The ectomycorrhizal contribution to tree nutrition

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Abstract

Trees can be associated with dozens of fungi helping them to acquire resources from forest soils. The most widespread mutualistic association in boreal and temperate forests is the ectomycorrhizal symbiosis. This symbiosis involves mushroom-forming fungi of basidiomycota, ascomycota, and some zygomycota clades and the roots of woody plant species, including oaks, poplars or pines. Although the impact of this association on ecosystem production and tree nutrition is investigated for about a century, our understanding on the molecular mechanisms that control water and nutrient fluxes between plant and fungal partners is still limited. Here, we review the recent knowledge on the ectomycorrhizal contribution to tree nutrition. We specifically highlight the molecular mechanisms driving the acquisition, translocation and release of water and nutrients in ectomycorrhizal systems. We particularly focus on the transport of macronutrients, including nitrogen, phosphorus, potassium, sulphur and calcium, micronutrients, and water by the symbiotic partner. We also provide background on the evolution, diversity, and importance of this symbiosis, identify knowledge gaps, and propose future research directions.

1. Introduction

The hydro-mineral nutrition of trees from boreal and temperate forests greatly depends on the mycelium of mycorrhizal fungi growing in the soil. These fungi can explore with their extra-radical mycelium a larger volume of soil than roots, take up water and nutrients from the soil, and translocate them to the intraradical mycelium inside of tree roots (Smith & Read, 2008). Among the different types of mycorrhizal associations, the ectomycorrhizal (ECM) symbiosis is the most important for forests of the northern hemisphere. ECM fungi are evolutionary closely related to saprotrophic fungi and mainly belong to the basidiomycota clade, but also include some ascomycota and zygomycota. Only 3–5% of land plants form this interaction, but ECM fungi play a critical role for forest ecosystems because they colonise up to 95% of tree short roots (Martin et al., 2001) and actively participate in nutrient cycling and carbon sequestration (Clemmensen et al., 2013; Marmeisse & Girlanda, 2016). Consequently, ECM associations greatly influence many forest ecosystems across temperate, boreal and some subtropical regions all around the globe (Finlay, 2008; Smith & Read, 2008). The majority of woody plants with high economic value are highly dependent on the ECM symbiosis, making a better understanding of these interactions critical for the establishment of a successful commercial agroforestry.

Plant and fungal tissues form ectomycorrhizas and mutually benefit from this interaction through the exchange of "goods". The fungus provides the plant with water, macronutrients, and micronutrients, whereas the host

transfers photosynthetically fixed carbon to its mycobionts. Although this mutualistic exchange of resources has been extensively described over the past decades, key information is still largely unknown. For example, we have only little information about (i) the proteins governing the acquisition of resources by the mycelium, and their release from the fungal cells to the plant roots, (ii) the functional characterisation and regulation of these transport proteins, and (iii) the molecular mechanisms driving the translocation of water and nutrients from extra-radical hyphae exploring the soil to intraradical mycelium in tree roots. Even though an increasing amount of genomic (Branco et al., 2015; Kohler et al., 2015; Martin et al., 2008, 2010; Martino et al., 2018; Mujic et al., 2017; Peter et al., 2016), transcriptomic (Doré et al., 2017; Hacquard et al., 2013), proteomic (Doré et al., 2017, 2015; Liang, Chen, Tang, & Shen, 2007) and metabolomic (Larsen et al., 2011) data is available, the validation of candidate players participating in resource exchange is hindered by the difficulty to genetically transform fungal and tree partners and the limited molecular tools that are currently available.

Here, we summarise the most recent knowledge on the diversity of this association and the transport of macronutrients (nitrogen, phosphorus, potassium, sulphur and calcium), micronutrients (primarily zinc), and water from the soil to the colonised tree roots. Aiming at highlighting the impact of ECM fungi on tree nutrition, we decided not to cover the transport of carbon from the plant to the fungal partners, since it was recently reviewed in a number of publications (Casieri et al., 2013; Courty, Doidy, Garcia, Wipf, & Zimmermann, 2016; Garcia, Doidy, Zimmermann, Wipf, & Courty, 2016). We particularly emphasise the molecular players of fungal and plant partners and the mechanisms that drive the hydro-mineral transport from soil to roots and propose research directions and future challenges in the study of ECM tree nutrition.

2. Evolution and diversity of ectomycorrhizas

2.1 Saprophytic to ectomycorrhizal fungal continuum

Fossil and molecular clock analyses suggest that ECM associations evolved about 100–200 million years ago (Heijden, Martin, Selosse, & Sanders, 2015; Kohler et al., 2015; Strullu-Derrien, Selosse, Kenrick, & Martin, 2018; Tedersoo, May, & Smith, 2010). Evidence from molecular, palaeographical, and palaeontological studies suggest that the evolution of ECM fungi approximately 180 million years ago coincided with (i) the origin of the Pinaceae lineage, which may represent the first group of ECM hosts, and (ii) the rapid diversification of angiosperms in the Jurassic and early Cretaceous period (Brundrett, 2002; Moyersoen, 2006; Strullu-

Derrien et al., 2018). Conversely, it is likely that the ability to interact with ECM fungi was a key step for the emergence of new host tree species and for the structure of ancient ecosystems (Quirk, Andrews, Leake, Banwart, & Beerling, 2014). Phylogenetic and phylogenomic studies revealed that ECM fungi have evolved independently 78–82 times from free-living saprotrophs in multiple fungal lineages (Eastwood et al., 2011; Floudas et al., 2012; Hibbett, Gilbert, & Donoghue, 2000; James et al., 2006; Tedersoo & Smith, 2017).

The shift from a saprophytic to a more mutualistic lifestyle was the result of a convergent loss of genes involved in the degradation of lignocellulose, including plant cell wall-degrading enzymes (PCWDEs), class II peroxidases or lignin oxidoreductases (Garcia & Ané, 2016; Kohler et al., 2015; Pellegrin, Morin, Martin, & Veneault-Fourrey, 2015; Tedersoo et al., 2010; Wolfe, Tulloss, & Pringle, 2012). Kohler et al. (2015) demonstrated that saprotrophic fungi, white-rot and litter decomposers, display on average 133 genes for PCWDEs, whereas brown-rot species have 81 genes and ECM species only 62 genes. Consequently, ECM fungi are generally less capable of utilising complex carbohydrates from forest soils, increasing their dependence for host plant photo-assimilates as a source of carbon (Section 3.1).

In addition, transcriptional profiling also revealed that the transition towards an ECM lifestyle resulted from the emergence of genes specifically expressed and/or regulated during the interaction with a host species. For example, Doré et al. (2017) reported that 3.6% of the genes from the ECM fungus *Hebeloma cylindrosporum* were significantly up-regulated during the symbiotic association with *Pinus pinaster*. Among them, many genes encode fungal effectors putatively involved in the molecular cross-talk between both symbionts and manipulation of plant defence responses (reviewed in Garcia, Delaux, Cope, & Ané, 2015; Martin, Uroz, & Barker, 2017; Mello & Balestrini, 2018), PCWDEs, and proteins responsible for the transport of nutrients (reviewed in Casieri et al., 2013; Garcia et al., 2016). Interestingly, orthologues of a large set of these up-regulated genes are also found in saprotrophic species, indicating their origin in saprotrophic fungi and their conservation by ECM fungi (Kohler et al., 2015). However, their precise role in the ECM symbiosis remains to be determined.

2.2 Diversity of the ectomycorrhizal symbiosis

Based on morphological, molecular and isotopic studies estimates, around 20,000 fungal species can form ectomycorrhizas (Tedersoo et al., 2012).

Although many of them are partly saprotrophic, they often rely on photosynthetically fixed carbon from their associated host trees for their sexual reproduction through the production of carpophores (Fig. 1). It has been estimated that around 6000 woody plant species from 30 families can interact with ECM fungi, including many economically important crop trees such as pines, beeches, oaks, eucalypts, dipterocarps or poplars (Heijden et al., 2015). Strict host specificity is rare in ECM fungi (Churchland & Grayston, 2014), and many ECM fungi can simultaneously colonise dozens of plants (Horton & van der Heijden, 2008). Interestingly, recent evidence also illustrated that ECM host specificity patterns depend on the mode of colonisation (Lofgren, Nguyen, & Kennedy, 2018), and that tree genetics influences the composition of ECM communities (Gehring, Sthultz, Flores-Rentería, Whipple, & Whitham, 2017). A low specificity can provide plants with a competitive advantage in the uptake of nutrients in comparison to non-host species, or plant species with a high specificity. This means that trees in forests are interconnected through the mycelium of many ECM fungi forming a living belowground network called the "Wood-Wide Web" (Newman, 1988; Simard et al., 1997).

3. Structure of ectomycorrhizas

3.1 Root colonisation by ectomycorrhizal fungi

Although ECM fungi penetrate into the roots of their host, their hyphae never enter the host cells, and the mycelium develops intercellularly in the root cortex (Fig. 1). Ectomycorrhizal roots can be distinguished into three structural components: extra-radical hyphae exploring the soil, hyphal mantle or sheath that encloses the root, and intra-radical hyphae, or the Hartig net, that forms a specialised interface with the host root (Fig. 2; Bonfante & Genre, 2010; Peterson & Massicotte, 2004). Interestingly, Hacquard et al. (2013) reported distinct transcriptional profiles between these three types of hyphae, indicating that all have different functions during the symbiosis.

