcripts of a small secreted protein that may play a role in early symbiotic signalling. Taken together, our results suggest that *Tulasnella* sp. SV6 may perceive information from orchid roots, leading to a readjustment of its transcriptomic and metabolomic profiles.

Keywords: orchid mycorrhiza, *Tulasnella*, *Serapias vomeracea*, pre-symbiotic events, interactions

5.1 Introduction

Symbioses between plants and microbes play a crucial role in the functioning of ecosystems, enhancing plant growth, health and productivity. The establishment of symbiotic relationships requires a communication system that enables plants and microbes to signal their reciprocal presence. Upon recognition of available symbiotic partners, metabolic pathways need to be activated to facilitate physical contact and establishment of the symbiosis. Among plant-microbe symbioses, mycorrhiza stands out as a particularly complex form of association that involves the roots of most terrestrial plant species and many soil fungi (Smith and Read, 2010; Genre et al., 2020; Shi et al., 2023). Pre-symbiotic events that occur before physical contact between mycorrhizal fungi and their host plant have been investigated mainly in arbuscular mycorrhiza (AM), the most ancient symbiosis in terrestrial plants (Genre et al., 2020). Here, plant and fungal chemical compounds involved in recognition are released in the rhizosphere. In particular, plants capable of forming AM release small molecules called strigolactones (SLs), phytohormones that are members of the large group of sesquiterpene lactones, initially discovered as inducers of seed germination in parasitic plants such as *Striga* and *Orobanche*

(Matusova et al., 2005). In the pre-symbiotic stages of the AM symbiosis, SLs can induce extensive hyphal branching in the germinating spores of AM fungi (Buée et al., 2000). Before branching, activation of AM fungal genes related to mitochondrial activity and lipid catabolism have been observed, as well as increased fungal respiration rate and mitochondrial density, suggesting a metabolic switch in the AM fungus upon perception of the plant SLs (Besserer et al., 2006, 2008; Lanfranco et al., 2018). In response to the host plant, AM fungi release two types of related signalling molecules, namely lipo-chito-oligosaccharides (LCOs, Maillet et al., 2011), and chito-oligosaccharides (COs, Genre et al., 2013). LCOs were first discovered as being released by symbiotic nitrogen-fixing rhizobia in the pre-symbiotic stages of the legume nodule symbiosis (Dénarié et al., 1996; Murray et al., 2011). The structurally similar LCOs produced by rhizobia and by AM fungi stimulate the host plant responses through the Common Symbiotic Signalling Pathway (CSSP), a shared signal transduction pathway (Kistner et al., 2005; Gutjahr and Parniske 2013; Genre and Russo, 2016). Intriguingly, core genes of the CSSP were revealed in the genome of plants forming different types of endosymbioses, *i.e.*, associations where the symbiotic microbe is hosted inside the plant cells (Delaux et al., 2014; Radhakrishnan et al., 2020). This genome

comparison included orchids, a large family of flowering plants forming typical endomycorrhizal associations with fungi mainly belonging to the Basidiomycetes (Smith and Read, 2010). Expression of *Bletilla striata* and *Dendrobium nobile* homologs of AM-related calcium and calmodulin-dependent protein kinase (CCaMK), a core gene of the CSSP, was activated during orchid mycorrhizal (ORM) symbiosis (Miura et al., 2018, Xing et al., 2020). These findings would indicate that early events in ORM may include chemical signals able to induce a response in the symbiotic partners. However, although *-omics* approaches have been employed to understand the complexity of the ORM interactions over the last years, the pre-symbiotic stages of plant-fungus interactions have been poorly investigated, and most studies have focused on the modulation of plant and fungal metabolism in established mycorrhiza (Perotto et al., 2014; Zhao et al., 2014; Fochi et al., 2017a,b; Miura et al., 2018; Ghirardo et al., 2020; Valadares et al., 2021; De Rose et al., 2023a,b).

In the natural environment, the germination of orchid seeds and the early stages of plant development typically require colonization by a fungal symbiont. This is due to the fact that the minute dust-like orchid seeds contain minimal or no stored energy reserves, which are provided by the ORM fungus (Arditti, 1992; Smith and Read,

2010). Nevertheless, asymbiotic seed germination and orchid growth can be achieved on artificial media that provide the plant with all the nutrients they require, including simple carbon sources (Knudson, 1922). In order to investigate pre-symbiotic events in ORM, we developed a dual *in vitro* cultivation system where the fungal isolate *Tulasnella* sp. SV6 was cultivated in the presence of asymbiotic plantlets of the terrestrial orchid host *Serapias vomeracea*. The genus *Tulasnella* (Tulasnellaceae, Agaricomycotina, Basidiomycetes) is among the most prevalent ORM fungi in both temperate and tropical climates (Dearnaley et al., 2012). In particular, this study focused on the pre-symbiotic responses of *Tulasnella* sp. SV6 to the presence of the orchid host. To this end, the fungal mycelium was collected before physical contact with the plantlet, and the fungal transcriptome and metabolome were studied to reveal possible remodeling of the fungal metabolism at this stage of the interaction.

5.2 Materials and methods

5.2.1 Biological material

Seeds of *Serapias vomeracea* (BURM.) BRIQ. were collected during summer 2021 from mature capsules of wild plants grown in the

locality of Cairo Montenotte (SV, Italy; Latitude 44.406944 N, Longitude 8.319795 E) and stored at 4°C. The fungal isolate SV6 (MUT4178), belonging to the *Tulasnella* genus, was originally isolated from *S. vomeracea* roots grown in Northern Italy (Girlanda et al., 2011) and stored in the Mycotheca Universitatis Taurinensis (MUT) at the University of Turin. The fungal isolate was grown and sub-cultured on solid oatmeal agar (OA, 0.3%) at 25°C in the dark. To assess fungal growth rate on the M551 medium, used in the co-inoculation experiment, the mycelium was transferred to Petri dishes containing solid M551 growth substrate and it was used as inoculum. A similar growth rate was observed for *Tulasnella* sp. SV6 on OA and M551 media (Fig. S1).

5.2.2 Asymbiotic germination of *S. vomeracea*

To obtain asymbiotic plantlets of *S. vomeracea*, seeds were surface sterilized with a solution of 1% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20 for 20 min, followed by three 5-min rinses with sterile distilled water, re-suspended in sterile water and placed on 9 cm Petri dishes containing solid BM1 culture medium (Van Waes and Deberg, 1986). Plates were incubated at 20°C in darkness. After 90 days, protocorms reached 3-4 mm in diameter and were exposed to natural light for additional 30 days at room temperature. Once a leaf

primordium formed at the top of the protocorms, they were transferred to 50 mL centrifuge tubes containing Malmgren Modified Terrestrial Orchid Medium (M551, PhytoTechnology Laboratories®) with sucrose 10% (w/v) at pH 5.8, prepared following the manufacturer's instructions, and grown at room temperature and natural light until seedling development. Once the seedlings showed developed leaf blades and rootlets, they were transferred to sterilized magenta jars containing fresh M551 medium. The procedure is shown in Figure S2. Just before the addition of the fungal mycelium, autoclaved shape-adapted cellulose membranes were positioned on top of the solid medium in the magenta jars, with a central hole for the orchid plantlet.

5.2.3 Experimental design and fungal sample preparation for transcriptomic and metabolomic analyses

To investigate the influence of *S. vomeracea* plantlets on the fungal transcriptome and metabolome, four plugs of *Tulasnella* sp. SV6 mycelium actively growing on M551 medium were placed at the four corners of the magenta jars containing asymbiotic orchid plantlets, on the autoclaved cellulose membranes (Fig. S3). The *Tulasnella* sp. SV6 mycelium was collected 5 days post inoculum (dpi), before the mycelium came in contact with the roots (PRESYMB sample). A

plug of *Tulasnella* sp. SV6 mycelium actively growing on M551 medium was also inoculated on autoclaved cellulose membranes placed on top of M551 medium in the Petri plates, to yield free-living mycelium (FLM sample) as asymbiotic control. Three biological replicates for each condition (PRESYMB and FLM) were prepared for transcriptomics, and four biological replicates for metabolomics. After sampling, all samples were frozen in liquid nitrogen and stored at -80°C.

5.2.4 Transcriptomic analysis

For the RNA-seq experiment, fungal mycelia for each tested condition (PRESYMB, FLM) were freeze-dried and finely homogenized with a TissueLyser (25 Hz, 1 min, twice, Qiagen Diagnostic GmbH, Hilden, Germany). RNA was extracted from ca. 80 mg of biological material from each biological replicate using the “pine tree” CTAB-based method (Chang et al., 1993). The RNA was then eluted in RNA-seq free water and quantified with NanoDrop 2000 and Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). IGA Technology Services (Udine, Italy) carried out the library preparation and RNA sequencing. In detail, Universal Plus mRNA-Seq kit (Tecan Genomics, Redwood City, CA) was used for library preparation, following the manufacturer’s instructions (library

type: fr-secondstrand). RNA samples were quantified and quality tested with an Agilent 2100 Bioanalyzer RNA assay (Agilent technologies, Santa Clara, CA) or by Caliper LabChip GX (PerkinElmer, Waltham, MA). Libraries were then sequenced on paired-end 150 bp mode on NovaSeq 6000 (Illumina, San Diego, CA).

Reads were aligned to the *Tulasnella calospora* AL13/4D v1.0 genome available at <https://mycocosm.jgi.doe.gov/Tulca1/> (Kohler et al., 2015) by using STAR v2.7.10a (Spliced Transcripts Alignment to a Reference; Dobin et al., 2013). To convert SAM data into BAM files and index them, the Samtools 1.11v was used (Li et al., 2009). Reads aligned on the reference genome were counted using HTseq-count 2.0.2v (Anders et al., 2015), and exon junctions and gene overlaps were mapped using the intersection method.

To identify differentially expressed genes (DEGs), raw read counts were imported into the DESeq2 tool version 1.34.0 (Love et al., 2014). Following default DESeq2 normalization of the count data (median of ratios method) and correction for multiple testing, DEG identification was carried out through Wald test using FLM samples as controls. The three biological replicates per condition were employed to compute read count variation. An adjusted p-value

threshold of 0.05 was employed to identify DEGs, that were functionally annotated *in silico* using Blast2GO v5.2.5 (Conesa et al., 2005), with their related Gene Ontology (GO) terms subsequently assigned. To perform a GO enrichment analysis and to provide a summary of the functions and pathways associated with the obtained sets of DEGs, Blast2GO v5.2.5 was employed.

5.2.5 Metabolomic analysis

A total of 8 frozen samples (four biological replicates for each condition; PRESYMB and FLM) were freeze-dried at -50°C under vacuum (Alpha 1-4 LDplus, Christ, Osterrode, Germany) and finely ground with TissueLyser (25 Hz, 1 min, Qiagen Diagnostic, Hilden, Germany). Approximately 10 mg of dried fine powder per sample was extracted following the protocol used by Bertić et al. (2021). Briefly, 800 µL of methanol/2-propanol/water solution (1/1/1, v/v/v) containing 50 µL of an internal standard (IS) mixture were added to the sample (Table S1). The mixture was vortexed for 1 min, sonicated for 10 min at 5°C in an ultrasonic bath and then centrifuged at 9300 *g* for 10 min at 5°C. The supernatant (400 µL) was recovered and the centrifugation process was repeated. The remaining 400 µL of supernatant was collected to obtain a total of 800 µL of the extracted metabolite solution. The supernatant was

dried by SpeedVac (Univapo 150H, Uniequip, Planegg, Germany) and re-dissolved in 350 μ L of 50% (v/v) acetonitrile in water. The solution was mixed and centrifuged at 9300 *g* for 10 min at 5°C, and 300 μ L of supernatant was collected and transferred in amber glass vials for metabolomics analysis.

Non-targeted metabolomic analysis was performed by ultra-performance liquid chromatography (UPLC) ultra-high resolution (UHR) tandem quadrupole/time-of-flight (QqToF) mass spectrometry (MS), as described in Bertić et al. (2021). The system consists of an Ultimate 3000RS UPLC (Thermo Fisher Scientific, Bremen, Germany), a Bruker Impact II (QqToF) and an Apollo II electrospray ionization source (Bruker Daltonics, Bremen, Germany). Metabolites were separated by reversed-phase liquid chromatography (RPLC) and by hydrophilic interaction liquid chromatography (HILIC), each run separately and eluted using H₂O + 0.1% (v/v) formic acid (solvent A) and acetonitrile + 0.1% (v/v) formic acid (solvent B) (see Bertić et al., 2021 for details). The spectra were acquired in both positive (+) and negative (–) ionization modes. Data were processed as outlined in Bertić et al. (2021), using the Metaboscape v4.0 software (Bruker Daltonics) and the parameters listed in Table S2.

Results of RP and HILIC analyses were manually merged and zero values were replaced with random numbers below the threshold value of 700 area unit. Data were normalized for the dry mycelium weight. Compounds were annotated as described in Bertić et al. (2021), matching MS/MS spectra using the libraries 'All Spectra' in MoNA (Mass Bank of North America, <https://mona.fiehnlab.ucdavis.edu>), MS-DIAL Lipids (<http://prime.psc.riken.jp>), MassBank (<https://massbank.eu/MassBank/>), Vaniya/Fiehn Natural Products Library (available through MoNA) and GNPS (<https://gnps.ucsd.edu>). Principal component analysis (PCA) was calculated using the normalized peak areas, logarithmically transformed and pareto scaled in MetaboAnalyst 6.0. Log₂FC were calculated using FLM as control, applying Student t-test and Benjamini–Hochberg correction to discriminate significant values (adj. p-value score <0.05).

