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Commentary

Nitrogen transport in the orchid mycorrhizal symbiosis – further evidence for a mutualistic association

Introduction

Mycorrhizas are symbioses integral to the health of plant-based ecosystems (Smith & Read, 2008). In a typical mycorrhizal association, fungi in or on plant roots pass soil-acquired inorganic nutrients and water to the plant host. In return, the host transfers excess photosynthate to the fungus. Orchid mycorrhizas were considered to be unusual symbioses in that during initial colonisation of the young, non-photosynthetic host, the fungus was thought to provide both inorganic and organic nutrition to the plant and to receive nothing in return for its services. That is until a significant new study by Fochi *et al.* (New Phytologist, this issue) investigating expression of fungal and plant nitrogen (N) transport and assimilation genes in mycorrhizas formed between the fungus *Tulasnella calospora* and the photosynthetic orchid, *Serapias vomeracea*. The research suggests, for the first time, flow of nutrients back to the fungal partner from the non-photosynthetic orchid host. Thus orchid mycorrhizas now appear to represent a true mutualism in both the early and mature stages of plant development (Cameron *et al.*, 2006) and they thus join the physiological ranks of the more extensively studied arbuscular mycorrhizal and ectomycorrhizal associations.

The Orchidaceae constitutes the largest of all plant families with estimates of more than 27,000 species (The Plant List, 2013). Orchids are typically pollinated by insects, with most having quite specific associations which can impact on rarity (Phillips *et al.*, 2011). The dust-like seed released from orchid fruits is wind dispersed to locations away from the parent plant. At this stage, a compatible mycorrhizal fungus, usually basidiomycete members of the Cantharellales and Sebacinales (Dearnaley *et al.*, 2012) grow into the tissues of the ungerminated seed or a pre-seedling stage known as a protocorm. Fungal coils or pelotons are produced inside the cells of the orchid by the mycorrhizal fungus (Fig. 1). Nutrients such as carbon (C), phosphorus (P) and N are transferred dynamically from the mycorrhizal fungus across the interfacial matrix, a new apoplastic space produced between the fungal peloton and the orchid cell membrane (Kuga *et al.*, 2014; Fig. 1). Fungal nutrients such as P, N and C also pass to the orchid from rupturing hyphae as the pelotons are digested by the host after a few days (Fig. 1). As the plant becomes photosynthetic, plant to fungus C flow becomes established (Cameron *et al.*, 2006; Fig. 1) finalising the life-stage dependent trophic switch from heterotrophic juvenile to autotrophic adult.

Nitrogen can be a limiting element in natural and agricultural ecosystems (Gress *et al.*, 2007). Organisms require N for protein and nucleic acid synthesis but also to manufacture a wide array of coenzymes. Plants may obtain N via direct uptake of nitrate from soils. Leguminous plants use bacteria in nodules to acquire amides and ureides. Many plants also use mycorrhizal fungi to take up N (Thirkell et al., 2016). In arbuscular mycorrhizas, fungi transfer ammonium to the host plant in exchange for photosynthate (Koegel *et al.*, 2015). In ectomycorrhizas, both organic and inorganic N forms appear to be transported to the plant host (reviewed in Muller *et al.*, 2007). Fungi are well known to transfer N to plants in orchid mycorrhizas and this possibly includes both organic and inorganic forms (Cameron *et al.*, 2006; Kuga *et al.*, 2014). Mycorrhizal fungi will target environmental N sources as demonstrated by in vitro growth experiments (Leigh *et al.*, 2008).

The common orchid mycorrhizal fungus, *Tulasnella calospora,* cannot use nitrate

Tulasnella calospora is a cosmopolitan orchid mycorrhizal fungus, colonising many photosynthetic species in Europe, North America, South America, Australia and Asia (reviewed in Dearnaley, 2007). Fochi *et al.* cultured *T. calospora* on a variety of media containing different sources of N. As evidenced by comparisons of mycelial dry weight, the fungus grew best on organic N sources such as glutamine and glutamic acid and ammonium-based media but grew poorly on media containing sodium nitrate. Searches of the *T. calospora* genomic database identified two functional ammonium transporters but no nitrate uptake and assimilation genes. Heterologous expression of these ammonium transporter cDNAs in yeast showed that they encoded functional genes. Using RNA-Seq analysis, one of the ammonium transporters, TcAMT2 was significantly upregulated in free living mycelium growing on ammonium. The discovery that *T. calospora* is unable to utilise environmental nitrate is interesting from a number of perspectives. Threatened orchids which exclusively associate with *T. calospora* eg. *Diurus*, *Drakaea* and *Thelymitra* spp. (Warcup, 1981; Nurfadilah *et al.*, 2013) will need appropriate N sources in *ex situ* growth media and in soils that are used for restoration work. The basis for some orchids to become weeds can perhaps be explained by a capacity to form mycorrhizal associations with fungi in addition to *Tulasnella calospora* (Bonnardeaux *et al.*, 2007), which in turn may belie a capacity to access multiple soil N sources.