Development of a functioning ectomycorrhiza follows a series of distinct steps that have extensively been reviewed (Balestrini & Kottke, 2016; Smith & Read, 2008). In brief, the symbiosis starts with an exchange of diffusible signals between host roots and the fungus, followed by the attachment of the hyphae to root surface, and the development of the fungal mantle. Finally, hyphae invade the root cortex and establish the Hartig net where nutrients are exchanged between both partners (Fig. 2). Fungal development within the host root is accompanied by changes in root architecture and

Fig. 1 Fruiting bodies of three model ECM fungi and ectomycorrhizas formed with their host tree. (A) *Laccaria bicolor* fruiting bodies under *Pseudotsuga menziesii* at Toll Bridge Park, Mount Hood, OR, USA, and (B) ectomycorrhizas after inoculation of *Populus trichocarpa* (Oak Ridge National Laboratory, Oak Ridge, TN, USA). (C) *Hebeloma cylindrosporum* fruiting bodies under *Pinus* sp. in the Landes forest, France, and (D) ectomycorrhiza in association with *Pinus pinaster* (Biochemistry and Plant Molecular Physiology Laboratory in Montpellier, France). (E) Fruiting body of the invasive European *Amanita muscaria* under *Quercus humboldtii* in Colombia, and (F) ectomycorrhizas on the same host species. *Images courtesy of Jay Chen and Jesse Labbé (A, B—Oak Ridge National Laboratory, USA), Claude Plassard and Jacques Guinberteau (C—French National Institute for Agricultural Research, INRA, France), Natalia Vargas Estupiñan and Anne Pringle (E, F—Universidad de Los Andes, Colombia, and University of Wisconsin-Madison, USA, respectively).*

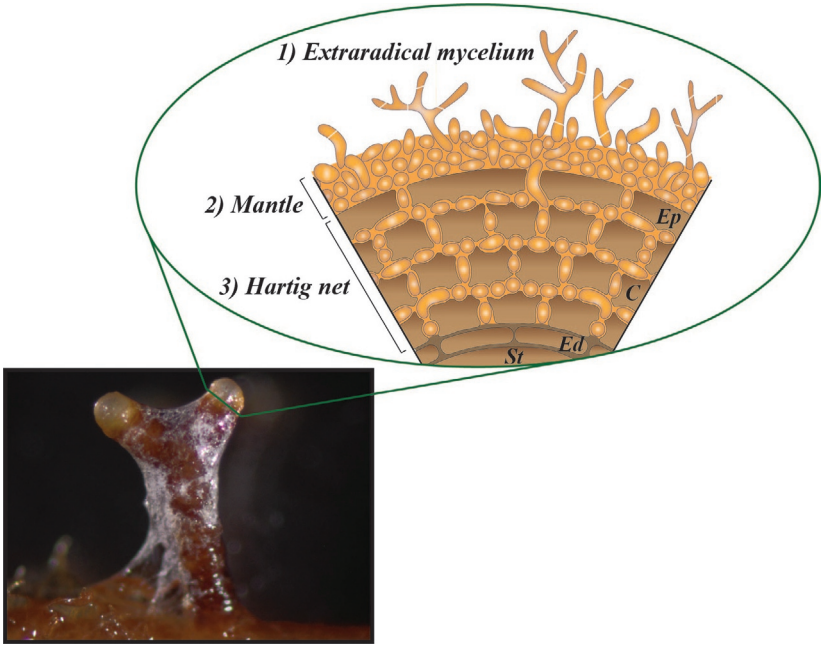

Fig. 2 Structure of a plant root colonised by an ectomycorrhizal fungus. Ectomycorrhizas can be divided into three structural components: (1) extra-radical mycelium with hyphae that explore the soil for nutrients and water, (2) hyphal mantle or sheath that encloses the root, and can represent a significant apoplastic barrier, and (3) the Hartig net in the root cortex, the site of nutrient and water exchange between both symbiotic partners. Abbreviations: *C*, cortex; *Ed*, endodermis; *Ep*, epidermis; *St*, stele.

morphology of the host by a reorganisation of the plant cytoskeleton (Timonen, Finlay, Olsson, & Söderström, 1996), and an increased development of lateral roots, and an inhibition of root hair formation (Ditengou, Béguiristain, & Lapeyrie, 2000).

3.2 The soil-fungus interface

The extra-radical mycelium of ECM fungi can represent up to 32% of total microbial biomass and 700–900 kg ha^{-1} in forest soils (Wallander, Nilsson, Hagerberg, & Bååth, 2001). It can be composed of individual hyphae with similar structure, or differentiated into rhizomorphs, complex aggregates of parallel hyphae (Agerer, 2001) that are involved in the long-distance transport of water and nutrients from hyphal tips to host roots. The structure of rhizomorphs can range from loose arrangements of relatively

undifferentiated hyphae to aggregates of highly differentiated hyphae (Smith & Read, 2008).

Another important feature of ectomycorrhizas is the fungal mantle encompassing the roots. Its structural composition ranges from relatively thin, loosely arranged assemblages of hyphae, to very thick, multi-layered and pseudoparenchymatous mantles. The surface of the mantle can be compact and smooth, or rough with numerous emerging hyphae, hyphal strands, or rhizomorphs (Agerer, 2001). The mantle isolates colonised roots from the soil, protects them against desiccation and pathogens, and can play a role in nutrient acquisition (Hacquard et al., 2013). Hyphal mantles also represent a significant apoplastic barrier for the entry of nutrients into the root cortex and can put nutrient uptake under the control of the fungal symplast (Behrmann & Heyser, 1992; Bücking, Kuhn, Schröder, & Heyser, 2002). Although poorly understood, the fungal mantle plays a key role in storage and partitioning of carbohydrates from the host plant, and nutrients acquired from the soil by extra-radical hyphae (Bücking, Liepold, & Ambilwade, 2012; Vesk, Ashford, Markovina, & Allaway, 2000).

3.3 The symbiotic plant-fungus interface

The intra-radical network of hyphae, or Hartig net, surrounds epidermal root cells in angiosperms, or both epidermal and cortical roots cells in gymnosperms up to the endodermis (Peterson & Massicotte, 2004; Smith & Read, 2008). Hyphae from the Hartig net display a complex and dense labyrinthine architecture, increasing the contact area between both partners and form an efficient interface for the bidirectional transport of nutrients (Finlay, 2008; Peterson & Massicotte, 2004). In the Hartig net, plant and fungal cells share a common apoplast in which solutes can freely move from one partner to another. The plasma membranes of both partners in the Hartig net show high activities of ATPases (Lei & Dexheimer, 1988) and many specific or up-regulated transport systems (Garcia et al., 2016), facilitating active or passive transport of nutrients and water across this mycorrhizal interface.

4. Importance of ectomycorrhizal fungi for forest ecosystems

ECM networks play a key role in the biochemical cycles of forest ecosystems and actively participate in carbon sequestration and mineral cycling (Clemmensen et al., 2013). For example, ECM fungi can contribute with up

to 80% to the total plant nitrogen (N) and phosphorus (P) uptake, increasing plant productivity (Casarin, Plassard, Hinsinger, & Arvieu, 2004; Chalot & Brun, 1998; Chalot et al., 2002; Perez-Moreno & Read, 2000; Torres Aquino & Plassard, 2004; Van Tichelen & Colpaert, 2000). ECM fungi can also reduce nutrient losses by leaching and denitrification (Midgley & Phillips, 2014). Besides their role in nutrient cycling, the occurrence of ECM associations can drastically improve seedling establishment (Nara, 2006), litter decomposition (Lindahl et al., 2006), soil formation, soil aggregation (Rillig & Mummey, 2006) and provides tolerance to numerous biotic and abiotic stresses (reviewed in Finlay, 2008). However, these effects are often variable depending on the fungal and plant species and environmental conditions (Heijden et al., 2015).

Plant availability of nutrients in boreal and temperate forest ecosystems is usually low and many nutrients are in organic form and integrated in humus rich soil organic matter (SOM) and litter in different stages of decomposition (Read & Perez-Moreno, 2003). SOM is composed of relatively small biomolecules originating from the decomposition of plant and microbial biomass such as lignocellulosic components (Berg & McClaugherty, 2014; Simpson et al., 2007). ECM fungi were long seen primarily as mutualists that absorb nutrients released during litter decomposition by saprotrophic fungi (Read & Perez-Moreno, 2003). However, recent analyses of fungal genomes revealed that ECM fungi still have the ability, even if limited, to decompose complex SOM (Kohler et al., 2015). For example, *H. cylindrosporum* and *Cortinarius glaucopus* still harbour genes coding for class II peroxidases involved in lignin modification and degradation (Kohler et al., 2015; Martin et al., 2017). Field and microcosm studies have shown that ECM systems can access nutrients sequestered in cellulose and polyphenols (Bending & Read, 1995; Read & Perez-Moreno, 2003). ECM fungi are also able to intensively exploit litter to reduce and uptake N and P (Perez-Moreno & Read, 2000), indicating that they are still able to degrade SOM.