5.3 Results

5.3.1 Transcriptomic profile of *Tulasnella* sp. SV6 during pre-symbiotic growth in the presence of *S. vomeracea* plantlets

Sequencing of RNA samples produced an average of 27,433,879

raw reads per sample (ranging from 24,260,989 to 32,378,146; Table S3). Overall, reads mapping on the *Tulasnella calospora* AL13/4D v1.0 reference genome resulted in an average mapping rate of 85.08%. The PCA of normalized read count data (Fig. 1) showed that RNA samples grouped into two distinct groups, the FLM and the PRESYMB samples being well separated by the first principal component (PC1; 94% of variance).

Gene expression in the *Tulasnella* sp. SV6 mycelium during pre-symbiotic (PRESYMB) and free-living (FLM) growth was compared to identify differentially expressed genes (DEGs) in the presence and in the absence of the orchid plantlet. A total of 1,669 genes (out of 19,659 *T. calospora* AL13/4D v.1 total genes) were found to be differentially regulated with adj. p -value < 0.05 . In Fig. 2, a Volcano plot shows the distribution of DEGs detected in PRESYMB. When compared to FLM, transcriptomic reprogramming during pre-symbiotic growth involved 1002 up-regulated and 667 down-regulated genes, respectively. The heatmap and hierarchical clustering in Fig. 3 depicts the significant relative changes in the expression of the top 500 genes showing the most divergent expression (gene expression patterns based on transcript count) between PRESYMB and FLM samples. The complete list of up-regulated and down-regulated DEGs in the PRESYMB sample, as

compared to the FLM sample, can be found in Table S4, and the top 20 up-regulated and down-regulated genes with a known putative function are listed in Table 1 and Table 2, respectively. The top 20 up-regulated DEGs all showed a $\text{Log}_2\text{FC} > 5$, the most up-regulated one coding for a putative glutathione S-transferase (transcript id: 31195, Log_2FC 9.27). A DEG coding for a small secreted protein was also strongly up-regulated (transcript id: 246100, Log_2FC 7.15). Among these top 20 up-regulated DEGs were also two putative aldo/keto reductases (transcript ids: 28543, Log_2FC 7.62 and 4711, Log_2FC 6.67), a tryptophan synthetase (transcript id: 185677, Log_2FC : 6.74), two putative homo-isocitrate dehydrogenase LYS12, involved in lysine biosynthesis (transcript id: 165648, Log_2FC : 5.15), a putative metallo-endopeptidase belonging to the M35 family (transcript id: 178320, Log_2FC : 6.67) and a tetratricopeptide repeat (TPR)-like protein (transcript id: 78871, Log_2FC : 6.70).

In addition to the tryptophan synthetase and the LYS12 genes, additional genes involved in the biosynthesis of amino acids were induced in the PRESYMB samples (Table S4), such as arginine (transcript id: 228403, Log_2FC : 3.16 and transcript id: 224256, Log_2FC : 2.16), asparagine (transcript id: 182504, Log_2FC : 2.38), histidine (transcript id: 233647, Log_2FC : 1.60), leucine (transcript id: 96719, Log_2FC : 2.01 and transcript id: 142459, Log_2FC : 2.59), and

lysine biosynthesis (transcript id: 109796, Log₂FC: 3.20 and transcript id: 240710, Log₂FC: 4.13). The shikimate pathway, leading to the synthesis of phenylalanine, tyrosine and tryptophan, was also activated in the PRESYMB sample (transcript id: 172333, Log₂FC: 4.11). Some genes coding for glycosyl hydrolases (GH) were also identified as being up-regulated. Three of them belong to the GH5 family (transcript ids: 93410, 188339 and 240794, Log₂FC 1.88, 1.76 and 1.75, respectively), *i.e.*, endoglucanases reported to be involved in plant-cell remodeling during early fungal colonization (Zhang et al., 2018), one to the GH32 family (transcript id: 23305, Log₂FC 3.97), which includes invertases, and one to the GH79 family (transcript id: 162910, Log₂FC 1.14).

The top 20 down-regulated DEGs with known putative functions in the PRESYMB samples are listed in Table 2. Among them, two genes coded for putative GPI-anchored cupredoxin (transcript id: 18717, Log₂FC: -8.64 and transcript id: 27833, Log₂FC: -5.74), the first being the most down-regulated gene in the PRESYMB samples. Two genes coding for cytochrome P450s were also among the top 20 down-regulated genes (transcript id: 17088, Log₂FC: -7.14 and transcript id: 34786, Log₂FC: -4.93, respectively), as well as some CAZymes, namely a rhamnogalacturonase B (transcript id: 12253, Log₂FC: -7.15), an endoglucanase GH5-1 (transcript id: 32580,

Log₂FC: -4.97) and a pectin lyase (transcript id: 25833, Log₂FC: -4.73).

A GO enrichment analysis was conducted to identify the biological processes, cellular compartments and molecular functions modulated in the ORM fungus during pre-symbiotic interactions with the orchid plantlet (Fig. 4). The GO terms enriched in the PRESYMB up-regulated transcriptome for biological processes were mainly related to ribosome biogenesis, organonitrogen compound biosynthetic process; the GO terms enriched for cellular components were nonmembrane-bound organelles, cytosol and ribosomes; the GO terms enriched for molecular functions were again related to ribosome and structural molecule activity (Fig. 4).

5.3.2 Metabolomic profiles of *Tulasnella* sp. SV6 during pre-symbiotic growth with *S. vomeracea* plantlets

The metabolomic profile of PRESYMB samples was compared with the profile of FLM samples by PCA (Fig. 5). The first two PCA components (PC1 and PC2) explained 83.4% of the total metabolome variance and showed separation between PRESYMB and FLM samples.

A total of 3372 mass features could be detected among the two different conditions. Significantly different abundances of mass

features (235 with an adj. p -value < 0.05) were detected in the PRESYMB samples with respect to FLM samples. The results are shown in the volcano plot in Figure 6, and the complete list of differentially accumulated mass features (DAMs) is provided in Table S5. Among them, 36 compounds were putatively annotated and are listed in Table 3. Most of them (30 out of 36 DAMs) were more abundant in the PRESYMB samples than in the FLM samples and include several lipids, mostly phospholipids such as phosphatidylcholines (PC) and lysophosphatidylcholines (LPC). Among the phospholipids accumulated in the PRESYMB samples were PC(18:3_20:5), PC(18:2_18:3), PC(16:0/0:0), PC(35:4), PC(18:1), PC(18:2), PC(9:0), LPC(14:0-SN1), LPC(18:3-SN1) and LPC(17:2-SN1). By contrast, a lower abundance in the PRESYMB samples, as compared to FLM, was observed for LPC(18:1) and phosphatidylethanolamine PE(O-15:1_3:0). Other lipids and lipid-like molecules more abundant in the PRESYMB than in the FLM samples were linolenic acid (Log₂FC: 3.57) and compounds annotated as valeryl-carnitine (Log₂FC of 2.64 and 1.83) or azelaic acid (Log₂FC:1.77). Other metabolites more abundant in the PRESYMB samples correspond to amino acids such as proline, pipecolinic acid, phenylalanine (Log₂FC of 6.22, 4.21 and 3.72, respectively), the dipeptides isoleucylvaline (Log₂FC: 3.18) and

leucylproline (Log₂FC: 3.62), as well as secondary metabolites like cinnamic acid (Log₂FC: 3.80) and two sesquiterpenoids like muurolladie-3-one and aspergillusene A (Log₂FC: 5.75 and 1.52 respectively). Among the metabolites that were less abundant in PRESYMB than in FLM samples, trehalose-6-phosphate showed the most negative Log₂FC (-4.28).

5.4 Discussion

The soil, the environment in which terrestrial plants anchor their roots, is a complex and densely populated ecosystem. It is home to a multitude of microbes that can have a neutral effect on the plant, or engage in symbiotic interactions that can be beneficial or harmful (Morgan et al., 2005; Raaijmakers et al., 2008; Wang et al., 2024). In order to establish beneficial interactions in this highly populated environment, plants release specific chemical signals that are perceived by potential microbial partners, thereby inducing a response that can include the release of microbial signals, directional growth and metabolic changes that facilitate the establishment of symbiosis (Ortíz-Castro et al., 2009; Bonfante and Requena, 2011; Genre et al., 2020). The chemical signals, released by plants and microbes, that are necessary for the establishment of

a symbiotic relationship have been identified and characterized in a limited number of interactions. These include the rhizobium/legume symbiosis and the arbuscular mycorrhiza (Limpens and Bisseling, 2003; MacLean et al., 2017; Crosino and Genre, 2022). Despite previous findings indicating that orchids may employ a genetic system of signal transduction similar to that identified in AM plants and legumes (Miura et al., 2018, Xing et al., 2020), the chemical signals involved in mutual recognition remain unknown, as do their influence on the cell organization and metabolism of the receiving partner. To the best of our knowledge, a single report suggests for SLs, the plant signal in AM (Kodama et al., 2022), a branch-inducing effect on *Armillaria mellea*, a fungus capable of forming ORM with the orchid *Gastrodia elata* (Yuan et al., 2018). However, *A. mellea* is an unusual ORM fungus, with a higher prevalence of reports associating it with root rot (Baumgartner et al., 2011).

In this study, we employed transcriptomics and metabolomics to examine the molecular alterations occurring in the ORM fungus *Tulasnella* sp. SV6 during the pre-symbiotic phase, preceding physical contact with the orchid roots. A transcriptomic comparison with the asymbiotic free-living mycelium, cultivated on the same medium in the absence of the orchid host, clearly showed substantial alterations in fungal gene expression in response to the

plant's presence. In particular, an increase in differentially expressed genes (DEGs) involved in protein biosynthesis was observed, as evidenced by the enrichment of several Gene Ontology (GO) terms related to ribosome biogenesis and functioning, as well as the synthesis of several amino acids. Increased amino acid biosynthesis was also supported by metabolomic data, showing a higher content of some amino acids such as proline, pipecolic acid (derived from lysine), phenylalanine, and some dipeptides. It is possible that some of the proteins induced in the ORM fungus during this pre-symbiotic stage may be involved in the symbiotic interaction. For example, one of the 20 most highly induced genes encodes a small secreted protein (transcript id: 246100, Log₂FC: 7.16). Small secreted proteins (SSPs) are a common feature of the secretome of fungi from all lifestyles, representing between 40% and 60% of the secreted proteins in some cases (Feldman et al., 2020). SSPs may play a fundamental role in communication within a hyphal colony or with surrounding microbes in saprotrophic fungi (Plett and Plett, 2022), but the complement of SSPs is typically increased in fungi interacting with plants, either as pathogens or as mutualistic symbionts (Kim et al., 2016). Novel functions for SSPs have been increasingly identified in plant-interacting fungi, where some

strongly induced SSPs are used to manipulate the host defence response and to facilitate plant colonization (Kloppholz et al., 2011; Plett et al., 2011). Although the function of the *Tulasnella* sp. SV6 SSP remains to be established, its strong transcriptional upregulation in the presence of the orchid plantlet makes it an interesting candidate for further investigations of the pre-symbiotic events in ORM. Additionally, two genes encoding RlpA-like proteins were markedly upregulated in the PRESYMB sample (transcript id: 243232, Log₂FC: 4.92 and transcript id: 240769, Log₂FC: 1.64). In the pathogenic fungus *Rhizoctonia solani*, a related protein was identified as an effector capable of suppressing the plant immune response and was found to be highly induced during early-stage infection of sugar beet seedlings (Charova et al., 2020).

Carbohydrate Active enZymes (CAZymes) represent a significant portion of the fungal secretome. They are primarily involved in the saprotrophic degradation of complex polymers present in the substrate (Bonnin and Pelloux, 2020; Gavande et al., 2023). However, fungal hyphae of mycorrhizal fungi interacting with plants must modulate the expression of these hydrolytic enzymes during interactions with the host (Veneault-Fourrey et al., 2014; Adamo et al., 2020; Chen et al. 2022). With regard to the repertoire of CAZymes regulated in the pre-symbiotic stage in *Tulasnella* sp.

SV6, it is noteworthy that, among the most down-regulated genes, were those encoding pectinolytic enzymes, namely a pectin lyase (transcript id: 25833, Log₂FC: -4.07) and a rhamnogalacturonase B (transcript id: 12253, Log₂FC: -7.15). The reason for the strong downregulation of these pectinolytic enzymes in the pre-symbiotic stage is unknown, but since pectin is a major component of the plant cell wall, we cannot exclude that some recognition event may have occurred and led to a reduction of the fungal degradative potential towards plant cell wall components.

Metabolomic analyses revealed significant alterations in the lipid composition of the fungal mycelium, characterized by an increased abundance of glycerophospholipids (GP) such as phosphatidylcholines (PC) and lysophosphatidylcholines (LPC). Phosphatidylcholines are the principal structural membrane lipids, synthesized via diverse metabolic pathways in the Basidiomycetes (Kotlova et al., 2022). The accumulation of these compounds, in conjunction with the increased protein biosynthesis, may indicate that the fungus is undergoing stimulation of fungal growth in response to the host plant and the need for membrane biogenesis. Our gene expression data corroborates the up-regulation of lipid biosynthesis, as DEGs coding for enzymes involved in phospholipid biosynthesis were up-regulated in the PRESYMB state (Table S4).