Molecular evidence that *T. calospora* transports amino acids to orchids

Cameron *et al.*, (2006) was first to suggest via ^{13}C-^{15}N isotope tracing experiments that the mycorrhizal fungus, *Ceratobasidium cornigerum* transfers organic N to orchid hosts. Contrastingly, a recent study by Kuga *et al.* (2014), utilising cellular level imaging of stable isotope tracers suggested that $^{15}NH_4{}^{15}NO_3$ passed from mycorrhizal fungus to orchid across intact pelotons. Fochi *et al.* investigated fungal and plant amino acid transport genes in free-living mycelium of *T. calospora* and asymbiotic and symbiotic protocorms of *S. vomeracea*. These investigations showed that a number of fungal amino acid transporters/permeases (TcAAT1, TcAAT2, TcAAT6) were significantly upregulated in the symbiotic situation. RT-PCR analyses of laser microdissected protocorm cells showed the presence of TcAAT1 transcripts in peloton-containing cells. Upregulation of plant amino acid transporters such as the permeases, SvAAP1 and SvAAP2 and a lysine-histidine transporter was shown in symbiotic protocorms. Fungal to orchid transfer of amino acids was also strongly suggested by assessment of fungal N assimilation gene expression. In symbiotic protocorms, fungal arginase and urease, enzymes involved in amino acid breakdown, were only weakly expressed. Additionally, a fungal argininosuccinate lyase, an enzyme involved in arginine biosynthesis, was upregulated in symbiosis. Thus orchids largely appear to acquire N in an organic form from their fungal partner. Further assessment of fungal to orchid inorganic N transfer is however, needed in light of the findings of Kuga *et al.* (2014).

Why do mycorrhizal fungi colonise the seeds and protocorms of orchids?

The research presented by Fochi *et al.* also provides a new perspective on orchid to fungal nutrient transport. In 2006, Cameron *et al.* significantly changed the landscape of orchid mycorrhizal physiology when it was shown, using $^{14}CO_2$ tracer experiments, that the photosynthetic orchid *Goodyera repens* transfers approximately 3% of fixed C to its fungal partner. For decades it was thought that early, non-photosynthetic orchid stages provided little reward for their fungal partners. Indeed, Leake *et al*. (2008) suggested that the impetus for fungal colonisation of non-photosynthetic plant tissues involved the concept of "give now but get more later", in that the C invested in orchid seedlings would be returned with interest from photosynthetic adults. In their study, Fochi *et al*. demonstrated upregulation of the plant ammonium transporter SvAMT1 in symbiotic protocorms of *Serapias vomeracea*. TcAMT2, a fungal ammonium transporter was also significantly upregulated in symbiosis and transcripts of both TcAMT2 and TcAMT1 could be found in laser microdissected peloton-containing cells. That the orchid passes ammonium back to its fungal partner in the *T.*

calospora-S. vomeracea system was also suggested by symbiotic upregulation of the fungal TcGS1 glutamine synthetase - an enzyme which scavenges ammonium to use in the synthesis of glutamine. These findings also cast light on how a compatible mycorrhizal fungus might detect an orchid seed or protocorm in soil. Orchid seeds have few reserves apart from lipid stores and protein bodies (Rasmussen, 1995). Following seed imbibition, these latter structures could potentially be catalysed to release ammonium into the environment and entice fungal colonisation.

A new model for nutrient transport in orchid mycorrhizas

In view of the study by Fochi *et al.* and other key work by researchers in the field eg. Cameron *et al.*, 2006; 2007; Bougoure *et al.*, 2013; Kuga *et al.*, 2014), a new model for nutrient transport in orchid mycorrhizas can be proposed (Fig. 1). In non-photosynthetic orchid stages such as seeds and protocorms, the plant exports NH_4^+ (Fochi *et al.*) and receives P, N and C (the latter two nutrients as fungal amino acids) from the fungal partner across intact membranes (Cameron *et al.*, 2006; 2007; Kuga *et al.*, 2014; Fochi *et al*.). Lysis of fungal pelotons also releases P, N and C to the plant (Bougoure *et al.*, 2013). In photosynthetic orchids, the plant exports sugars to the fungus (Cameron *et al.*, 2006) and receives only P from the fungal partner across intact membranes as fungus to plant, amino acid transport at this location discontinues in photosynthetic orchids (Cameron unpublished). Fungal P, N and C from lysed hyphae continues to be taken up by the mature orchid host.