Studies on saprotrophic fungi revealed that white-rot and brown-rot fungi use distinct mechanisms to decompose lignocellulose (Hatakka & Hammel, 2010). White-rot fungi rely on enzymatic systems for the degradation of lignocellulose, whereas brown-rot fungi employ a non-enzymatic mechanism due to their lack of oxidative enzymes and PCWDEs (Eastwood et al., 2011; Floudas et al., 2012). This non-enzymatic mechanism, called the Fenton reaction ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + OH$), allows the release of sequestered carbon without the need for complete lignin degradation (Arantes, Jellison, & Goodell, 2012). Interestingly, the ECM fungus

Paxillus involutus can also assimilate organic N from the decomposition of lignocellulosic material in SOM through a Fenton-based oxidation mechanism (Op De Beeck, Troein, Peterson, Persson, & Tunlid, 2018; Rineau et al., 2012). However, ECM fungi are not able to acquire significant amounts of carbon from SOM, and primarily rely on the carbon provided by their host (Lindahl & Tunlid, 2015). Rineau et al. (2013) demonstrated that adding glucose to organic material stimulated the expression of transcripts for enzymes involved in the Fenton reaction. This indicates that ECM fungi use carbon resources derived from the host to mobilise N and P trapped in recalcitrant organic matter complexes (Tunlid, Floudas, Koide, & Rineau, 2016). This is further supported by both the overall reduction of genes coding for PCWDEs in ECM species (Kohler et al., 2015) and field studies showing that less than 2% of carbon in the fungal biomass originated from litter decomposition (Treseder, Torn, & Masiello, 2006). However, some studies demonstrated that ECM fungi express genes encoding for carbohydrate-active enzymes (CAZymes) upon growth on SOM (Shah et al., 2015). In conclusion, the importance of SOM decomposition for the acquisition of carbon is still under debate for ECM fungi, and further observations are necessary to determine the contribution of carbon derived from SOM decomposition to the nutrition of ECM fungi.

5. The hydro-mineral nutrition of ectomycorrhizal trees

5.1 The symbiotic acquisition of nitrogen

N is one of the most important growth-limiting factors in boreal and temperate forests, and ECM fungi play a critical role in its acquisition, assimilation and transport to their host plants (Franklin, Näsholm, Högberg, & Högberg, 2014). N is available in the soil in an organic form such as peptides or amino acids from decomposing plant or animal matter, or in an inorganic form through N fixation, precipitation, or surface runoff. Proteins for the uptake of inorganic and organic N sources have been characterised in both extra-radical and intra-radical hyphae of ectomycorrhizas, highlighting the importance of ECM for tree N nutrition (Fig. 3).

5.1.1 Inorganic nitrogen uptake by fungal hyphae

5.1.1.1 Ammonium transporters

Ammonium (NH_4^+) is the major source of N for ECM fungi even though the soil concentration of NH_4^+ in forest soils is generally lower than that of nitrate (NO_3^-). NH_4^+ is often preferred by ECM fungi due to its lower

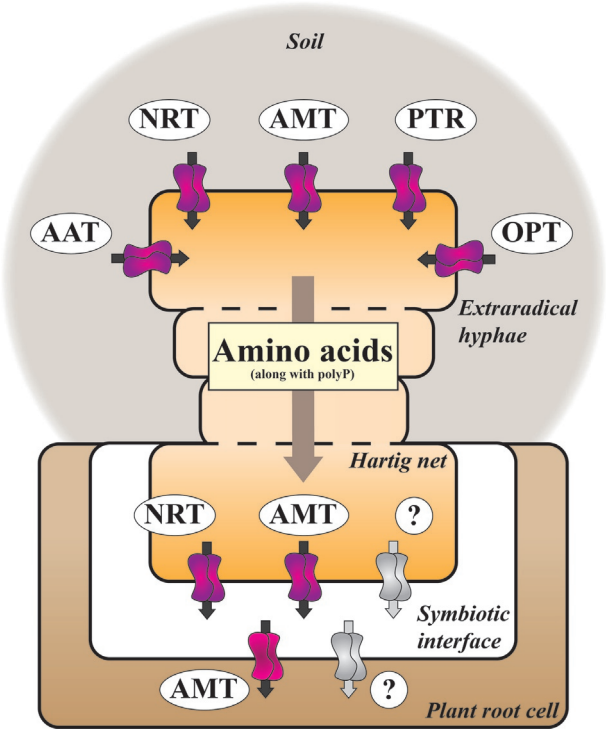

Fig. 3 Schematic representation of the molecular players governing the transport of nitrogen in ectomycorrhizas. Transport proteins are localised in the extra-radical hyphae for the uptake of ammonium (AMT), nitrate (NRT), amino acids (AAT), peptides (PTR) and oligopeptides (OPT). After uptake, nitrogen is translocated from extra-radical hyphae to the Hartig net in vacuoles presumably associated with polyphosphates (polyP). Fungal nitrate (NRT) and ammonium (AMT) transporters are localised in the fungal membrane and release nitrogen into the symbiotic interface. Other forms of nitrogen could also be transferred into the mycorrhizal interface through currently unidentified proteins. Nitrogen is taken up by plant cortical cells from the symbiotic interface via ammonium transporters (AMT). Other forms of nitrogen could also be released to the interface and taken up, but the transporters are currently unknown.

assimilation costs compared to the reduction of NO_3^- to NH_4^+ (Courty, Smith, Koegel, Redecker, & Wipf, 2015). Hyphal NH_4^+ acquisition can account for up to 45% of the total plant N uptake under N deficiency (Javelle, André, Marini, & Chalot, 2003). ECM fungi express high- and low-affinity transporters of the AMT family that take up NH_4^+ under low or high N supply conditions, respectively (Fig. 3; Garcia et al., 2016).

A bioinformatical analysis suggests the existence of eight putative AMTs, three high-affinity and five low-affinity transporters, in the transcriptome of *Laccaria bicolor* (Lucic et al., 2008). Laser capture microdissection and

microarray analysis demonstrated three putative NH_4^+ uptake transporters in ectomycorrhizas of *Tuber melanosporum*, and one of these transporters is specifically upregulated in the fungal mantle, but down-regulated in the Hartig net (Hacquard et al., 2013). High-affinity AMTs have been described for several ECM fungi, including *H. cylindrosporum* (*HcAMT1* and *HcAMT2*: Javelle, André, et al., 2003; Javelle, Morel, et al., 2003; Javelle et al., 2001), *Amanita muscaria* (*AmAMT2*: Willmann, Weiß, & Nehls, 2007), and *Tuber borchii* (*TbAMT1*: Montanini, Moretto, Soragni, Percudani, & Ottonello, 2002).

In general, the expression levels of these transporters are regulated by the external N supply conditions. The high-affinity NH_4^+ transporter of *A. muscaria AmAMT2*, for example, is highly expressed under low N supply conditions, but down-regulated when easily metabolisable N sources, such as NH_4^+ or amino acids, are readily available (Willmann et al., 2007). Transcript levels of *AmAMT2* are also high when NO_3^- is supplied, indicating that *A. muscaria* cannot utilise NO_3^- as a N source. The fact that *AmAMT2* is highly expressed in the extra-radical mycelium but down-regulated in both mantle and Hartig net of ECM poplar roots indicates that AmAMT2 is involved in NH_4^+ acquisition from the soil but not in its transfer towards the host (Willmann et al., 2007). The expression levels of *AMT1* and *AMT2* of *Hebeloma crustuliniforme* are not only controlled by the external availability of N sources but also by the internal levels of glutamine (Javelle, Morel, et al., 2003). Glutamine serves as an essential amino N donor for the synthesis of many essential metabolites, such as nucleic acids, amino sugars, histidine, tyrosine, asparagine, and various co-factors. The intracellular concentrations of glutamine have also been shown in *Arabidopsis* and *Saccharomyces cerevisiae* to repress the expression of AMTs (Rawat, Silim, Kronzucker, Siddiqi, & Glass, 1999; Slaughter, McKernan, & Saita, 1990). In contrast, the low-affinity AMT of *H. cylindrosporum HcAMT3* is not affected by the internal glutamine levels, what could ensure a basal level of NH_4^+ uptake under different N supply conditions (Javelle, Morel, et al., 2003).

Interestingly, some aquaporins from *L. bicolor* (LbAQPs) that are normally involved in water transport (Section 5.5) can also facilitate the membrane transport of other small molecules, such as urea and NH_4^+/NH_3^- (Dietz, von Bülow, Beitz, & Nehls, 2011; Selle et al., 2005). Candidate aquaporins that could play a role for N uptake were also found in *T. melanosporum* (Hacquard et al., 2013).

5.1.1.2 Nitrate transporters

Only a few NO_3^- transporters have so far been identified in ECM fungi (Fig. 3). Their low representation in fungal genomes is likely due to the fungal preference for NH_4^+. NH_4^+ is generally energetically favoured by fungi, since NO_3^- first needs to be reduced to nitrite and eventually to NH_4^+ by the activity of NO_3^- and nitrite reductases, respectively. Fungal uptake of secondary N sources, such as NO_3^-, is closely regulated by the fungal high affinity NO_3^- assimilation cluster (fhant-AC), which consists of a high-affinity transporter (*Nrt*), a reductase (*nr*), and a ferredoxin-independent nitrite reductase (NAD(P)-H-*nir*) (Jargeat, Gay, Debaud, & Marmeisse, 2000; Jargeat et al., 2003; Kemppainen & Pardo, 2013). All genes within this cluster share the same regulation pattern that is generally characterised by a down-regulation of their transcript levels by NH_4^+. NO_3^- availability on the other hand leads to an up-regulation of the fhant-AC gene cluster but does not neutralise its strong suppression by NH_4^+ (Kemppainen, Alvarez Crespo, & Pardo, 2010).