For instance, a NAD(P)H-dependent glycerol-3-phosphate dehydrogenase, which generates a key precursor for phospholipid synthesis (transcript id: 3905, Log₂FC: 4.13) was identified. Additionally, the data revealed the presence of a probable phospholipid-transporting ATPase IIB (transcript id: 109520, Log₂FC: 2.47), as well as two phosphatidylserine decarboxylases (transcript id: 241123, Log₂FC: 2.07; transcript id: 31445, Log₂FC: 1.24).

The largest metabolomic differences observed in a previous study by Ghirardo et al. (2020) were also related to changes in the *Tulasnella* lipid content during asymbiotic and symbiotic growth. In that study, the ORM fungal mycelium was collected near orchid protocorms in which the mycorrhizal association was already established (i.e., a symbiotic condition), and compared with the free-living mycelium. By comparing our results with those of Ghirardo et al. (2020), PCs were found to increase their content in the *Tulasnella* mycelium in both symbiotic and pre-symbiotic conditions, as compared to the FLM. However, other lipids that increased their content in the fungal mycelium collected outside mycorrhizal protocorms (i.e., the symbiotic stage) were not found to increase in the mycelium collected in the pre-symbiotic stage. For example, a strong increase in sphingolipids (SP) and

lysophosphatidylethanolamine (LPE) was detected in the symbiotic stage (Ghirardo et al., 2020), whereas we found no significant changes for these lipids in the pre-symbiotic ORM mycelium. Interestingly, the expression of a gene coding for a putative alkaline ceramidase, an enzyme that catalyzes the hydrolysis of ceramides into sphingosine, the precursor of sphingolipids, was downregulated in the pre-symbiotic stage (transcript id: 185418, Log₂FC: -1.49). Further experiments are required to understand to what extent the different growth media and plant developmental stages (i.e., protocorms or adult plants) may influence the biosynthesis and accumulation of lipids in the ORM fungal mycelium, but the current data from this study and the previous one by Ghirardo et al. (2020) suggest that lipid composition may change in the external mycelium once the symbiosis with the plant is established.

Lipids are of significance not only as constituents of cellular membranes, but also as signalling molecules. Lysophosphatidylcholines play a significant role as signals and growth regulators in plants (Okazaki and Saito, 2014), however their function in fungi remains less well-understood. A LPC (14:0-SN1) was found to be significantly accumulated in the PRESymb samples (m.f. rp2307, Log₂FC: 6.57). It is noteworthy that LPC has been observed to induce the expression of mycorrhiza-specific phosphate

transporters in *Solanum tuberosum* roots colonized by AM fungi (Drissner et al., 2007; Vijayakumar et al., 2016). However, whether the LPC signal originates from the plant, the fungus, or a combination of both remains to be established.

Inositol phosphates are generated from phosphatidylinositol-4,5-bisphosphate (PIP₂) through the action of phospholipase C and serve as recognized secondary messenger molecules in signal transduction and lipid signalling (Munnik and Testerink, 2009). Two up-regulated DEGs coding for phospholipase C were identified in the *Tulasnella* sp. SV6 transcriptome, (transcript id: 31235, Log₂FC: 2.06; transcript id: 21638, Log₂FC: 1.92). The role of phospholipase C has been investigated in saprotrophic and pathogenic fungi belonging to diverse taxa, primarily through the disruption of PLC genes. The phenotype of these defective mutants indicates that phospholipases C in fungi are necessary for a number of cellular functions mainly related to fungal growth and hyphal development (Barman et al., 2018). A reduction in virulence was also typically observed in PLC mutants of pathogenic fungi. The up-regulation of the two *Tulasnella* sp. SV6 phospholipase C genes in the pre-symbiotic stage is therefore consistent with the general increase in biosynthetic activity, as revealed by both transcriptomics and metabolomics.

In conclusion, our findings demonstrate that the ORM fungus *Tulasnella* sp. SV6 exhibits alterations in its transcriptomic and metabolomic profiles when grown in the presence of the host plant *S. vomeracea*, before any physical contact with the host occurs. In comparison to the free-living mycelium, the fungus in the PRESYMB stage activates a biosynthetic apparatus involving protein and lipid biosynthesis. It is yet to be determined whether these changes are part of a specific response of the ORM fungus to symbiosis-related signalling molecules released by the host plant, or whether they reflect some modifications caused by the plant in the dual co-cultivation system, such as the release of nutrient-rich exudates or volatiles. Further investigations are required to address these questions.

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Declarations

Ethics approval and consent to participate

Not applicable.

Adherence to national and international regulations

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author Contributions

S.P., R.B. and J.-P.S. conceived and designed the research. A.G., S.D.R. and F.S. conducted all wet lab experiments and analysed the data. S.P., S.D.R. and R.B. wrote the manuscript. All authors reviewed the manuscript.

5.5 Tables

Table 1. List of top 20 up-regulated DEGs in the PRESymb samples.

Transcript id	Log ₂ FC	Log ₂ FC SE	Adj. p-value	Description
31195	9.27	1.70	1.75E-06	glutathione S-transferase family protein
230917	7.73	2.18	4.74E-03	S1/P1 nuclease-domain-containing protein
28543	7.62	0.88	1.21E-15	Aldo/keto reductase
246100	7.16	0.64	6.76E-26	small secreted protein
104862	6.96	1.62	3.29E-04	flavo protein WrbA
21363	6.87	0.68	3.68E-21	reverse transcriptase domain-containing protein
185677	6.75	1.38	3.07E-05	tryptophan synthetase
78871	6.70	1.47	1.24E-04	TPR-like protein
4711	6.67	0.52	1.31E-34	putative aldo-keto reductase
178320	6.67	1.66	9.44E-04	M35 family metallo-endopeptidase
21793	6.58	1.48	1.84E-04	DJ-1/PfpI family protein
32761	6.35	0.65	6.60E-20	minor allergen Alt a 7
106210	5.84	0.73	1.76E-13	60S ribosomal protein L15
20463	5.78	1.58	3.35E-03	hypothetical protein SCP_0703600
17873	5.75	0.70	3.44E-14	FAD/NAD(P)-binding domain-containing protein
243502	5.56	0.79	2.04E-10	hypothetical protein CB0940_10658
5838	5.32	0.85	2.22E-08	probable nucleolar protein 5-2
165648	5.16	1.67	1.82E-02	putative LYS12 - Homo-isocitrate dehydrogenase
8665	5.10	0.83	4.13E-08	protein FAR1-RELATED SEQUENCE 5-like
9090	5.08	1.59	1.34E-02	GPN-loop GTPase QQT2 isoform X2

Table 2. List of top 20 down-regulated DEGs in the PRESymb samples.

Transcript id	Log ₂ FC	Log ₂ FC SE	Adj. p-value	Description
18717	-8.64	0.75	2.47E-27	putative GPI-anchored cupredoxin
18719	-7.27	0.89	5.48E-14	putative extracellular serine-rich protein
12253	-7.15	1.31	1.95E-06	Rhamnogalacturonase B
17088	-7.14	1.40	9.77E-06	cytochrome P450
30377	-6.12	0.66	9.79E-18	hypothetical protein COCCADRAFT_36097
26982	-5.80	1.55	2.51E-03	WD40 repeat-like protein
29516	-5.79	1.47	1.20E-03	recombinase, putative
244650	-5.78	1.34	3.07E-04	2-oxoglutarate metabolism-related protein, putative
27833	-5.74	0.79	5.26E-11	putative GPI-anchored cupredoxin
30761	-5.70	1.43	1.04E-03	S1/P1 nuclease-domain-containing protein
20821	-5.65	0.41	1.68E-38	Hsp20/alpha crystallin family protein
4369	-5.51	0.64	1.89E-15	tyrosinase
241343	-5.38	0.50	7.51E-24	Di-copper centre-containing
92348	-5.28	1.00	4.12E-06	serine/arginine repetitive matrix protein 1 isoform X3
20659	-5.14	1.57	1.10E-02	Bet v1-like protein
241500	-5.04	0.62	1.38E-13	uncharacterized protein BJ212DRAFT_1482028
32580	-4.97	1.51	1.08E-02	Endoglucanase gh5-1
34786	-4.93	0.80	3.97E-08	cytochrome P450
17292	-4.77	0.53	3.91E-17	UMTA protein
25833	-4.73	1.67	3.53E-02	pectin lyase fold/virulence factor

Table 3. List of annotated m.f. detected in the PRESYMB condition. as compared to FLM.

Id	RT [min]	m/z meas .	Name	Molecular Formula	Log ₂ FC	P-adj. value
rp3 812	25.9 1	802. 5343	PC 18:3_20:5	C46H76N O8P	6.9 5	0.013 455
rp3 813	25.9 2	780. 5524	PC 18:2_18:3	C44H78N O8P	6.5 0	0.015 172
rp1 009	12.3 4	116.0 704	PROLINE	C5H9NO 2	6.2 2	0.001 088
rp1 603	17.6 1	219. 1745	MUUROLLADIE-3-ONE	C15H22O	5.7 4	0.017 328
rp2 551	19.1 6	496. 339	PC (16:0/0:0)	C24H50N O7P	5.3 8	0.002 478
rp3 828	26.4 9	768. 5526	PC 35:4	C43H78N O8P	5.2 7	0.039 068
rp3 11	6.82	298. 0964	5-S METHYLTHIOADENOSINE; LC-TDDA; CE30	C11H15N 5O3S	4.6 4	0.001 01
h17 7	11.6 8	130. 085	PIPECOLINIC ACID	C6H11N O2	4.2 1	0.038 573
rp2 871	20.4 1	524. 3702	1-STEAROYL-SN- GLYCERO-3- PHOSPHOCHOLINE	C26H54N O7P	4.0 9	0.001 435
rp8 3	4.77	149. 0592	CINNAMIC ACID	C9H8O2	3.7 9	0.002 891
rp8 2	4.77	166. 086	PHENYLALANINE	C9H11N O2	3.7 2	0.002 891
h16 8	5.25	534. 2946	PC (16:0/0:0)	C24H50N O7P	3.5 9	0.016 078
h19	6.16	104. 0699	4-AMINO BUTANOATE	C4H9NO 2	3.5 7	0.039 64
rp2 923	20.6 9	279. 2312	LINOLENIC ACID	C18H30O 2	3.5 6	0.009 063
rp2 286	18.4 4	518. 3237	LPC 18:3-SN1	C26H48N O7P	3.5 1	0.044 945
rp1 821	17.8 6	536. 3341	PC 18:1	C26H50N O8P	3.3 4	0.020 771
rp2 13	6.2	231. 1703	ISOLEUCYLVALINE	C11H22N 2O3	3.1 7	0.039 899
rp1 875	17.9 3	534. 3195	PC 18:2	C26H48N O8P	3.1 5	0.002 815
rp3 119	21.8 6	282. 2786	LAUROCAPRAM	C18H35N O	2.9 3	0.009 728
rp5 40	8.24	246. 1699	VALERYL-CARNITINE; AIF; CE30; CORRDEC	C12H23N O4	2.6 3	0.009 931

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rp2 318	18.4 8	506. 3224	LPC 17:2-SN1	C25H48N O7P	2.5 5	0.010 345
rp2 462	18.8 4	267. 1718	TRI- ISOBUTYLPHOSPHATE	C12H27O 4P	2.1 7	0.018 705
rp5 68	8.57	246. 1699	VALERYL-CARNITINE; AIF; CE30; CORRDEC	C12H23N O4	1.8 2	0.034 181
rp1 138	13.9	211.0 947	AZELAIC ACID	C9H16O4	1.7 6	0.006 521
rp3 315	22.4 2	287. 1994	4-ANDROSTENE-3.17- DIONE	C19H26O 2	1.6 9	0.006 685
rp9 21	11.3 4	412. 2092	PC 9:0	C17H34N O8P	1.6 5	0.015 172
rp2 469	18.8 6	235. 1689	ASPERGILLUSENES A	C15H22O 2	1.5 2	0.026 676
rp2 552	19.1 7	522. 3546	LPC 18:1	C26H52N O7P	- 1.5 7	0.026 265
rp2 548	19.1 5	480. 3079	PE O-15:1_3:0	C23H46N O7P	- 2.8 8	0.006 521
rp2 690	19.6 4	355. 283	MONOLINOLEIN	C21H38O 4	- 3.1 4	0.013 566
h60	11.0 7	191. 019	CITRIC ACID	C6H8O7	- 3.2 7	0.014 65
h50	3.27	162. 1118	CARNITINE	C7H15N O3	- 3.4 4	0.039 068
h19 7	15.9 4	421. 0754	TREHALOSE-6- PHOSPHATE	C12H23O 14P	- 4.2 8	0.034 181

5.6 Figures

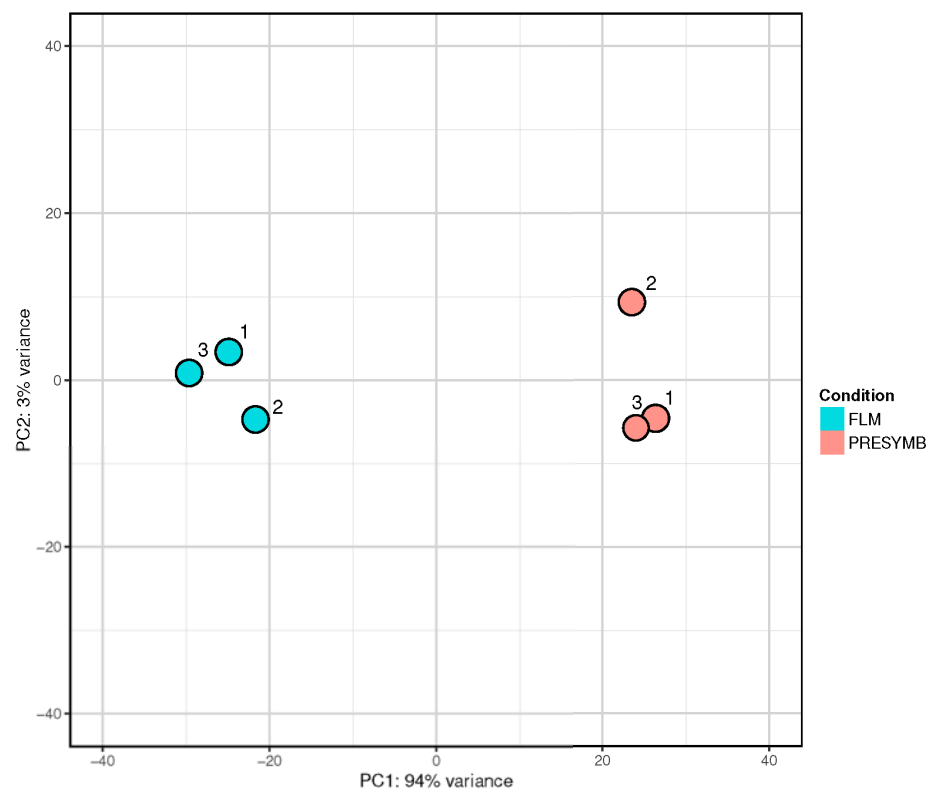


Figure 1. Principal components analysis (PCA) of normalized read counts of all samples used in RNAseq experiment.