Implications of bidirectional nutrient transfer in young and adult orchids

The research of Fochi *et al*. suggests that orchid mycorrhizas represent a true mutualism in that both symbionts benefit at all stages of the association. Such a finding reinforces an old ecological adage, in that in nature there is "no such thing as a free lunch" and thus orchid seeds and protocorms must pay for the nutrients that the fungal partner provides. As the orchid mycorrhizal system is easy to study *in vitro* (ie. both symbionts are axenically culturable), this suggests that it could provide a useful model system to investigate biological aspects of the more difficult to manipulate, arbuscular and ectomycorrhizal associations. Continued investigations of orchid mycorrhizas may assist with unravelling the intricacies of other mycorrhizal systems and ultimately improve the health and sustainability of natural, horticultural and agricultural systems.

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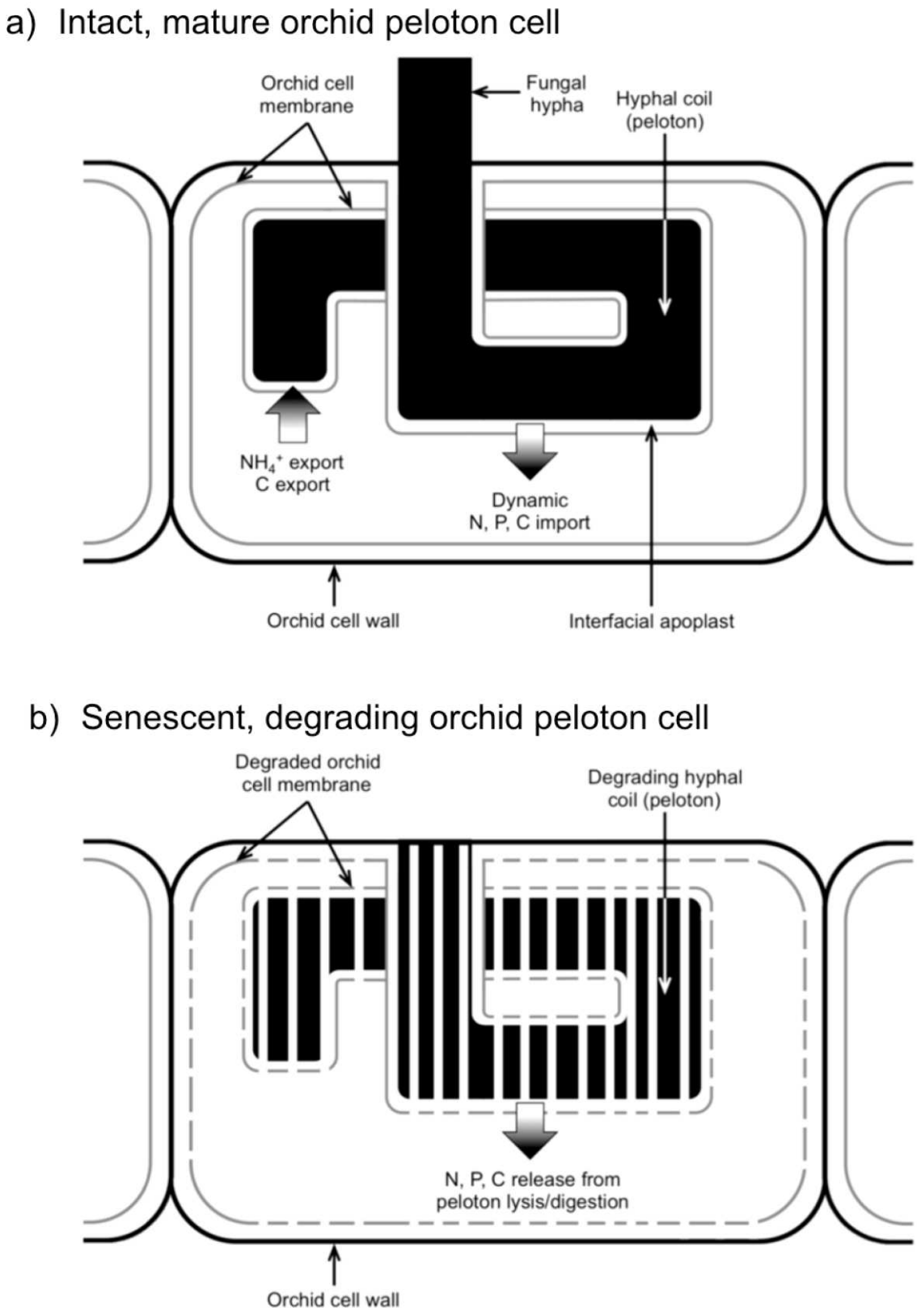

Fig. 1 Model of nutrient transport in orchid mycorrhizas. a) In orchid cells containing intact, mature pelotons, the plant exports NH_4^+ to the fungus in non-photosynthetic stages and C in photosynthetic stages. The plant imports N, P & C from the fungus across intact membranes. b) In orchid cells containing senescent pelotons, the plant receives N, P and C as the hyphal coils are digested.