HcNRT2 of *H. cylindrosporum* was the first high-affinity NO_3^- transporter that was characterised in ECM fungi. Consistent with the general characteristics of the fhant-AC, *HcNRT2* is not induced by NO_3^- but is suppressed by an external supply of NH_4^+ (Jargeat et al., 2003). Interestingly, fungal strains with only a minimal detectable transcription of *LbNrt* after silencing were affected in their colonisation of poplar and showed only a loose hyphal growth on the root surface, and no Hartig net development (Kemppainen & Pardo, 2013). This illustrates that despite the fact that ECM fungi generally prefer NH_4^+ over NO_3^-, expression of this transporter and an uncompromised N metabolism of the fungal partner is necessary for the stability of the ECM symbiosis.

TbNRT2 is a high-affinity NO_3^- transporter of *T. borchii* with a transport activity also for nitrite. *TbNRT2* is up-regulated by NO_3^- and nitrite but also by N starvation through a NO_3^- independent de-repression mechanism. In contrast, the adjacent NO_3^- reductase in the same gene cluster is only expressed at low levels under N-starvation, and requires NO_3^- for induction (Montanini et al., 2006). This dual expression pattern of *TbNRT* could provide *T. borchii* with an advantage for N uptake under fluctuating N supply conditions. Though the NO_3^- independent gene expression pattern would lead to higher costs for NO_3^- assimilation when NH_4^+ is available, it would also ensure a certain level of N uptake when both inorganic N sources are limited.

Many ECM fungi have the ability to grow on media with NO_3^- as the sole N source (Nygren et al., 2008), but the contribution of NO_3^- to tree nutrition is still under debate. Since ECM fungi generally favour NH_4^+ as N source, it was generally believed that NO_3^- plays only a minor role for the N nutrition of trees. However, NO_3^- has a higher mobility in soils than other N sources, and a recent study suggests that NO_3^- may play a more prominent role for N nutrition than previously been expected. In the presence of mass flow, NO_3^- can represent the dominating form of mobile N even in nutrient rich soils, and plant transpiration can be an important driver for NO_3^- fluxes and N acquisition of boreal forests (Oyewole, Inselsbacher, Näsholm, & Jämtgårda, 2017).

5.1.2 Organic nitrogen uptake by fungal hyphae

5.1.2.1 Amino acid transporters

In addition to NH_4^+ and NO_3^-, other forms of N sources can be taken up and assimilated by fungal hyphae and transferred to host plants through the fungal mantle and Hartig net, including amino acids, peptides and other organic N sources (Fig. 3). Amino acids can dominate soil N fluxes in both nutrient rich and poor forest ecosystems and can thereby form an important N source for ECM fungi (Oyewole et al., 2017). Recently, it has been shown that N deposition and the shift from inorganic to organic N sources in the soil is a determining factor for the diversity of ECM communities (van der Linde et al., 2018). Fungi that use organic N, such as *Cortinarius*, *Piloderma* and *Tricholoma* species, show a low abundance at sites with high N deposition, while species that rely mainly on inorganic N sources, such as *Laccaria* and *Elaphomyces* species, become dominant species in these ECM communities.

AmAPP1 was the first amino acid transporter that was identified in the ECM fungus *A. muscaria* (Nehls, Kleber, Wiese, & Hampp, 1999). *AmAPP1* encodes a general amino acid transporter with a preference and high affinity for basic and aromatic amino acids and a lower affinity for acidic or neutral amino acids. Furthermore, its expression is enhanced by the absence of inorganic N sources but repressed when inorganic N sources are available (Nehls et al., 1999). *HcGAP1* from *H. cylindrosporum* codes for a general amino acid permease, like *AmAAP1*, and is down-regulated under high N supply but up-regulated under N limitation (Wipf, Benjdia, Tegeder, & Frommer, 2002). Most fungal amino acid transporters belong to the amino-acid-polyamine-organocation (APC) superfamily that mediates the transfer of a broad spectrum of amino acids (Casieri et al., 2013). Bioinformatical analysis revealed an expansion of the APC superfamily

(29 gene models of 4 different families) in *L. bicolor*, compared to basidiomycetes with a saprobic or parasitic lifestyle. This could indicate a higher capability of ECM fungi to utilise organic N sources, including amino acids, and could be related to their dual lifestyle (symbiotic vs. saprobic) (Lucic et al., 2008).

5.1.2.2 Peptide transporters

H. cylindrosporum can take up di- and tripeptides and utilise them as sole N source (Benjdia et al., 2006). After uptake, peptides can be rapidly converted by peptidases and utilised as source for N, amino acids or carbon. Several fungal peptide and oligopeptide transporters (PTRs and OPTs, respectively) have been identified in genomes of ECM fungi, and two di- and tripeptide transporters of *H. cylindrosporum*, *HcPTR2A* and *HcPTR2B*, have so far been functionally characterised (Benjdia et al., 2006). *HcPTR2A* is involved in high-affinity uptake of peptides under N limiting conditions. *HcPTR2A* is strongly expressed under N limitation, or when only NO_3^- is available as N source, but down-regulated when other organic sources become available. *HcPTR2A* and *HcGAP1* (Wipf et al., 2002) show similar expression patterns and are both controlled by a system that senses external N availabilities, but are also regulated by a feedback mechanism that is dependent on internal amino acid pools. In contrast, *HcPTR2B* is constitutively expressed, and ensures a basal level of peptide uptake activity under various N supply conditions (Benjdia et al., 2006). In the same fungus, the oligopeptide transporter *HcOPT1* is also constitutively expressed, but this transporter is not fully characterised yet (Lambilliotte et al., 2004; Müller et al., 2007).

In the genome of *L. bicolor*, two genes coding for putative peptide transporters and eight for oligopeptide transporters were also found (Lucic et al., 2008). Similar to *HcPRT2b*, both peptide transporters are constitutively expressed, indicating that they could facilitate a basal uptake of peptides under various N supply conditions. Of the nine putative oligopeptide transporters (*LbOPTs*), four of them are constitutively expressed in all tissues, two are specifically upregulated in fungal fruitbodies, and two in fruitbodies and ECM mycelium. This suggests that all fungal hyphae might have the capability for peptide uptake either for fungal nutrition or for tissue redistribution (Lucic et al., 2008).

5.1.2.3 Urea transporters

Urea is another important nutrient in forest soils and one of the most abundant nitrogenous compounds found in ECM fungi (Smith & Read, 2008).

Urea can represent up to 73% of the total soluble N compounds in extraradical mycelium of the *P. involutus–Betula pendula* symbiosis under acidic soil conditions or when N is limited (Morel et al., 2005). Urea is first metabolised to NH_4^+ by urease activity and then converted into glutamine by glutamine synthetase (Morel et al., 2008). Microarrays showed that the urease coding gene was up-regulated in *L. bicolor* (Martin et al., 2008). *PiDUR3* encodes a urea transporter of *P. involutus*, and has been characterised as a permease, transmembrane transport protein of the sodium: solute symporter (SSS) family. This transporter family catalyses the uptake of a wide range of solutes, such as sugars, amino acids, nucleosides, urea or anions via Na^+ symport. Urea uptake activity by this transporter is highly pH-dependent, and external protons stimulate urea transport (Morel et al., 2008). High glutamine levels in the cell lead to lower transcript levels and a catabolic repression of *PiDUR3* (Morel et al., 2008). The repression of *PiDUR3* by high intracellular glutamine concentrations reduces the import of urea when NH_4^+, the favoured N source, is available (Morel et al., 2008). In *T. melanosporum*, three putative transporters were identified that could participate in the transport of NH_4^+, urea, and water (Hacquard et al., 2013). In *L. bicolor*, aquaporins were identified that are also permeable for urea (Dietz et al., 2011; Hacquard et al., 2013; Selle et al., 2005).

5.1.3 Nitrogen metabolism in ectomycorrhizal fungi

In plants and fungi, absorbed NH_4^+ is first assimilated into the key N donors glutamate and glutamine via the glutamine synthetase/glutamate synthesis (GS/GOGAT) pathway or the glutamate dehydrogenase (GDH) pathway (Chalot, Blaudez, & Brun, 2006). In the GS/GOGAT pathway, glutamate is aminated into glutamine by glutamine synthetase (GS), and the amino group of glutamine is transferred to 2-oxoglutarate to yield two molecules of glutamate by glutamate synthase. In the GDH pathway, glutamate dehydrogenase (NADP-GDH) catalyses the reversible amination of 2-oxoglutarate to glutamate. Most organisms possess both pathways to produce glutamate.