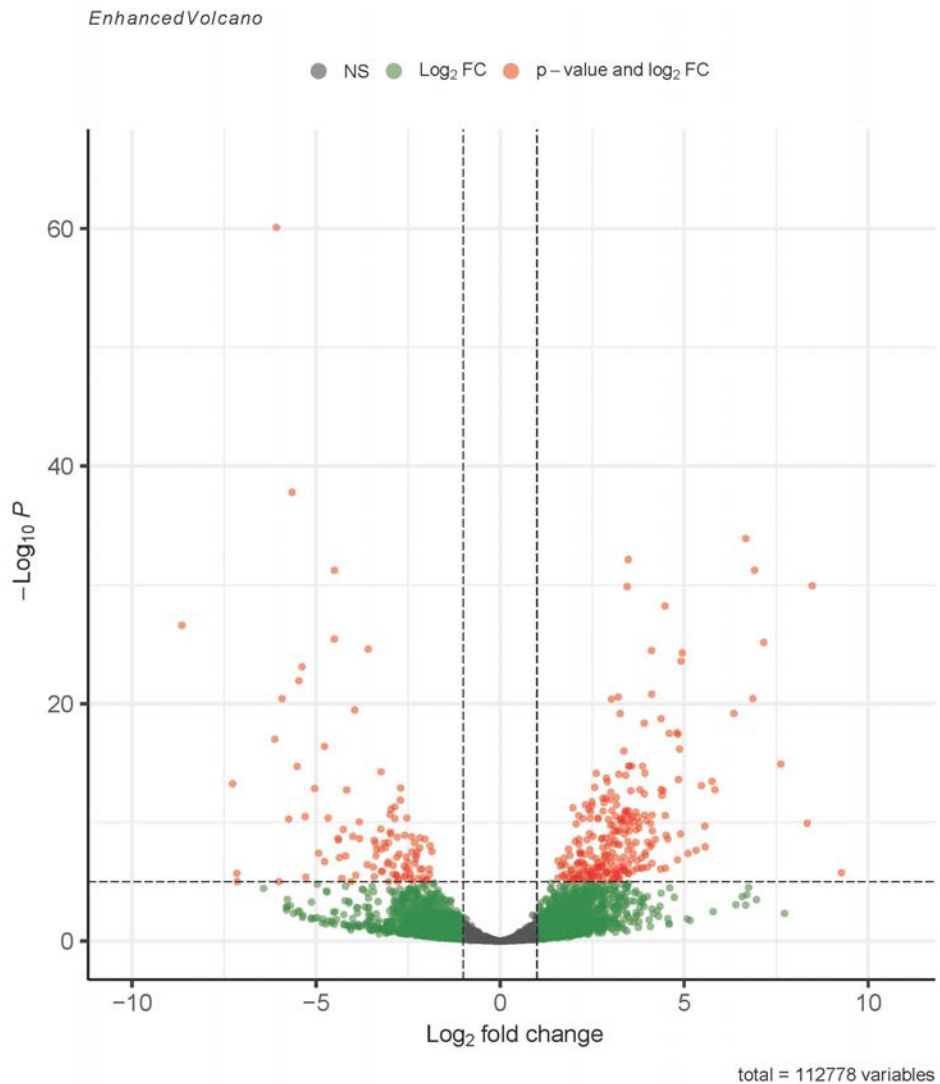


Figure 2. Volcano plots showing identified DEGs in the PRESymb sample as compared with the FLM sample (control condition). Significant up-and down-regulated genes were represented by red dots ($\text{Log}_2\text{FC} > 1$ or < -1 , p-adjusted value < 0.05). Green dots represent genes with a $\text{Log}_2\text{FC} > 1$ or < -1 but a not-significant p-adjusted value (p adjusted value > 0.05). Grey dots represented genes showing a Log_2FC ranging from -1 and 1 and a not-significant p-adjusted value (p-adjusted value > 0.05).

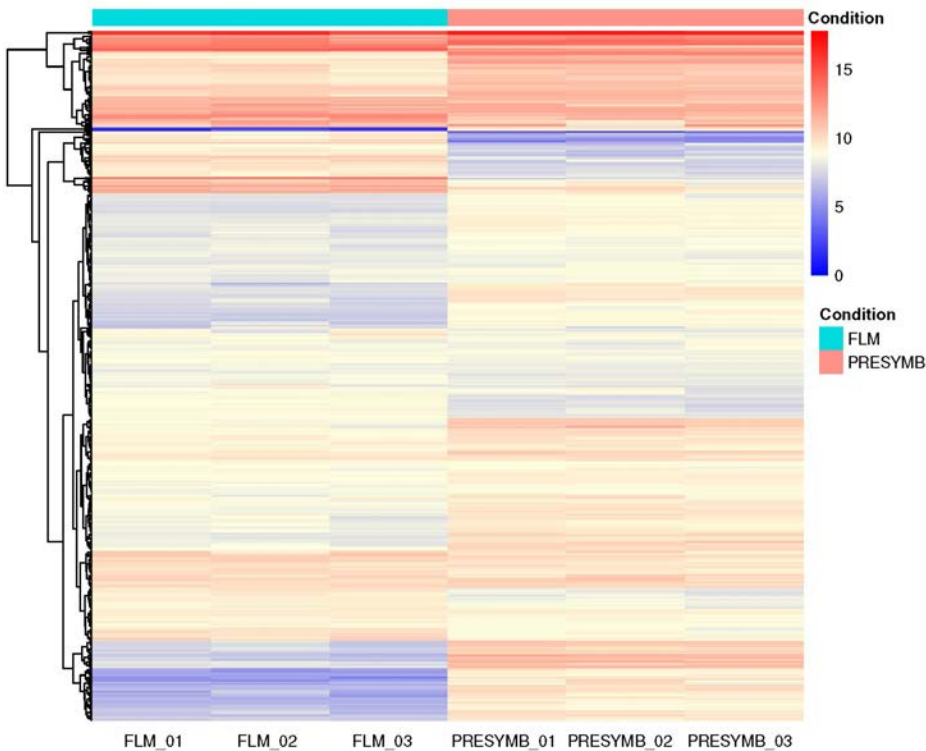


Figure 3. Heatmap and hierarchical clustering of the 500 genes showing the most divergent expression in the three biological replicates of PRESYMB and FLM, using the McQuitty algorithm. The heatmap shows the gene expression patterns in the two conditions, with red indicating up-regulated and blue representing down-regulated genes. Different color intensity indicates different levels of expression based on Log₂FC.

Omics approaches to investigate pre-symbiotic responses of the mycorrhizal fungus *Tulasnella sp. SV6* to the orchid host *Serapias vomeracea*

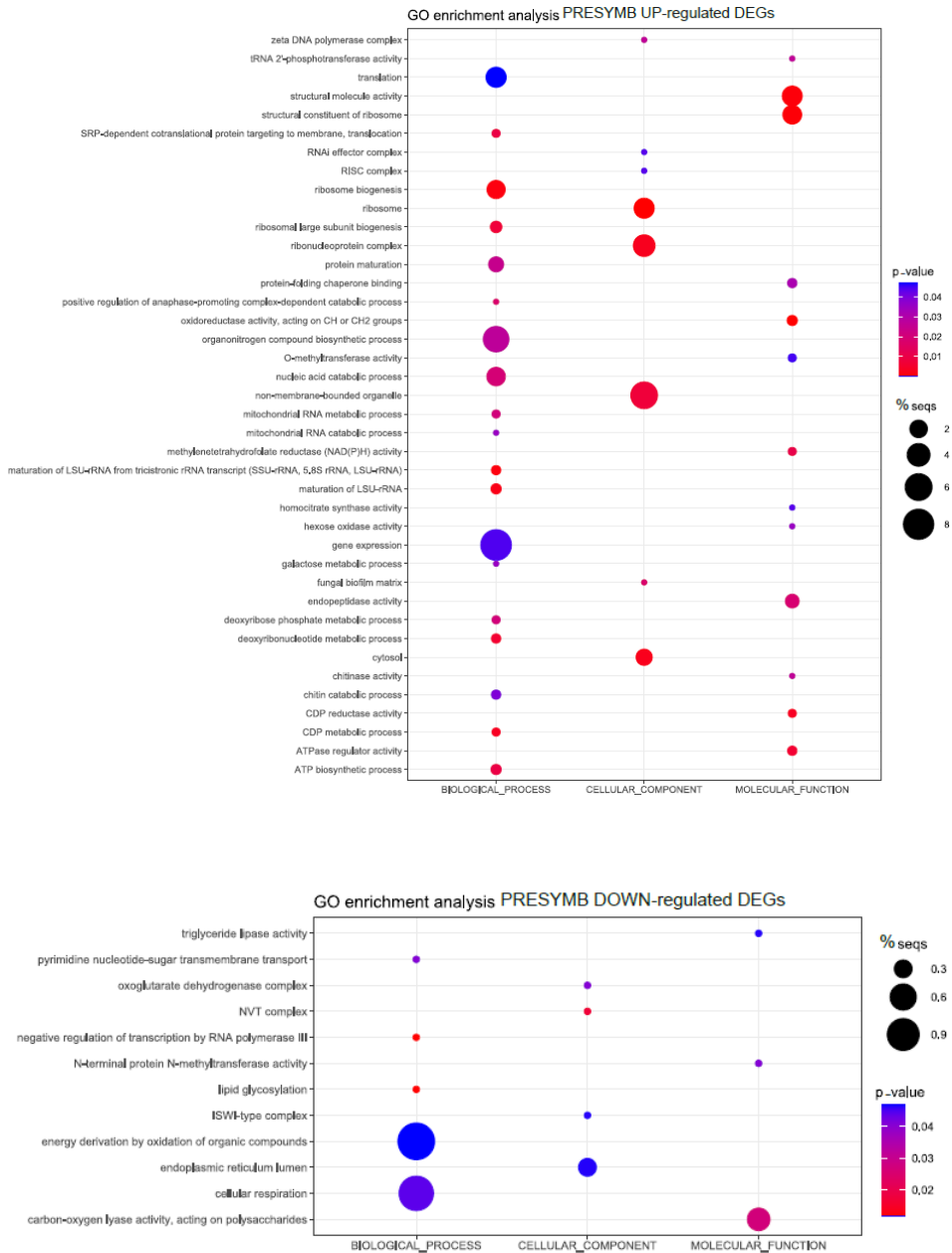


Figure 4. Bubble plots showing GO-enriched terms classified as Biological Process (BP) in detected up and down-regulated DEGs. The x-axis shows the fold enrichment values, i.e., the percentage of genes in the selected DEG list belonging to a pathway divided by the corresponding percentage in all reference gene list, and the y-axis reports the GO terms. Sizes of bubbles are proportional

to the number of genes assigned to the related GO term, while bubble color indicates the significance of the enriched term (False Discovery Rate values) as calculated by the enrichment analysis by Blast2GO.

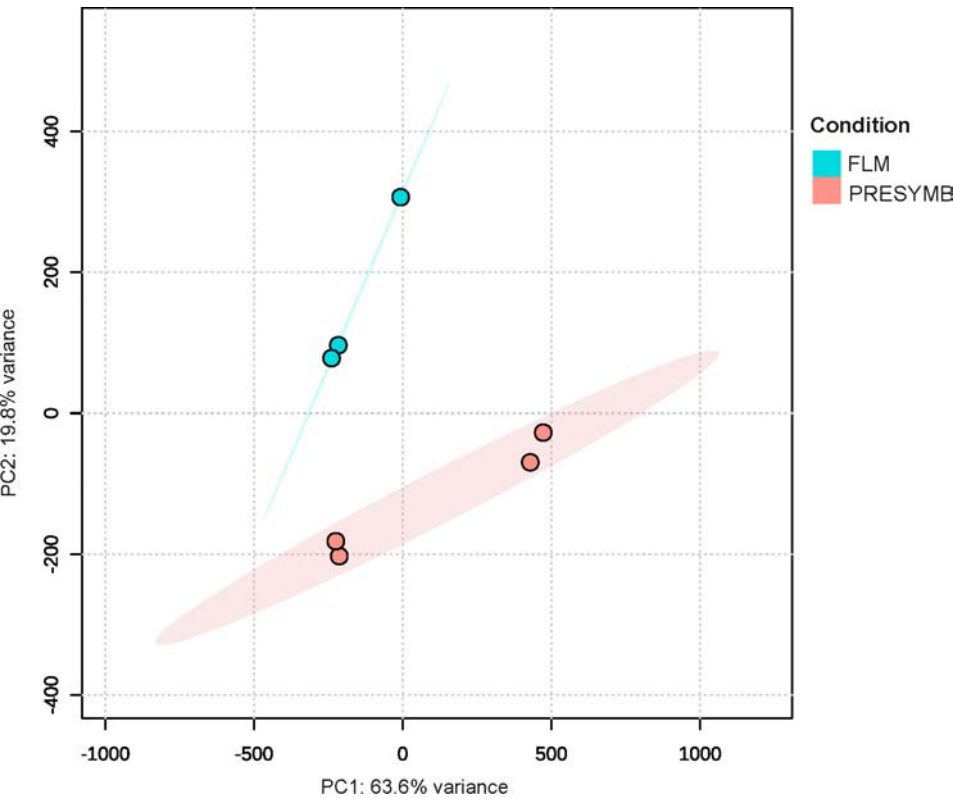


Figure 5. Principal components analysis (PCA) of metabolite-related mass features (m.f.) measurements in all samples.

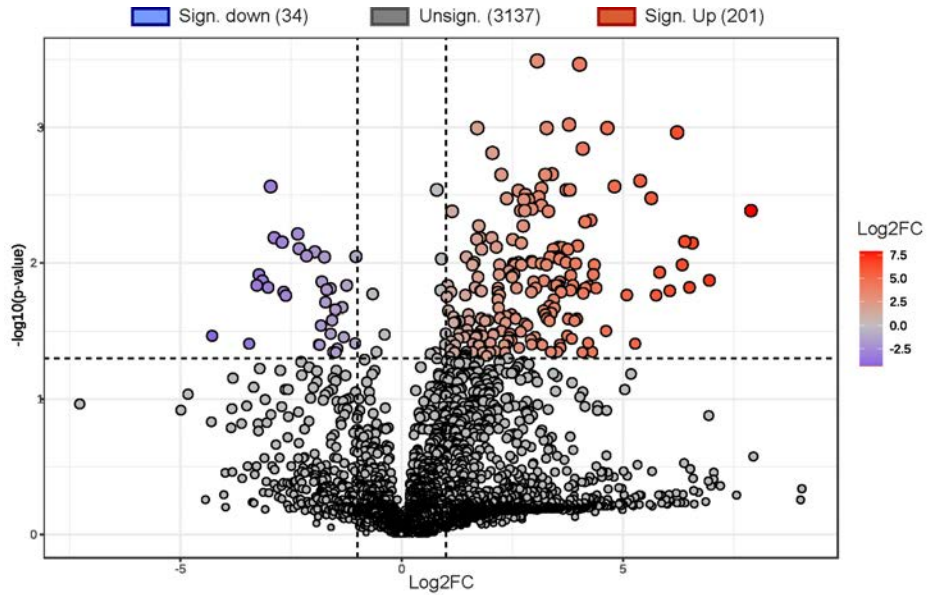
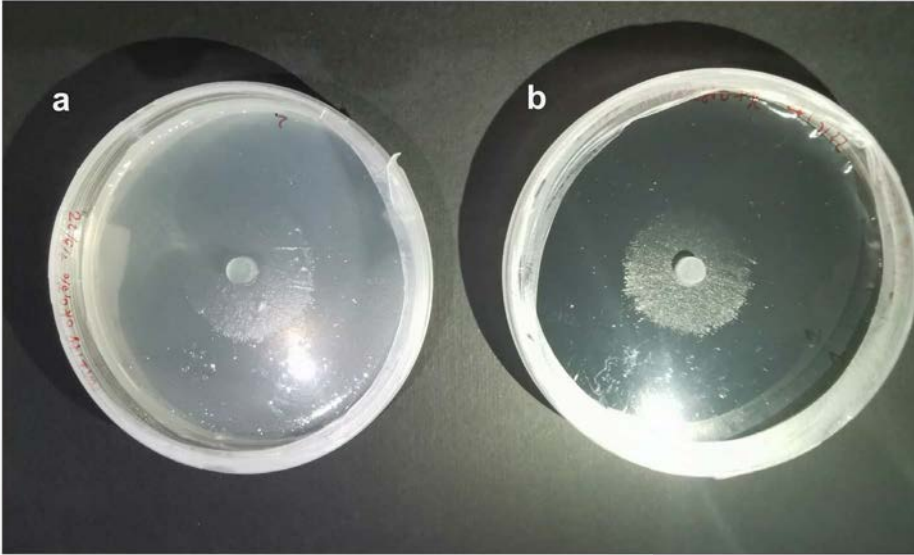
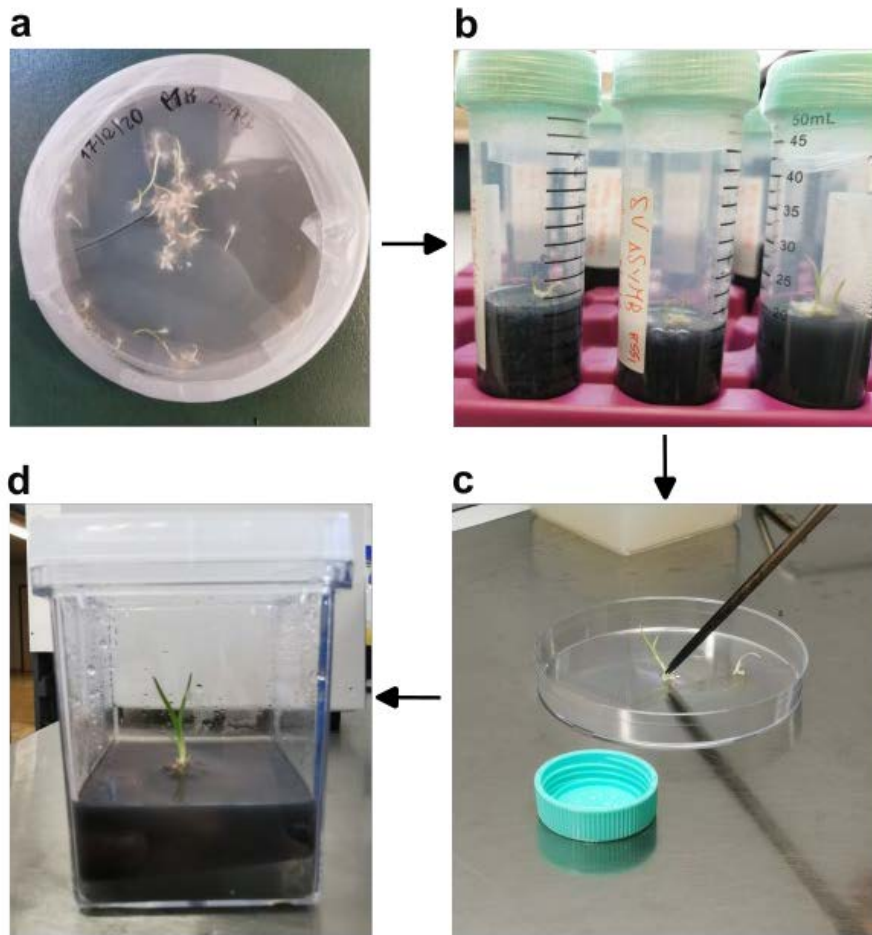


Figure 6. Volcano plot showing identified m.f. in PRESYMB as compared to FLM (control condition). Significant up- and down- accumulated m.f. were represented by red dots ($\text{Log}_2\text{FC} > 1$, p-adjusted value < 0.05) and blue dots ($\text{Log}_2\text{FC} < -1$, p-adjusted value < 0.05). Grey dots represented m.f. showing a not-significant p-adjusted value (p-adj > 0.05).

5.7 Supplementary materials



Supplementary figure 1. *Tulasnella* sp. SV6 growing on 0.3% Oat Agar (a) and on M551 (b) media in Petri dishes.



Supplementary figure 2. The different growth stages of *S. vomeracea* grown asymbiotically. a) Protocorms with leaf primordia 120 days after germination; b) *S. vomeracea* seedlings transferred in Falcon tubes 140 days after germination; c) transfer of *S. vomeracea* seedlings to magenta jars under sterile conditions; d) asymbiotic *S. vomeracea* plantlets in magenta jars.

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CHAPTER 6

Conclusions and perspectives

Orchid mycorrhiza (ORM) is considered a founding event in the evolution of Orchidaceae, contributing to the ecological adaptability and diversification of species within the family (Rasmussen and Rasmussen, 2014). This symbiosis involves a variety of fungal taxa and influence plant population and community dynamics through mechanisms such as soil nutrient partitioning and differential mycorrhizal benefits (Tedersoo et al., 2020). The involvement of diverse fungal partners and intricate ecological requirements of orchids makes the ORM interactions extremely complex. Many authors have focused their research on the taxonomic diversity of ORM fungi in orchids with different trophic strategies, adaptations or life stages, and their potential for orchid conservation (Reiter et al., 2020; Calevo et al., 2021; Hartvig et al., 2024). Advances in high-throughput molecular identification techniques have been applied to this field and have increased our knowledge of ORM fungal diversity and its ecological dynamics (Li et al., 2021; Lofgren and Stajich, 2021).

By contrast, less attention has been devoted to the study of the molecular basis of plant-fungus interactions in ORM. As reviewed in Chapter 2, the application of *-omics* approaches has provided deeper insights into the molecular mechanisms and nutrient transfer processes involved in these interactions. Despite these advances,

many aspects of the ORM symbiosis remain unexplored. In this context, the purpose of my research project was to use transcriptomics and metabolomics approaches to address unresolved questions and to uncover novel characteristics of this complex relationship.

Whole-genome sequencing offers the most detailed view into the genetic functions of an organisms, and a protocol to improve DNA extraction and quality for the genome sequencing of *Tulasnella* sp. SV6 and of two additional ORM fungal isolates has been described in the appendix.

6.1 Nutrients translocation in ORM during mycoheterotrophic stages: a winning strategy for orchid?

Orchid growth, nutrient acquisition and survival rely on ORM fungi, especially during plant seed germination and early development (Favre-Godal et al., 2020; Leng et al., 2024). In this thesis, I have used some *-omics* techniques to address plant-fungus interaction in OrM during presymbiotic events as well as in fully established mycorrhiza. The main results of these investigations are summarized in Figure 1.

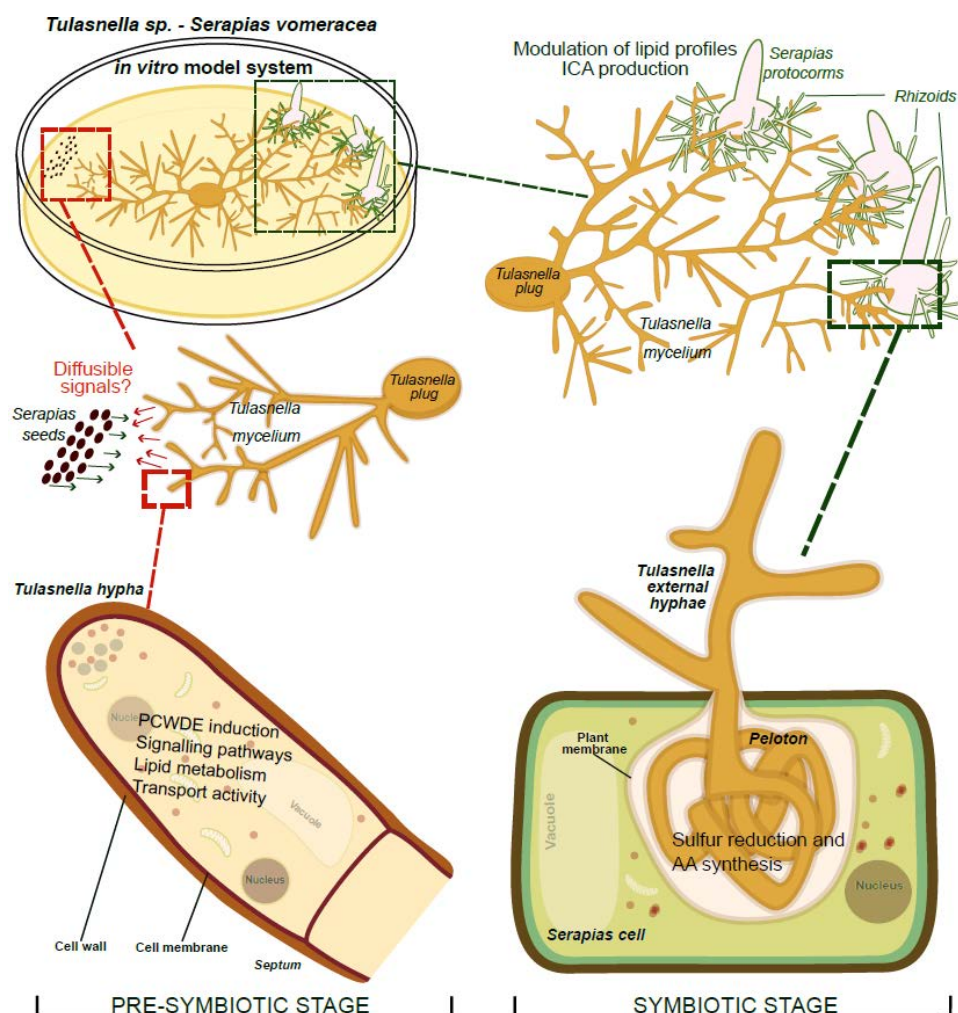


Figure 1. Figure providing an overview of the results obtained. AA, amino acids; ICA, Indole-3-carboxylic acid; PCWDE, plant cell wall degrading enzymes.