In *H. cylindrosporum* GS and GDH activity can be found, and the expression of both genes is up-regulated by low NH_4^+ availabilities in the soil, but GDH is down-regulated under high NH_4^+ availabilities. However, not all ECM fungi have a functioning NADP-GDH pathway. GDH-positive fungi such as *H. cylindrosporum* have both GS and GDH activities, while GDH-negative fungi such as *P. involutus*, *A. muscaria*, and *Suillus bovinus* have only GS activities (Morel, Buée, Chalot, & Brun, 2006). The authors suggest that the evolutionary loss of GDH in some fungi is an adaptation to their environment. GDH-negative fungi are common in humus rich soils or

soil layers, in which organic N sources such as amino acids are the dominant N sources. Direct uptake of amino acids including glutamate from the environment would make GDH activities dispensable (Morel et al., 2006). Genes involved in the GS/GOGAT pathway, the urea cycle, and the provision of carbon skeletons for NH_4^+ assimilation via β-oxidation and the glyoxylate cycle have been shown to be highly expressed in rhizomorphs and the extra-radical mycelium of *P. involutus* (Wright, Johansson, Le Quéré, Söderström, & Tunlid, 2005).

Another unique feature in *L. bicolor* is the capability to synthesise and utilise allantoin via urea. This capability is represented by uric acid oxidase, allantoinase, and allantoicase proteins encoded and expressed in the genome (Larsen et al., 2011). Allantoate can be produced by purine and pyrimidine catabolism and transported by an allantoate (*DAL5*) transporter or degraded into urea by the enzymatic activity of allantoicase (Morel et al., 2005). Allantoin can be used by some fungi, bacteria, and plants as carbon and N source, and it has been suggested that allantoin or a derivative of the allantoin pathway may contribute to the fungal capability to provide N to the host plant (Larsen et al., 2011). In several ECM fungi, allantoate/allantoin transporters have been identified (Larsen et al., 2011; Rineau et al., 2013).

5.1.4 Nitrogen transport across the mycorrhizal interface to the host

NH_4^+, amino acids and peptides have been proposed as candidates for N transport across the mycorrhizal interface to the host plant in ECM symbiosis. Amino acids were long seen as the main N source that is transferred across the symbiotic interface. This assumption was mainly based on the capability of ECM fungi to take up amino acids from the soil. However, a transport of amino acids across the mycorrhizal interface after uptake of inorganic N sources and assimilation in the extra-radical mycelium would also result in a flux of carbon skeletons back to the plant host. So far, no clear candidate for fungal amino acids efflux carriers has been identified in the genome of ECM fungi that could be involved in amino acid release from the Hartig net hyphae (Nehls & Plassard, 2018). However, in both *L. bicolor* and *H. cylindrosporum* genomes and mycorrhizal root tips, homologues of yeast AQR1 (Acids Quinidine Resistance 1) were identified (Casieri et al., 2013). AQR1 serves in yeast as an amino acid excretion transporter, that is particularly expressed when amino acids accumulate intracellularly, and is involved in the secretion of homoserine, threonine, and other amino acids from the cell (Velasco, Tenreiro, Calderon, & André, 2004). Whether this transporter could facilitate a secretion of amino acids from the Hartig net hyphae into the mycorrhizal interface needs to be elucidated.

There is increasing evidence, however, that the transport of NH_4^+/NH_3 across the interface may play a more significant role for N transport than previously has been suggested. Amino acids could be assimilated in the extra-radical mycelium, transferred to the Hartig net hyphae, presumably via polyphosphates (polyPs) (Bücking & Heyser, 1999), and broken down to release NH_4^+ into the symbiotic interface.

It has been suggested that N compounds are released into the mycorrhizal apoplast either via passive diffusion, active transport, or exocytosis (Chalot et al., 2006). Potential candidates for active transport proteins for the release NH_4^+ into the mycorrhizal interface could be ATO proteins (for ammonia transport outward) or aquaporins. ATO proteins have been identified in *A. muscaria* (Selle et al., 2005) and *L. bicolor* (Lucic et al., 2008). Fungal aquaporins generally facilitate water transport across membranes (see below), but some are also permeable for NH_4^+. Fungal aquaporins have been identified in *L. bicolor* and *T. melanosporum*, and have been shown to play a key role for ectomycorrhiza development and functioning (Dietz et al., 2011; Hacquard et al., 2013). In addition, voltage-dependent cation channels could be involved in the release of inorganic NH_4^+ to the apoplast (Chalot et al., 2006).

The proposed NH_4^+ transport is consistent with (1) the observed decrease in the amino acid pools (Blaudez, Chalot, Dizengremel, & Botton, 1998) and a strong repression of the GS expression in hyphae of the Hartig net (Wright et al., 2005); (2) the high expression of genes for ammonia-permeable aquaporins in basidiomycota (*L. bicolor*, Dietz et al., 2011) and ascomycota (*Cenococcum geophilum*, Peter et al., 2016); and (3) the up-regulation of plant NH_4^+ transporters.

There is also evidence that an allantoin transporter gene or allantoin permease is expressed in *L. bicolor* and *T. melanosporum* (Hacquard et al., 2013; Martin et al., 2008) which could be involved in the export of allantoate into the symbiotic interface. Additional investigation is needed to determine the contribution of different N sources to N transport in the ECM symbiosis and the precise function and localisation of these proteins in the different symbiotic tissues.

5.2 The symbiotic acquisition of phosphorus

P limits the productivity of plants in many terrestrial ecosystems, particularly in forests (Batjes, 1997). Orthophosphate ions (HPO_4^{2-}; $H_2PO_4^-$; P_i) are the main forms of P taken up by roots from the soil solution. The

concentration of free P_i in the soil is generally very low, and often does not exceed 1–10 μM (Hinsinger, 2001). This is due to its strong affinity for combining with cations and clays, resulting in the formation of insoluble P complexes unavailable for plants (Bieleski, 1973; Hinsinger, 2001). Organic P (P_o) can represent the major form of P in soils, particularly in tropical and temperate forests, and can contribute with up to 30–65%, and sometimes to even more than 90% to the total soil P (Condron & Tiessen, 2005; Tibbett, 2002). Most of the P_o is in the form of phosphate esters (C–O–P bond), such as phosphate monoesters (e.g., sugar phosphates) and phosphate diesters (nucleic acids, phospholipids; Turner, 2008), but access to these sources requires specific hydraulic capacities. To cope with this very low availability, trees acquire a significant part of their P from the symbiosis with ECM fungi. ECM associations are therefore determinant factors for the functioning of forest ecosystems and facilitate P movements and cycling.

5.2.1 Inorganic phosphate acquisition by fungal hyphae

Active absorption of P results in the generation of depletion zones around plant roots. Mycelium of ECM fungi allows trees to overcome this issue by providing roots with access to a larger volume of soil, beyond depletion zones (Cairney, 2011; Smith & Read, 2008). Van Tichelen and Colpaert (2000) demonstrated that the P_i uptake capacity of pine roots was strongly increased in plants with ECM associations, compared to non-mycorrhizal plants. Accumulation of P in ECM plants is correlated to the soil exploration capacity of external hyphae, indicating that P can be directly transferred from the fungus to the roots (Torres Aquino & Plassard, 2004).

At the molecular level, extra-radical hyphae acquire P_i by expressing plasma membrane transporters (Fig. 4; Becquer, Trap, Irshad, Ali, & Claude, 2014; Casieri et al., 2013; Garcia et al., 2016). Several genes putatively encoding P_i transporters have been identified in ECM fungi (Casieri et al., 2013; Kohler et al., 2015) based on the homology with *PHO84* and *PHO89* P transporters from yeast (Bun-Ya, Nishimura, Harashima, & Oshima, 1991; Martinez & Persson, 1998). Most sequenced ECM fungi harbour three to five putative H^+/P_i symporters belonging to the *Pht1* subfamily, suggesting that the ability of ECM fungal cells to take up P_i is dependent on the external pH. So far, only one Na^+/P_i symporter from the *Pht2* family has been identified in the ECM fungus, *T. melanosporum*, suggesting that at least in this fungal species an additional pathway for the acquisition of P might exist (Casieri et al., 2013). Among all P_i transporters

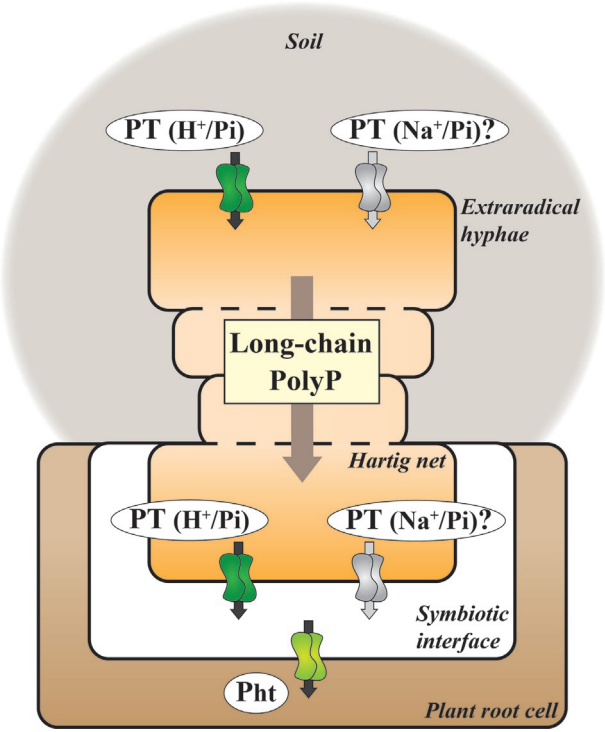

Fig. 4 Schematic representation of the molecular processes that control the transport of inorganic phosphate (P_i) in ectomycorrhizas. P_i is acquired from the soil by extra-radical hyphae through H^+/P_i and possibly Na^+/P_i PT transporters. P_i is condensed into short or long-chain polyphosphates (polyPs) in the fungal vacuoles. PolyPs are translocated from the extra-radical hyphae to the Hartig net via motile tubular vacuoles. Fungal H^+/P_i and potentially Na^+/P_i PT transporters are localised at the fungal membrane at the mycorrhizal interface for the release of P_i. P_i is taken up from the symbiotic interface by plant cortical cells through transporters belonging to the Pht family.