Early research has shown that an ORM isolate (at that time erroneously identified as *Rhizoctonia solani*), could break down polysaccharides like cellulose and supply organic carbon (C) to orchid protocorms of *Dactylorhiza purpurella*, likely in the form of

carbohydrates (Smith, 1967). Further studies have revealed that nitrogen (N) is also transferred to the photosynthetic orchid *Goodyera repens* when amino acids like glycine are provided to the ORM fungus (Cameron et al., 2006). The full complement of genetic functions of an organism can be revealed by the sequencing of its complete genome (Blaxter et al., 2022). The genome of the isolate *T. calospora* AL13, the first ORM fungus to be sequenced (Kohler et al., 2015), revealed two ammonium transporters and several amino acid transporters, but lack of a nitrate assimilation system (Fochi et al., 2017a). These authors suggested that this ORM fungus may transfer N to the orchid host mainly in organic forms, since many plants amino acids transporters were up-regulated in *S. vomeracea* protocorms colonized by *T. calospora* AL13, specifically in protocorm cells containing viable fungal pelotons (Fochi et al., 2017a,b).

Thanks to a collaboration with Prof. Yukari Kuga of the University of Hiroshima (Japan), advanced techniques like secondary ion mass spectrometry (SIMS) were employed in this thesis to investigate further nutrient transfer to the host plant in the same ORM model system already used by Fochi et al. (2017). The SIMS technique can be used to localize stable isotopes inside cells and tissues, and was already used to demonstrate, inside mycorrhizal protocorms of the

orchid *Spiranthes sinensis*, the transfer and localization of ^{13}C and ^{15}N provided to the ORM fungus *Ceratobasidium* sp. (Kuga et al. 2014). In this thesis (Chapter 3), we demonstrate that *T. calospora* can assist the orchid *S. vomeracea* in nutrition by taking up sulfur (S), C, and N from the substrate and by translocating these elements to the host plant (De Rose et al., 2023). The SIMS technique indicated in fact that labeled ^{34}S , ^{15}N , and ^{13}C , provided to the external ORM fungal mycelium, were translocated to the intracellular fungal hyphae and to the host cells, where they labelled the plant nuclei. No information was previously available regarding S uptake and metabolism in ORM, and these findings provided a first demonstration that *T. calospora* actively supplies S to the host plant. The parallel transcriptomic investigations of plant and fungal genes putatively involved in S uptake and metabolism, also provided clues about the form of S translocated to the plant in ORM. Plants and fungi acquire oxidized inorganic S forms from the external environment, but before they can introduce S into organic compounds they must reduce it through the reductive sulfate assimilation (RSA) pathway (Marzluf, 1997; Koprivova and Kopriva, 2016). This pathway, although not identical in plants and fungi, involves in both cases the expression of permeases, transporters and enzymes for S acquisition and assimilation (Marzluf, 1997;

Koprivova and Kopriva, 2016). In this thesis, a strong upregulation of the fungal RSA pathway was observed in mycorrhizal protocorms, as compared to the free-living mycelium, while the plant RSA pathway was not significantly activated in symbiosis. Together with the SIMS data, that indicate a linear relationship between C, N and S transfer to the plant, these results prompted the hypothesis that these elements may be part of the same organic compound, likely a S-containing amino acid or a short peptide such as glutathione.

Although further studies are required to determine the specific molecular forms of N and S translocated to the plant, transfer of organic molecules containing these two essential elements could represent a successful evolutionary adaptation during the early mycoheterotrophic development of orchids. Photosynthetic plants typically take up S as sulfate and N as nitrate, subsequently reducing them within their cells through energy-consuming processes (Kopřiva et al., 2024; Li et al., 2020; Vivian et al., 2021). The provision of both S and N in organic forms by ORM fungi would be extremely useful for a plant that cannot convert light energy through photosynthesis, and the ORM fungus would take all the energetic cost associated with N and S reduction. In addition, provision of organic forms of N and S would have a double value

because it would also provide C, highly required during the mycoheterotrophic stages for orchid metabolism and growth.

Many aspects of S metabolism in ORM remain to be clarified. The set of plant genes analyzed in Chapter 3 were limited, due to the lack of a genome sequence for *S. vomeracea*, and did not provide a complete picture of S metabolism in this orchid species. Additionally, the molecular mechanisms regulating S sensing and homeostasis in plants remain in general poorly understood (Kopriva et al., 2019; Li et al., 2020). Further research is also needed to elucidate the complete mechanism that regulates fungal S uptake and transfer to the plant. Another intriguing question is S translocation to adult *S. vomeracea* plants, and in general to adult orchids. Adult photosynthetic orchids can convert light energy through photosynthesis, and it would be interesting to investigate if they show a preference for inorganic S forms, or whether they maintain the same strategy as mycoheterotrophic protocorms. Despite all open questions, these novel data on S metabolism in mycorrhizal *S. vomeracea* protocorms pose intriguing interpretations of the mechanisms that orchids have evolved, and that allow them to grow during the achlorophyllous stages.

6.2 Remodelling of fungal metabolism during early establishment of ORM symbiosis

In Chapters 4 and 5, the *S. vomeracea*-*Tulasnella* sp.SV6 *in vitro* model system has been used to reveal possible reactions of the fungus to the presence of the plant during the early stages of the interaction, even before physical contact occurred. Recent research on the molecular mechanisms underlying the ORM symbiosis employed genomic, transcriptomic and proteomic approaches to identify in the plant possible involvement of signaling molecules, responses to mycorrhizal factors and immune system regulation (Favre-Godal et al., 2020), although these aspects remain poorly understood.

Very little is also known about the strategies that ORM fungi might employ to establish symbiosis with their host. Significant metabolic changes were observed in the mycelium of *T. calospora* AL13 collected outside established mycorrhizal protocorms of *S. vomeracea*, particularly related to lipid metabolism (Ghirardo et al. 2020). As compared to other *omics* approaches, metabolomics provides direct information on the biochemical condition of cells (Ghirardo et al., 2020). In this PhD project, the results of metabolomics were confronted with those of transcriptomics to

investigate the metabolic remodeling occurring in the fungal partner before the establishment of the ORM symbiosis. The integration of data from gene expression and metabolite profiling revealed early changes induced in the *Tulasnella* sp. SV6 isolate when co-cultivated with either *S. vomeracea* seeds or plantlets. In particular, the up-regulation of several genes related to signal transduction mechanisms prior to the development of the symbiosis, as well as significant changes in fungal amino acid and lipid profile, indicates that the proximity of the orchid host is relevant for the remodulation of the primary metabolic activity of the fungal partners.

It remains to be established if the metabolic remodeling observed in the ORM fungus depends on the perception of specific signaling molecules produced by the host, or whether it represents a generic response to growth conditions that may have been altered in the *in vitro* co-cultivation system. In this sense, the proteome and metabolome may offer insights into plant-microbe interactions, as they not only add extra levels of regulation but also often contain the final products of the regulatory processes (Feussner and Polle, 2015). Interactions between proteins and endogenous metabolites are a hallmark of all cellular processes, from metabolism to signaling; in metabolism, enzymes interact with metabolites to catalyze chemical reactions, while in signaling, chemical

compounds act as co-factors to mediate protein function (Li et al., 2021). Protein–metabolite interactions thus serve as a bridge between transcriptomics and metabolomics, providing insights that single datasets alone cannot capture.

Proteomics can be used to investigate changes in the cellular proteome, in the secreted proteome (i.e, the secretome), in protein interactions, and in post transcriptional and translational modifications (Liu et al., 2022). In *Oncidium sphacelatum* mycorrhizal protocorms, a proteomic approach allowed the detection of highly expressed proteins implicated in stress response, protein–protein interaction, and saccharide and protein biosynthesis in the ORM fungus *Ceratobasidium* sp. (Valadares et al., 2014). During symbiotic seed germination of the orchid *Dendrobium officinale*, comparative proteomic and transcriptomic analyses allowed the identification of some key plant proteins induced during fungal colonization of orchid seeds and involved in lipid and carbohydrate metabolism (Chen et al., 2017).

The use of proteomic tools may allow to outline possible links between the dataset obtained in this PhD project, as well as to further investigate the gene and protein regulatory networks active during the early stages of *S. vomeracea* - *Tulasnella* sp. symbiotic interactions. In particular, the secretome of *Tulasnella* sp. could

reveal, in addition to the small secreted proteins identified in Chapters 4 and 5, specific features and mechanisms in which they are involved both before and after symbiosis establishment. New advanced workflows can broaden the scope of “exoproteomics”. Very recently, a workflow called EXCRETE (enhanced exoproteome characterization by mass spectrometry, Russo et al., 2024), was used for characterize the microbial extracellular proteome. In addition, since post- transcriptional and translational modifications (PTMs) create significant variety, complexity, and diversity in gene products, a thorough analysis of PTMs within the fungal regulatory network using methods such as mass-spectrometry-based proteomics could be useful to reveal the metabolic pathways they regulate (Jensen, 2004; Aebersold and Mann, 2016). These modifications are essential for fungal stress response, host adaptation, and antifungal resistance in pathogenic species like *Candida*, *Aspergillus*, and *Cryptococcus* (Retanal et al., 2021). Metabolic processes are strongly influenced by circadian regulation at the post-transcriptional level In *Neurospora crassa* (Hurley et al., 2018), and the involvement of PTMs in the synthesis of secondary metabolites in the genus *Aspergillus* and other fungi was extensively reviewed (Yang et al., 2022).

Recently, McDonald et al. (2022) discussed the role of protein phosphorylation, one of most studied PTMs, in the regulation of transporters at the symbiotic interface in legume–rhizobia, AM and ECM symbioses, but information on the impact of these regulatory levels on metabolism is still missing, especially in ORM. Understanding how the proteome modifications influence the fungal–orchid relationship would be highly valuable.

Thus, there is currently only indirect evidence that potential signaling molecules may be produced by the plant and perceived by the fungus during the early stages of ORM symbiosis. Given the scarcity of information on ORM, possible similarities with AM can provide a critical basis for studying ORM interactions. The establishment of AM symbiosis has been extensively studied, revealing a complex exchange of molecular signals between plants and fungi (Bonfante and Requena, 2011). Plants release strigolactones (SLs), which stimulate fungal metabolism and branching (López-Ráez et al., 2015). A single report suggested that SLs may induce branching on the mycelium of *Armillaria mellea*, a symbiont of the orchid *Gastrodia elata* (Yuan et al., 2018), but this observation should be verified.

A dialogue between symbiotic partners involves an exchange of molecular signals. In response to the plant signals, AM fungi

produce Myc factors, chitin-derived molecules that trigger symbiotic responses in host plants (Crosino and Genre, 2022). This chemical dialogue enables mutual recognition and orchestrates symbiotic programs (López-Ráez, 2015).

An interesting discovery was the identification, in some orchid species, of a set of genes involved in the so called Common Symbiotic Signaling Pathway (CSSP), also found in the genome of angiosperms that form AM or other endosymbioses (Delaux et al., 2014; Radhakrishnan et al., 2020). In AM plants, the CSSP transduces the signals produced by the fungus and enables the downstream activation of symbiotic genes that facilitate fungal accommodation and nutrient exchange (Stougaard, 2001; Kistner et al., 2005; Genre and Russo, 2016). One of these genes (BsCCaMK), identified in the transcriptome of the orchid *Bletilla striata*, retained functional characteristics similar to the gene in AM plants because it was able to complement a *L. japonicus ccamk* mutant (Miura et al. 2018). This finding suggests that ORM fungi, like AM fungi, may release chitin-derived molecules related to Myc factors (Crosino & Genre, 2022). There is currently no evidence on the existence and nature of ORM fungal signals able to trigger symbiotic responses in the host plant, but research has shown that Myc factors, particularly lipo-chitooligosaccharides (LCOs), are not

exclusive to AM fungi or even to symbiotic fungi (Rush et al., 2020). *In vitro* cultures of fungal species across different lineages revealed how the production of LCOs is more broadly distributed among the Kingdom Fungi than previously understood, and how they act as regulators of fungal developmental processes (Rush et al., 2020; Delaux and Gutjahr, 2024). Interestingly, in the liverwort *Marchantia paleacea*, signalling induced by Myc factors was not essential for the establishment of AM symbiosis, suggesting the existence of as yet uncharacterized AM-fungi signals (Teyssier et al., 2024). Additional fungal signals may be involved in triggering the symbiotic program for establishing and maintaining plant-fungal symbioses, and among the different existing types of molecules in nature, lipids are interesting candidates. In AM associations, lipids can act in fact as signaling molecules, carbon sources for fungi, and components of plant-derived membranes surrounding arbuscules (Kameoka and Gutjahr, 2022). Lipids are also involved in general in rhizosphere interactions, operating as chemical signals to regulate the plant's defense mechanisms in response to perception or contact with either beneficial or phytopathogenic microbes (Macabuhay et al., 2022). In particular, because of their structural similarities across bacterial, fungal, and plant taxa, fatty acids and oxylipins have the potential to be used in cross-kingdom

communication (Beccaccioli et al., 2022). In ectomycorrhizal colonization of oak roots, oxylipins are induced as part of the plant's defense response (Sebastiana et al., 2021). Fungal-derived oxylipins, such as 5,8-diHODE, act as signaling molecules that trigger developmental processes like lateral branching in *Aspergillus* species and appressorium formation in *Magnaporthe grisea* (Niu et al., 2020).