identified to date in ECM fungi, only two proteins from *H. cylindrosporum* (HcPT1.1 and HcPT2), one from *Boletus edulis* (BePT), one from *Rhizopogon luteolus* (RlPT) and one from *Leucocortinarius bulbiger* (LbPT) have been characterised by heterologous expression in yeast (Tatry et al., 2009; Wang, Li, Wu, & Zhao, 2014; Zheng et al., 2016). Of the two P_i transporters initially found in the extra-radical mycelium of *H. cylindrosporum*, HcPT1.1 mediates P_i uptake under P limiting conditions and HcPT2 under high P availabilities (Becquer, Garcia, Amenc et al., 2018; Garcia et al., 2013; Tatry et al., 2009). Another H^+/P_i transporter, HcPT1.2, has also been recently described in *H. cylindrosporum* as participating in P_i uptake from the external medium under high and low P_i conditions (Becquer, Garcia, & Plassard, 2018).

5.2.2 Mineral and organic P mobilisation from the rhizosphere

To release P_i from insoluble minerals, ECM fungi secrete low-molecular-weight organic acids (mainly oxalate) and protons (Courty et al., 2010; Rineau & Garbaye, 2010). This capability of ECM fungi and the resulting P accumulation in host tissues were demonstrated by using apatite (Casarin et al., 2004; Smits, Bonneville, Haward, & Leake, 2008; Wallander, Wickman, & Jacks, 1997) or rock phosphate (Liu, Loganathan, Hedley, & Grace, 2008) as a sole source of P. There is a strong positive correlation between the available P_i levels, P accumulation in colonised plants, and the oxalate concentrations near ECM root tips (Wallander, 2000). Given that the ability of ECM fungi to release organic acids depends on the fungal species and the host species involved, a high variability in the improvement of P nutrition by ECM fungi can be observed (Arvieu, Leprince, & Plassard, 2003; Casarin et al., 2004; Courty et al., 2010; Lapeyrie, Ranger, & Vairelles, 1991).

ECM fungi are also able to produce phosphatases to remove P_i groups from P_o compounds (Plassard & Dell, 2010). ECM fungi can deplete P from birch, pine or beech litters (Perez-Moreno & Read, 2000), from pollen (Read & Perez-Moreno, 2003), and even from nematode necromass (Perez-Moreno & Read, 2002). Dependent on the external source of P that was examined in microcosm experiments, a reduction of 30–97% of the initial litter P content was measured, and 7–33% of this released P was transferred to the host plant through ECM fungi (Quiquampoix & Mousain, 2005). It was suggested that phosphatases released by ECM fungi more likely play a role for the release of P_i from P_o compounds contained in fresh organic matter rather than P that is associated with mineral soil particles (Ali, Louche, Legname, Duchemin, & Plassard, 2009). Phosphatase activities have been shown to be enhanced at low P_i levels in both axenic cultures (Tibbett, Sanders, & Cairney, 1998) and in soils (Louche et al., 2010). This enhancement was also observed when the host plant was in demand of P (Alvarez et al., 2012; van Aarle & Plassard, 2010), suggesting that molecular signals coming from the plant could trigger the expression and excretion of phosphatases by the fungal partner. Lastly, Rennenberg and Herschbach (2013) proposed that ECM fungi could also be able to absorb P_o. However, the molecular players controlling P_o acquisition, movement, and delivery to the host, including transporters and regulators, are still unknown.

5.2.3 Synthesis and regulation of fungal polyphosphates

After uptake, P_i is rapidly incorporated into the active P pool, including phosphorylated primary metabolites, structural molecules and nucleic acids,

or condensed into polyP chains stored in vacuoles (Ashford, Ryde, & Barrow, 1994). PolyP chains are linear polymers of three (short-chained, $\leq$20 units) to hundreds of P_i residues (long-chained $\geq$21 units) connected by energy-rich phospho-anhydride bonds (Wood & Clark, 1988). Mobile short-chained polyP can be detected in fungal tissues using nuclear magnetic resonance (NMR) spectroscopy (Pfeffer, Bago, & Shachar-Hill, 2001). Metachromatic granules in ECM fungi have also been observed to consist of mobile and immobile short- and long-chained polyP by either chemical treatments (Ashford, Peterson, Dwarte, & Chilvers, 1986; Ashford, Vesk, Orlovich, Markovina, & Allaway, 1999) or energy dispersive X-ray microanalysis (Bücking & Heyser, 2000).

The different steps involved in P uptake and transfer are the (i) preferential accumulation and transport of newly absorbed P_i as long-chained polyP by P-starved mycelia, (ii) conversion of long-chained polyP pool into shorter chain lengths in the Hartig net, (iii) hydrolysis of short-chained polyP to P_i to replenish the metabolically active P_i pool in fungal hyphae, (iv) specific release of fungal P_i into the symbiotic interface, and (v) uptake by cortical cells (Torres-Aquino et al., 2017). It has been assumed that short-chained or long-chained polyP could be transferred from the extra-radical mycelium to Hartig net hyphae through a motile tubular vacuole system (Fig. 4) (Bücking & Heyser, 1999; Cairney, 2011). Mobilisation of long-chained polyP is probably under the control of a specific signal released by host plant cells, although its characterisation remains to be determined (Torres-Aquino et al., 2017).

The carbohydrate supply of the host plant serves as an important trigger for fungal P transport. Long-chained polyPs accumulated in the Hartig net hyphae and were not remobilised when photosynthetic activity of the host and carbohydrate transfer to the fungal partner were reduced (Bücking & Heyser, 2003). It has been suggested that there is a bidirectional transport of carbohydrates and of P_i across the same interface (Bücking & Heyser, 2001), and that the fungal degradation of polyPs in the Hartig net plays an important role for the control of the P_i flux across the mycorrhizal interface. Transcripts of an endopolyphosphatase and exopolyphosphatase and their respective activities that could be involved in the breakdown of long-chained polyPs were demonstrated in arbuscular mycorrhizal fungi (Ezawa, Smith, & Smith, 2002; Tisserant et al., 2011), but never in ECM fungi. However, orthologues of these enzymes can be found in the genome of *H. cylindrosporum*, suggesting that comparable mechanisms might exist in ECM symbiosis (Plassard, pers. comm.).

5.2.4 Phosphate delivery to colonised roots

Although it is known for more than half of a century that ECM fungi transfer P to host trees (Melin & Nilsson, 1950), the molecular mechanisms that control the allocation from fungi to root cells remain largely unknown (Becquer et al., 2014; Nehls & Plassard, 2018). Usually, P_i is transported across membranes by H^+/P_i or Na^+/P_i symporters depending on proton-motive force and electrochemical gradients for P_i (Preuss, Huang, & Tyerman, 2011). Since passive transport proteins that could act as channels have not yet been identified, the release of P_i from P_i-rich fungal cells towards the P_i-poor mycorrhizal interface is puzzling. However, a recent *in silico* analysis in arbuscular mycorrhizal interactions predicted that proton-coupled transporters might not only be suited for the uptake of nutrients but also for their release towards the host plant (Schott et al., 2016). Consequently, P_i transporters may transport their substrate along coupled electrochemical gradients without rectification preferences (Ai et al., 2009; Cubero et al., 2009; Fristedt, Weinander, Martinsson, & Persson, 1999; Hürlimann, Pinson, Stadler-Waibel, Zeeman, & Freimoser, 2009; Preuss, Huang, Gilliham, & Tyerman, 2010). For example, a homologous high affinity phosphate transporter (Pho84) has been identified in yeast that ensures a bidirectional transport of P_i along localised pH and P_i gradients at each side of the membrane (Fristedt et al., 1999). Similarly, Schott et al. (2016) proposed a model that high P_i concentrations in the fungal cytosol might be a factor that stimulates P_i fluxes from fungus to the plant through transporters. A P_i gradient which stimulates P_i efflux may be the result of a facilitated polyP degradation within the Hartig net (Torres-Aquino et al., 2017) triggered by the host plant supply with carbohydrates (Bücking, 2004). Anion channels could also mediate P_i efflux. In the genome of *H. cylindrosporum*, two anion channels of ClC family are present but have not yet functionally described.