In Chapter 4, a strong modulation of lipid metabolism was described, with biosynthesis of unsaturated fatty acids significantly enriched in both stages T2 and T3. The lipid remodelling observed in PRESYMB stage (Chapter 5) involved an increased abundance of glycerophospholipids (GP) such as phosphatidylcholines (PC) and lysophosphatidylcholines (LPC) or the polyunsaturated fatty acid (PUFA) linolenic acid. In fungi, PUFAs such as linolenic acid released from membrane phospholipids are mobilized through the action of phospholipases and subsequently oxidized by enzymes including lipoxygenase (LOX) and dioxygenase (DOX) to synthesize oxylipins such as hydroxy-octadecadienoic acid (HODE) (Beccaccioli et al., 2022). It would be of great interest to investigate the presence of HODE and of the diverse class of fungal oxylipins in the model system *Tulasnella sp.-S.vomeracea* using, for example,

targeted metabolomics as well as transcriptomics and proteomics approaches to detect their biosynthetic enzymes.

The substantial increase, in *T. calospora* sp. SV6, of a compound putatively annotated as indole-3-carboxylic acid (ICA), both before and during the establishment of the ORM symbiosis with *S. vomeracea* seeds (Chapter 4), may suggest a possible involvement in the early stimulation of symbiosis, similarly to what has been observed in ECM (Wu et al., 2021). However, the chemical nature and the role of this metabolite in ORM should be further explored, bearing in mind that the genomes of many ECM plants, like the Pinaceae, lack the core genes of the CSSP (Radhakrishnan et al., 2020) and ECM establishment must rely on other mechanisms of fungal signals recognition and transduction.

A peculiar feature of ORM fungi is their rich complement of plant cell wall degrading enzymes (PCWDEs), as compared to AM and ECM fungi (Kohler et al. 2015). ORM fungi exhibit diverse ecological strategies, including saprotrophic behavior, with gene expression of plant cell wall degrading enzymes finely regulated during symbiosis (Adamo et al., 2020). Many of these PCWDEs have been found to be up-regulated during symbiosis (Kohler et al., 2015; Anasontzis et al. 2019; Villa-Rivera et al. 2021; Chen et al., 2022), and they have been suggested to facilitate the penetration of host cells by the

fungus and thus contribute to establishment of the symbiosis. It was therefore intriguing to find a significant up-regulation of many *Tulasnella* PCWDEs genes during the early stages of co-cultivation with the orchid host. This suggests that the production of enzymes that might facilitate fungal penetration and host cell colonization is already induced prior to physical contact with the plant, and may be part of a pre-symbiotic reprogramming of the fungus in preparation for symbiosis.

In conclusion, although further research is needed to elucidate the functioning and complex signaling mechanisms underlying the ORM relationship, the results described in this PhD thesis represent a step forward in the understanding of the nutrient exchange and molecular communication between orchids and their symbiotic fungi, thus contributing to decipher the complexity of this relationship.

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7. APPENDIX

Set up of an improved protocol to obtain *Tulasnella* sp. high molecular weight (HMW) DNA suitable for long-read high-throughput sequencing technologies

7.1 Introduction

As described in earlier chapters, orchid plants rely on their symbiotic fungi for the provision of essential nutrients, particularly during seed germination and early stages of plant development. Therefore, ORM fungi represent a determining factor for orchid growth, distribution and population dynamics (Li et al., 2021).

The kingdom Fungi is estimated to include between 1.5 to 5 million species, although the well-described species currently stands at around 120,000 (Hawksworth and Lücking, 2017). The significance of fungi as “ecological driving force” (Bahram and Netherway, 2022) is continuously increasing due to their crucial role in ecosystems, establishing connections across various biological and ecological levels. Their roles as decomposers, pathogens, or mutualistic symbionts have the potential to significantly impact the macroecology and evolution of organisms interacting with them (Bahram and Netherway, 2022) and investigations at all these levels requires new methods and resources. An example is functional genomics, that can quickly reveal potential functions of many genes discovered solely through genome sequencing (Grigoriev et al., 2014). Over the past decade, DNA sequencing has undergone a revolution (Koren and Phillippy, 2015), advancing the study of fungal genomes and contributing to the improvement of our understanding

of their evolution and diversity. From the first complete genome sequencing of the yeast *Saccharomyces cerevisiae* by Goffeau et al. (1996), in fact, more of 1000 fungal genomes were submitted to sequence databases mainly obtained using second generation short-read DNA sequencing (Petersen et al., 2022). Concerning ORM fungi, the first isolates from terrestrial orchids to be sequenced were the *T. calospora* AL13 isolate (Kohler et al., 2015), also used in this PhD project (De Rose et al., 2023), and a *Ceratobasidium* sp -anastomosis Group 1 (Miyauchi et al., 2020), but more isolate have been added thanks to sequencing projects supported by the U.S. Department of Energy (DOE) Joint Genome Institute (JGI) in California, USA. Among these projects, the 1000 Fungal Genomes Project has significantly contributed to our understanding of fungal diversity, evolution, and ecological roles by generating high-quality reference genomes for a wide range of fungal species (Grigoriev et al., 2014).

7.1.1 Genome sequencing technologies and challenges

Different technologies can be used for genome sequencing. Short-read high-throughput sequencing (HTS) platforms like Illumina, Ion Torrent, and DNBseq offer high-quality reads for DNA fragments smaller than 550 bases (Runnel et al., 2022), and have allowed significant insights into microbial ecology and function (Tedersoo et

al., 2021). Short reads enable various analyses like strain typing, outbreak tracing, and pan-genome surveys, improved by the incorporation of finished genomes. However, recent research has predominantly yielded unfinished genomes, relying instead on a limited number of finished references for annotation and mapping (Koren and Phillippy, 2015). Long-read sequencing technology offers the potential to change this by enabling cost-effective completion of microbial genomes in just a few days, eliminating manual intervention in the assembly process (Koren and Phillippy, 2015). Pacific Biosciences (PacBio) has been a leader in providing longer reads (Koren and Phillippy, 2015), and combined with Oxford Nanopore Technologies (ONT), these technologies offer efficient and cost-effective options for large-scale sequencing (Karin et al., 2023). In addition, they provide high-quality, state-of-the-art alternatives for investigating hypotheses about microbiome structure and function, as well as for assembling eukaryotic genomes from complex environmental DNA samples (Tedersoo et al., 2021).

DNA extraction and DNA fragmentation during library preparation are key factors which affect the read length distribution (Petersen et al., 2022); in particular, extracting high-quality, high-molecular-weight (HMW) DNA is a crucial step in genome sequencing

(Fauchery et al., 2022). In filamentous fungi, the composition, heterogeneity and structural complexity of the cell wall architecture represents a challenge for the isolation of HMW DNA, since several obstacles exist in devising universal extraction protocols suitable for diverse fungal species (Fauchery et al., 2022). Conventionally, to achieve high DNA yields, robust mechanical disruption methods (e.g. vortex use) are employed to break the strong fungal cell wall during cell lysis. However, such procedures impact the size distribution of the extracted DNA fragments, rendering them unsuitable for long-read sequencing applications. As a result, optimized protocols tailored to specific fungal groups or species are often required to obtain high-quality, high-molecular-weight (HMW) DNA suitable for long-read sequencing.

Here, a modified protocol has been designed to obtain fungal HMW DNA suitable for PacBio sequencing PacBio Multiplexed >10kb with Blue Pippin Size Selection. This genome sequencing technology is employed by the DOE JGI (USA) to carry out the 1000 Fungal Genomes Project, an initiative dedicated to capturing the genomic diversity within the fungal kingdom, with a specific focus on phylogenetic diversity (<https://1000.fungalgenomes.org/>). The setup of a modified protocol of HMW DNA extraction and purification from

the ORM fungal isolates was necessary because of previous failures in passing the quality control for PacBio sequencing. Although the DNeasy Plant Mini Kit (Qiagen) has been successfully adapted for fungal HMW DNA extraction in multiple studies (Lee et al., 2017; Serna-Domínguez et al., 2018; Bellemare et al., 2018), its performance for DNA extraction from *Tulasnella* strains kit was not equally efficient. In particular, my contribution involved the extraction of HMW DNA suitable for PacBio sequencing and high-quality RNA suitable for Illumina RNASeq with PolyA Selection from three distinct fungal symbionts associated with orchids from the *Tulasnella* genus: *Tulasnella* sp. SV6, used in this PhD project, *Tulasnella irregularis*, and *Tulasnella helicospora*. These fungi were isolated from Italian orchid roots collected in the field. Their RNA and DNA were then sent to the U.S. Department of Energy (DOE) Joint Genome Institute (JGI) in California, USA, for sequencing and subsequent integration into the 1000 Fungal Genomes Project.

7.2 Materials

7.2.1 Fungal material

The three fungal isolates, all belonging to the *Tulasnella* genus, were:

- *Tulasnella* sp. isolate SV6 (MUT4178), obtained from *Serapias vomeracea* roots collected in North Italy (Girlanda et al., 2011);
- *Tulasnella irregularis* isolate AM1.12e (MUT 6698), obtained from *Anacamptis morio* roots collected in South Italy (unpublished);
- *Tulasnella helicospora* isolate OP6.3d (MUT 6699), obtained from *Orchis patens* roots collected in North Italy (Calevo et al., 2020),

These isolates were grown on solid MEA 2% medium to obtain starting cultures (Fig. 1). In particular *T. calospora* sp.SV6 was grown for 10 days, *T. irregularis* and *T. helicospora* were grown for 15 days to obtain similar growth.

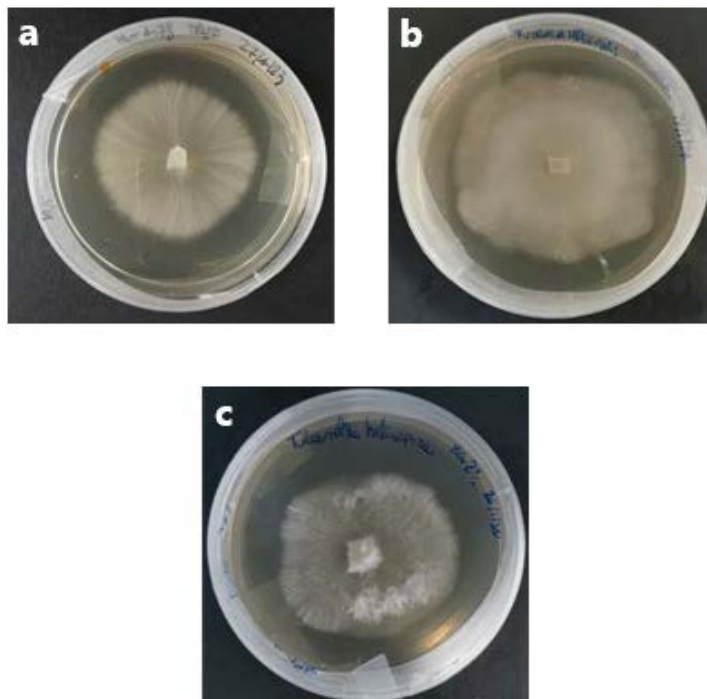


Figure 1. Fungal mycelia growing on solid MEA. a) *Tulasnella* sp.SV6, 10 dpi; b) *T. irregularis*, 15 dpi; c) *T. helicospora*, 15 dpi. dpi, days post inoculation

Three plugs (5 × 5mm) were then excised axenically from the fungal cultures on MEA and inoculated into flasks containing 100 ml of liquid 2% malt extract medium under a laminar flow hood. Liquid cultures were grown at room temperature (25°C) for 15 days in the dark, shaking at 150 rpm. For each fungal isolate, the mycelium was harvested under a laminar flow hood by filtration on sterile filter paper, dried and lyophilized overnight in a freeze-dryer. Before RNA and HMW DNA extraction, the mycelia were grounded to a fine powder with mortar and pestle in liquid nitrogen and stored at -80°C.

7.2.2 Media and reagents

The following culture media were used: 2%MEA solid medium (20g Malt extract and 15g Agar for microbiology, dissolved in 1L MilliQ water) and 2% ME liquid medium (20g Malt extract dissolved in 1L MilliQ water). The reagents and suppliers are indicated in Table 1.

The basic reagents used in the course of DNA extraction are listed in Table 1, whereas the commercial kits for DNA purification and RNA/DNA quantification are listed in Table 2.