Supporting the view of a bidirectional P_i transport, two H^+/P_i transporters from the ECM fungus *H. cylindrosporum*, HcPT1.1 and HcPT2, were localised in both the extra-radical mycelium and within the Hartig net (Becquer, Garcia, Amenc et al., 2018; Garcia et al., 2013). Although HcPT1.1 seems to be primarily involved in P_i acquisition under low P_i conditions (Tatry et al., 2009), the key role of HcPT2 in both P_i uptake from the soil and release to the symbiotic apoplast has recently been revealed (Becquer, Garcia, Amenc et al., 2018). The efflux of P_i from fungus to plant through HcPT2 was dependent on an unknown signal originating from the host (Becquer, Garcia, Amenc et al., 2018). The artificial down-regulation

of *HcPT2* inhibits ectomycorrhiza formation, indicating that a less cooperative fungus can be sanctioned by the host and that P_i itself can be a key regulator of ectomycorrhiza formation (Garcia et al., 2015). However, we cannot exclude that a transport of P_i could also be mediated by other mechanisms, such as vesicular trafficking or the activity of other transport proteins (Becquer, Garcia, Amenc et al., 2018; Becquer, Garcia, & Plassard, 2018).

Plant cortical cells surrounded by the Hartig net need to specifically express and/or regulate transporters to take up P_i that has been delivered by the fungus to the symbiotic interface. To date, no plant transporter ensuring this role has been functionally characterised, but some candidates have been identified in poplar (Loth-Pereda et al., 2011) and pine (Plassard, pers. comm.). Although *PtPT9* and *PtPT12* were up-regulated in poplar roots colonised by the ECM fungus *L. bicolor* and the arbuscular mycorrhizal fungus *R. irregularis* (Loth-Pereda et al., 2011), further studies are necessary to assess their importance in these symbioses.

5.3 The symbiotic acquisition of potassium

Potassium (K^+) is one of the most important macronutrients for plant growth. This cation is essential for various physiological and cellular functions and for resistance against environmental stressors. In plant roots, K^+ is taken up by specialised high or low affinity transport systems (Anschütz, Becker, & Shabala, 2014; Nieves-Cordones, Alemán, Martínez, & Rubio, 2014; Shabala & Pottosin, 2014). Since K^+ is a major constituent of many rock-forming minerals, its availability in natural ecosystems is often drastically limited (Zörb, Senbayram, & Peiter, 2014). Due to their physiological and biochemical properties, fungal hyphae can better solubilise K^+ from rocks than plant roots. ECM fungi can solubilise K^+ through the secretion of protons and organic acids, and thereby significantly contribute to host plant K^+ nutrition (Rygiewicz & Bledsoe, 1984). For example, amount of exchangeable K^+ through weathering found in soils colonised with *Piloderma* spp. was higher than in non-ECM soils (Arocena, Glowa, Massicotte, & Lavkulich, 1999). The ECM fungus *Suillus variegatus* also increases the concentration of organic acids in soils when biotite is used as K^+ source. However, no significant effect on plant K^+ nutrition was observed in seedlings that were colonised with this fungus compared to non-mycorrhizal control plants (Wallander & Wickman, 1999). *P. involutus* is also able to weather muscovite, to release K^+ and to enhance plant nutrition (Van Schöll, Smits, & Hoffland, 2006). However, not all

ECM fungal species show this capability, indicating that the effect on K^+ nutrition can vary depending on the fungus involved in this interaction and on environmental conditions. For example, it has been reported that *P. involutus* contributes 5–6% of total K^+ of its host *Picea abies* (Jentschke, Brandes, Kuhn, Schröder, & Godbold, 2001). Another fungus, *Pisolithus albus*, enhanced K^+ acquisition of *Acacia spirorbis* and *Eucalyptus globulus* by 21–38% when grown in a New Caledonian topsoil that was contaminated with toxic metals (Jourand et al., 2014).

Under K^+ limiting conditions, *P. pinaster* seedlings colonised by *H. cylindrosporum* showed an up to 35% higher K^+ content (Garcia et al., 2014). Insights into the molecular players that are involved in K^+ transport in the ECM symbiosis have become possible by the increasing number of sequencing programs for ECM fungi, as cited above. Consequently, four families of putative K^+ transport systems have recently been identified in ECM fungi (Garcia & Zimmermann, 2014). The current model suggests that transporters are responsible for K^+ absorption from the soil by extra-radical or mantle hyphae, while ion channels contribute to the release of K^+ from the Hartig net into the mycorrhizal interface (Fig. 5).

5.3.1 Potassium uptake from the soil through fungal transporters

So far, two conserved families of K^+ transporters have been identified in fungi, Trk (Trk/Ktr/HKT; Transporter of K^+) and HAK (KT/KUP/HAK; High-Affinity K^+ uptake) (Benito, Garciadeblás, Fraile-Escanciano, & Rodríguez-Navarro, 2011). Trk transporters were first identified in *S. cerevisiae* (Gaber, Styles, & Fink, 1988; Ko & Gaber, 1991) and were later also described for bacteria, plants, and fungi as K^+/H^+ or Na^+/K^+ symporters, or Na^+ uniporters (Corratgé-Faillie et al., 2010; Rodrı´guez-Navarro, 2000). This functional diversity indicates that Trk proteins may also be involved in other cellular functions than K^+ transport, including the acquisition of Na^+ and its compartmentalization in the vacuole for detoxification.

In all genomes of ECM fungi that have so far been sequenced, at least one member of the Trk family has been identified (Garcia & Zimmermann, 2014). In *H. cylindrosporum*, two K^+ transporters HcTrk1 and HcTrk2 were found but only one of them (HcTrk1) has so far been functionally characterised and studied in ECM roots (Corratgé et al., 2007; Garcia et al., 2014). Due to its localisation at uptake active sites of ECM roots of *P. pinaster*, it has been suggested that HcTrk1 plays a major role in K^+ acquisition from soils. Colonisation by *H. cylindrosporum* increased the K^+ contents in the shoots of pine seedlings by about 35% (Garcia et al., 2014).

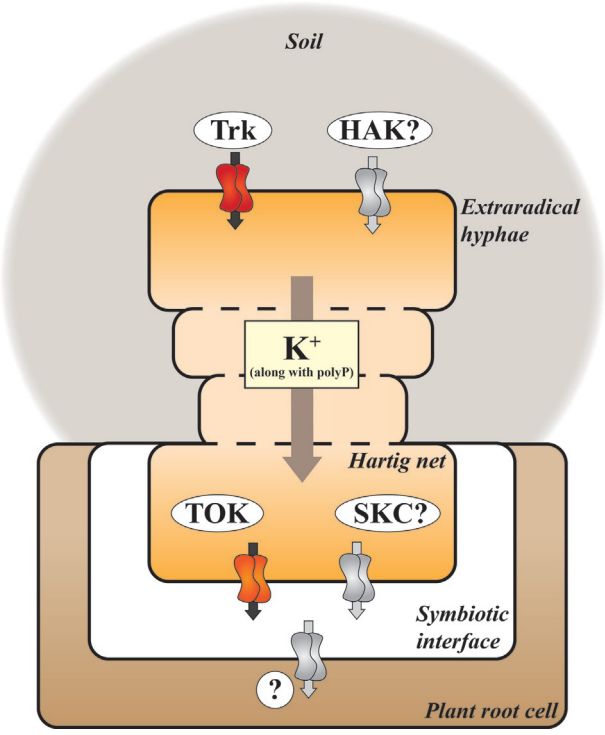

Fig. 5 Schematic representation of the molecular mechanisms that control the transport of potassium (K^+) in ectomycorrhizas. K^+ is acquired from the soil by Trk and possibly HAK transporters localised in extra-radical hyphae. K^+ is translocated from the extra-radical hyphae to the Hartig net in vacuoles in association with polyPs. TOK and potentially SKC channels are localised at the symbiotic interface and release K^+ into the mycorrhizal interface. K^+ is transported from the symbiotic interface to plant cortical cells through unidentified transporters.

However, this effect was not observed in seedlings that were colonised by transgenic fungal strains overexpressing *HcTrk1* (Garcia et al., 2014). It is likely that the overexpression of *HcTrk1* triggered an ectopic accumulation of the corresponding proteins in the Hartig net that resulted in a competition with plant cells for K^+ acquisition from the symbiotic interface (Garcia et al., 2014). Similarly, plants that were colonised with these overexpressing strains displayed reduced P concentrations, indicating that K^+ homeostasis is a crucial component for fungal P allocation to the host. K^+ serves as one of the major counter-ions for the formation and stabilisation of polyPs in ECM fungi (Bücking & Heyser, 1999).

HAK transporters are well characterised in fungi and plants and are involved in K^+ uptake under deficient conditions (Cabrera, Álvarez, Martín, Siverio, & Ramos, 2012; Rivetta, Allen, Slayman, & Slayman, 2013). One to four HAK members were found in the genome of ECM fungi (Garcia & Zimmermann, 2014), but their role for K^+ transport in the symbiosis is still unknown. Based on their function in other organisms and their level of expression in ECM tissues, we can hypothesise that they might play a significant role for the K^+ nutrition of ECM plants under K^+ limitation (Garcia and Zimmermann, unpublished).