Table 1. Reagents used for fungal growth or DNA extraction and RNA/DNA quality control, with indications of the supplier and catalogue number

Product	Supplier	Catalog number
Oxoid Malt extract	Thermo Fisher Scientific™	LP0039
Agar for microbiology	Duchefa Biochemie	9002-18-0
RNase A (100 µg/ml)	Qiagen	1069223
Isopropanol	Sigma-Aldrich	34863

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Ethanol absolute	Sigma-Aldrich	107017
β -mercaptoethanol	Sigma-Aldrich	M6250
Proteinase K	Roche	03 115836 001
GelRed	Sigma-Aldrich	SCT123
Ethidium Bromide	Sigma-Aldrich	E8751

Table 2. Commercial kits used for DNA extraction, purification and RNA/DNA quantification, with indications of the supplier and catalogue number

Product	Supplier	Catalog number
QIAGEN Genomic-Tips 20/G	Qiagen	10223
Genomic Buffer Set	Qiagen	19060
Qubit™ 1X dsDNA HS Assay Kit	Thermo Fisher Scientific™	Q33230
Qubit™ RNA BR Assay Kit	Thermo Fisher Scientific™	Q10210
Qubit™ RNA IQ Assay Kit	Thermo Fisher Scientific™	Q33221

7.3 Methods

7.3.1 Extraction and purification of HMW DNA

The extraction and purification of HMW DNA was performed according to the method described in Petersen et al. (2022) and made use of the Qiagen Kits (Table 2), according to the manufacturer's protocol with some modifications.

The following steps were performed to extract HMW DNA from the fungal samples:

1. 60 mg of lyophilized and ground mycelium were placed into 2 mL Eppendorf tubes and mixed to 1 mL lysis Buffer Y1 containing 1 μ L of β -mercaptoethanol, flicked for 2-3 minutes

- until the solution was homogeneous and incubated at 37°C for 1 h.
2. The Eppendorf tubes were centrifuged at 5,000 x g for 10 min at room temperature.
 3. The supernatant was removed and the pellet was carefully resuspended in 2 mL digestion Buffer G2 containing 4 µL RNase A (Qiagen) and 20 µL Proteinase K (Roche) by mixing the tubes up and down. The mixtures were incubated at 50°C for 2 h.
 4. The tubes were centrifuged at 5,000 x g for 10 min at room temperature and the supernatant was retained and processed for DNA purification.

The DNA purification steps were performed using the QIAGEN Genomic-Tips 20/G (Table 2). In detail:

1. The QIAGEN Genomic-Tips 20/G were equilibrated with 1 mL of equilibration Buffer QBT from the Genomic Buffer Set, using gravity flow.
2. The supernatant obtained after centrifugation of samples in Buffer G2 and containing the HMW DNA (see step 4 of DNA extraction) was loaded onto the equilibrated QIAGEN

Genomic-Tips 20/G. This step was repeated until all HMW DNA was loaded.

3. The HMW DNA was washed with 1 mL of washing Buffer QC from the Genomic Buffer Set and this step was repeated three times.
4. The HMW DNA was eluted in a 1.5 mL Eppendorf tube with 1 mL of elution Buffer QF (pre-heated to 50°C) from the Genomic Buffer Set.
5. The eluted HMW DNA was precipitated by mixing it with 0.7 volumes isopropanol and carefully mixed by inverting the tubes several times (Fig. 2).



Figure 2. Precipitation of HMW DNA from *Tulasnella* sp.SV6 after addition of isopropanol.

6. The mixture was incubated for 30 min at room temperature.
7. The precipitated HMW DNA was centrifuged for 15 min at 14,000 x g and the supernatant was removed without disrupting the pellet.
8. The pellet was washed with 1 mL ice-cold ethanol 70% without disrupting it and centrifuged for 5 min at 14,000 x g
9. The ethanol was removed and the step 12 was repeated.
10. The ethanol was carefully removed and the pellet was dried for 5 minutes under chemical hood at room temperature.
11. Finally, the pellet was dissolved in 55 μ L of DNase free water and the tubes were flipped gently to help resuspension.

7.3.2 Extraction and purification of high-quality RNA

The extraction and purification of RNA for annotation of the fungal genomes was conducted as described in **Chapter 2**, following the method of Chang et al. (1993).

7.4 Results

7.4.1 Quality control of HMW DNA

After the extraction procedure, some parameters were measured on NanoDrop One (ThermoFisher), namely DNA concentration, A260/A230 ratio, and A260/A280 ratio. Following these first

measurements, the concentration of DNA was measured using Qubit 4.0 (Invitrogen) with a Qubit™ dsDNA HS Assay Kit (Table 2). The results for the three fungal isolates are summarized in Table 3.

Table 3. Summary of HMW DNA measurements.

Species	DNA concentration (Nanodrop) (ng/μL)	DNA concentration (Qubit) (ng/μL)	A260/230 ratio	A260/280 ratio
<i>Tulasnella</i> sp. SV6	150.3	174.0	2.13	1.81
<i>T. irregularis</i>	92.5	21.7	2.20	1.70
<i>T. helicospora</i>	166.0	29.2	2.10	1.70

An aliquot of the DNA sample was tested on a denaturing 0.8% agarose gel stained with GelRed® (Table 2) in order to confirm the integrity of the DNA prior to shipment. The electrophoresis was performed in TAE 5x buffer for 40 minutes at 100 Volts (Fig. 3). A strong and sharp band of HMW DNA in the *Tulasnella* sp. SV6 sample was observed (Fig. 3). By contrast, the HMW DNA band was rather weak for *T. irregularis*, as also indicated by direct DNA measurements (Table 3). A great difference in the DNA concentration was measured by NanoDrop or Qubit for the *T. helicospora* sample (Table 3). When separated on the agarose gel, this sample showed a strong band of HMW DNA, but also some DNA degradation (Figure 3).

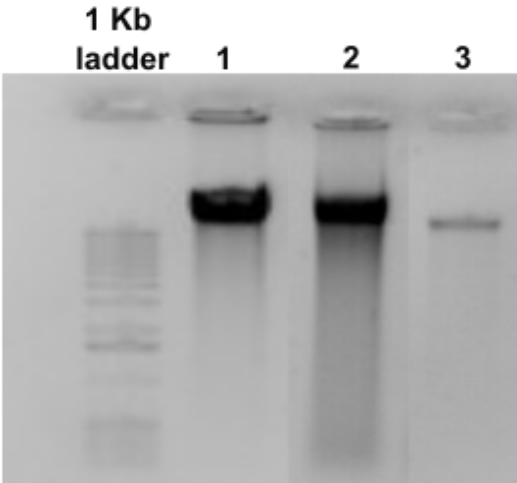


Figure 3. Agarose gel showing the separation of HMW DNA the after 40 minutes. The three samples are from 1) *Tulasnella* sp. SV6, 2) *T. helicospora*, and 3) *T. irregularis*. The DNA was stained with gelRed.

7.4.2 Quality control of total RNA

After the extraction procedure, some parameters were measured on NanoDrop One (ThermoFisher), namely RNA concentration, A260/A230 ratio and A260/A280 ratio. In addition, the RNA concentration was measured on Qubit 4.0 (Invitrogen-) using the Qubit™ RNA BR Assay Kit (Tabel 2). Integrity and quality of the extracted RNA were checked with the IQ RNA Kit (Table 2). The results are summarized in Table 4.

Table 4. Summary of RNA measurements.

Specie	RNA concentration (Nanodrop) (ng/μL)	RNA concentration (Qubit) (ng/μL)	A260/280 ratio	A260/230 ratio	RNA Integrity & Quality (IQ) Number
<i>Tulasnella</i> sp. SV6	241.0	226.0	1.99	1.83	10.0
<i>T. irregularis</i>	328.3	159.0	2.06	2.11	10.0
<i>T. helicospora</i>	119.3	72.0	2.03	1.95	10.0

To further check the RNA integrity before shipment, an aliquot of the RNA samples was run on a denaturing 1.2% agarose gel stained with ethidium bromide (EtBr). Gel separation was in TAE 5x buffer for 30 minutes at 80 Volts. Figure 4 shows the two clear bands corresponding to the 28S and the 18S rRNAs bands.

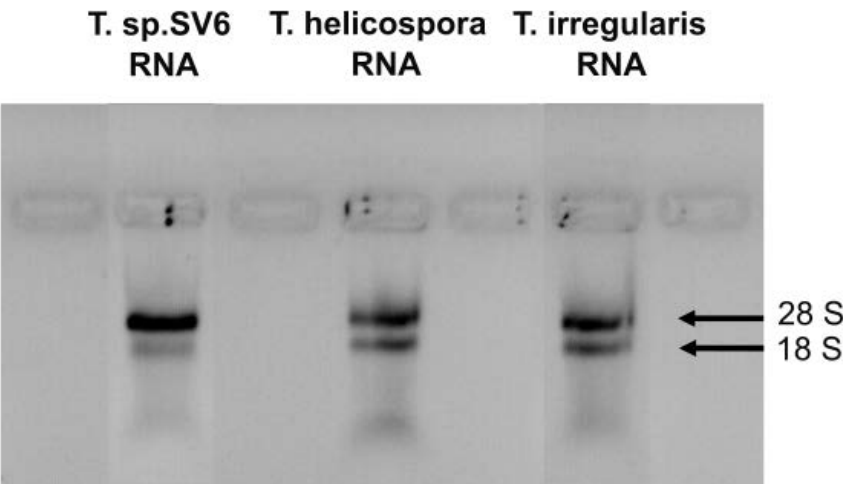


Figure 4. Agarose gel showing the separation of RNA after 30 minutes. From the left: *T. irregularis*, *T. helicospora* and *Tulasnella* sp.SV6. The 18S and 28S rRNA bands are visible in all samples. The RNA was stained with ethidium bromide.

7.4.3. Quality control by the Joint Genome Institute

The HMW DNA and RNA samples were sent to JGI and were subjected to their quality control procedure. The results are illustrated in Table 5 and confirm that the best sample (in terms of amount and quality of HMW DNA), was *Tulasnella* sp. SV6. Although all samples passed the JGI quality control, the amount of material for *T. helicospora* and *T. irregularis* was insufficient (and therefore unsuitable) for PacBio Multiplexed >10kb w/ Blue Pippin Size Selection, and was processed for PacBio Multiplexed 6-10 kb Ultra-Low Input.

In general, the amount of DNA and RNA measured at the JGI in all three samples was lower than the amount we measured by NanoDrop or Qubit. In addition, only 68% of HMW DNA from *T. helicospora* and *T. irregularis* sample was above 20kb, which made these sample not suitable for PacBio Multiplexed >10kb w/ Blue Pippin Size Selection.

Set up of an improved protocol to obtain *Tulasnella* sp. high molecular weight (HMW) DNA suitable for long-read high-throughput sequencing technologies

Table 5. Summary of measurements and comments by JGI following quality control of the HMW DNA and RNA samples

Sequencing Project Name	Comments	Initial Measured Quantity	Collaborator Reported Quantity	Library Type
<i>Tulasnella</i> sp. SV6 HMW DNA		2559.16 ng	3210.00 ng	PacBio Multiplexed >10kb w/ Blue Pippin Size Selection
<i>Tulasnella</i> sp. SV6 RNA	FA run shows elevated baseline indicating sample degradation.	2667.50 ng	4520.00 ng	Illumina RNASeq w/PolyA Selection
<i>Tulasnella helicospora</i> OP6.3d HMW DNA	FA run shows sample degradation, Not suitable for PacBio Multiplexed >10kb w/ Blue Pippin Size Selection due to insufficient material, Could be suitable for PacBio Multiplexed 6-10 kb Ultra-Low Input.	590.24 ng	876.00 ng	PacBio Multiplexed 6-10kb Ultra-Low Input
<i>Tulasnella helicospora</i> OP6.3d RNA		1123.25 ng	2078.00 ng	Illumina RNASeq w/PolyA Selection
<i>Tulasnella irregularis</i> AM1.12e HMW DNA	68% of sample is above 20kb, Not suitable for PacBio Multiplexed >10kb w/ Blue Pippin Size Selection due to insufficient material, Could be suitable for PacBio Multiplexed 6-10 kb Ultra-Low Input.	424.94 ng	813.00 ng	PacBio Multiplexed 6-10kb Ultra-Low Input
<i>Tulasnella irregularis</i> AM1.12e RNA	FA run shows elevated baseline indicating sample degradation.	548.15 ng	944.00 ng	Illumina RNASeq w/PolyA Selection

7.5 Concluding remarks

The protocol described in this appendix was modified with respect to previous protocols that failed the quality control at JGI. The different DNA extraction technology used in the Genomic DNA Buffer Set (Qiagen), based on anion-exchange, have revealed more sensitivity respect to the previously silica-membrane technology (used in DNeasy Plant Mini Kit, Qiagen).

In particular, it is interesting to note that, by eliminating the use of a vortex, the DNA maintained a better integrity. The challenge now will be to further improve this HMW DNA extraction protocol, in order to make it more efficient for all *Tulasnella* strains.

In the meanwhile, all three fungal isolates have been successfully sequenced at the JGI, and although the data are not yet publicly available at this date because of the need of some final checks and information, they can be already accessed in a reserved area of the Mycocosm website. These data will be instrumental for further investigations on the diversity and functional roles of ORM fungi and, together with the sequencing of the plant genome, they may boost research on the molecular aspects of this fascinating symbiosis.

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