Besides Trk and HAK transporters, members of the ACU P-type ATPase family (alkali cation uptake ATPase) with K^+ and Na^+ transport activities were found in most fungal genomes (Benito et al., 2011; Benito, Garciadeblás, Schreier, & Rodríguez-Navarro, 2004). However, their roles in both free-living mycelia or symbiotic conditions are still unknown.

5.3.2 Potassium movements within the fungus

Although the long-distance transport of K^+ from uptake to release sites of ectomycorrhizas is only poorly understood, it seems closely related to the movements of polyP through fungal hyphae (Benito & González-Guerrero, 2014). K^+ has been detected by elemental analyses in polyP granules of fungal hyphae and has been identified as one of the major counter-ions (Bücking & Heyser, 1999). The correlation between P_i and K^+ concentrations in hyphae of *H. cylindrosporum* supports the view of a joined transport within the fungus (Garcia et al., 2014). This is currently the only mechanism hypothesised so far since no diffusion process along concentration gradients was observed in fungi (Fig. 5).

5.3.3 Potassium transfer from the Hartig net to colonised roots—The missing step

Although the symbiotic transport of K^+ from the Hartig net to root cortical cells remains largely unknown, promising candidates have been identified in EST libraries (Lambilliotte et al., 2004) and fungal genomes (Kohler et al., 2015). The two most promising candidate families that could be involved in the release of K^+ from fungal hyphae into the mycorrhizal apoplast are Shaker-like K^+ channels (SKC) and tandem-pore outward-rectifying K^+ (TOK) channels (Garcia & Zimmermann, 2014). SKCs belong to the voltage-gated ion channel (VIC) superfamily, and only members from

animal and plant species have so far been characterised (Papazian, Schwarz, Tempel, Jan, & Jan, 1987; Sentenac et al., 1992). These channels have also been found in fungi, including ECM fungi, from basal species (neocallimastigomycota, chytridiomycota, mucoromycota, zoopagomycota) to basidiomycota, but not in ascomycota (Garcia & Zimmermann, 2014). It has been hypothesised that SKC proteins might have a role in K^+ release across the fungal plasma membrane towards the apoplast, but no report describes their involvement in ECM symbiosis, and no ECM fungal member has so far been functionally characterised.

Members of the fungal-specific TOK channel family are also promising candidates for K^+ efflux in ectomycorrhizas (Garcia & Zimmermann, 2014; Guerrero-Galán, Delteil, et al., 2018, Guerrero-Galán, Garcia, Houdinet, & Zimmermann, 2018). These channels harbour in each polypeptide two pore domains in tandem that are assembled as dimers. They are described as outward rectifiers in yeast (Ketchum, Joiner, Sellers, Kaczmarek, & Goldstein, 1995), and as ion channels, rectification drives the current preferentially in one direction. Interestingly, small inward currents can also be observed dependent on the K^+ concentration and membrane potential. Inward currents can explain the uptake of K^+, the growth and observed complementation of TOK yeast mutants (Bertl et al., 2003). Two TOK channel subfamilies have been found in ascomycota and basidiomycota, including some ECM species (Garcia & Zimmermann, 2014).

Three TOK channels HcTOK1, HcTOK2.1 and HcTOK2.2 have been identified in *H. cylindrosporum* based on their sequence similarities and secondary structures. Given the nature of already studied TOK channels in yeast (Ketchum et al., 1995) and *Neurospora crassa* (Roberts, 2003), their homologues in *H. cylindrosporum* could be interesting candidates for K^+ transfer from the fungus to plant apoplast. Recent functional studies have demonstrated outward K^+ currents in *Xenopus laevis* oocytes that express HcTOK1 and HcTOK2.1, and growth complementation by HcTOK1 and HcTOK2.2 in yeast mutants (Guerrero-Galán, Delteil, et al., 2018). An enhanced expression of *HcTOK2.2* in ECM roots and its localisation in the Hartig net region implies that this channel could be involved in the K^+ transfer towards host roots. Consistently, pine seedlings that were colonised with transgenic fungal strains overexpressing *HcTOK2.2* displayed a larger accumulation of K^+ in their shoots than control plants (Fig. 5; Guerrero-Galán, Delteil, et al., 2018). It has also recently been demonstrated that the plasma membrane channel *HcTOK1* plays a key role in fungal K^+ homeostasis, and ultimately in the symbiotic transfer of K^+ to *P. pinaster* (Guerrero-Galán, Garcia, et al., 2018).

K^+ released at the symbiotic interface needs to be taken up by transport systems that are localised at the plasma membrane of plant cortical cells. Dozens of K^+ transporters from various model plant species have been characterised, but only very few were identified in the roots of trees and so far no transporter has been identified that is involved in the uptake of K^+ from the mycorrhizal interface. A SKOR-type outward Shaker-like K^+ channel was highly up-regulated in the roots of *Pinus sylvestris* that were colonised by the ECM fungi *C. geophilum*, *Suillus granulatus* and *Rhizopogon roseolus* (Peter et al., 2016). Typically, these channels mediate K^+ loading to the xylem, suggesting that they might have a similar role in colonised roots. More research is needed to clearly identify those plant transport systems in the plasma membrane of cortical cells responsible for the uptake of K^+ from the symbiotic interface.

5.4 The symbiotic acquisition of other ions

ECM fungi are also able to provide their host with other essential ions and can protect the roots against high metal concentrations or against salt stress (Colpaert, Wevers, Krznaric, & Adriaensen, 2011). This protective function from the fungal partner can partly be explained by the presence of a fungal sheath surrounding the roots (Bücking et al., 2002; Jentschke & Godbold, 2000), but also by the contribution of highly selective ion transport systems.

5.4.1 Other macronutrients

An improved nutrition by ECM fungi with other macronutrients such as magnesium, calcium, and sulphur has also been reported (Casieri et al., 2013). However, in comparison to N, P and K^+, the molecular mechanisms that control these transport processes are largely unknown. Organic acid secretion by ECM fungi can also mobilise other essential nutrients by mineral weathering and can for example release calcium from apatite (Blum et al., 2002; Landeweert, Hoffland, Finlay, Kuyper, & van Breemen, 2001). Formation of calcium oxalate crystals in colonised eucalypt roots indicates a higher calcium uptake of ECM plants (Pylro et al., 2013). A symbiotic transport of magnesium, an essential element for forest productivity that is particularly limiting in acidified forest soils, has also been observed in *P. abies* seedlings colonised by *P. involutus* (Jentschke & Godbold, 2000). *P. involutus* led also to an improved calcium and magnesium nutrition in *Populus deltoides* seedlings under aluminium stress (Khosla, Kaur, & Sudhakara Reddy, 2009). In nickel-rich soils from New Caledonia, calcium acquisition was significantly enhanced in *A. spirorbis* and *E. globulus* plants by the ECM fungus *P. albus*, while magnesium uptake

was significantly reduced (Jourand et al., 2014). A decrease in sulphur starvation of ECM plants has also been observed, and sulphate and methionine permeases have been found in fungal genomes (Casieri et al., 2013; Garcia et al., 2016).

5.4.2 Micronutrients

ECM fungi can also transfer micronutrients, including iron, zinc (Zn^{2+}), copper, and manganese from the soil to its host root. At the same time, ECM fungi can also protect their host against toxic concentrations of these metals in their environment (Colpaert & van Assche, 1987; Colpaert et al., 2011; Galli, Schüepp, & Brunold, 1994). This requires a tight regulation of cytosolic concentrations of these micronutrients in fungal hyphae through specialised transport systems. Recent studies demonstrated the improvement of plant micronutrient nutrition by ECM fungi and identified several of the involved fungal transporters. For example, Zn^{2+} is acquired from the soil through transporters belonging to the ZIP family (Zrt/Irt-like proteins). SlZRT1 of *Suillus luteus* is expressed at the plasma membrane and facilitates the cellular uptake of Zn from the soil (Fig. 6; Coninx et al., 2017). When Zn^{2+} concentrations in the environment are too high, *S. bovinus* is able to limit its transfer to the host plant *P. sylvestris* and protect the plant against its toxic effects (Adriaensen, Vangronsveld, & Colpaert, 2006; Bücking & Heyser, 1994). Involved in the protection against toxic metal concentrations could be the accumulation and detoxification of Zn^{2+} in fungal vacuoles mediated by vacuolar transporters such as SlZnT1, a Cation Diffusion Facilitator (CDF) that has been identified in *S. luteus* (Ruytinx et al., 2017). High concentrations of Zn^{2+} in the fungal vacuole could be detoxified by binding to long-chained polyPs (Bücking & Heyser, 1999). Interestingly, *H. cylindrosporum* stores excess Zn^{2+} in ER-derived vesicles, probably through the activity of HcZnT1 (Blaudez & Chalot, 2011). Similar transporters have been identified in *Russula atropurpurea* that could mediate both vacuolar Zn^{2+} storage (RaCDF1) and bidirectional metal transport (RaCDF2) at the plasma membrane (Sácký, Leonhardt, & Kotrba, 2016).

5.5 The symbiotic acquisition of water

It is known for almost a century that ECM fungi can help trees in the acquisition of water, particularly during the recovering after a drought period (Cromer, 1935; Goss, 1960; Reid, 1978; Zevora, 1955). Transport of water from ECM fungi to their host plant has been demonstrated for the first time using tritiated water in associations between *S. bovinus* and

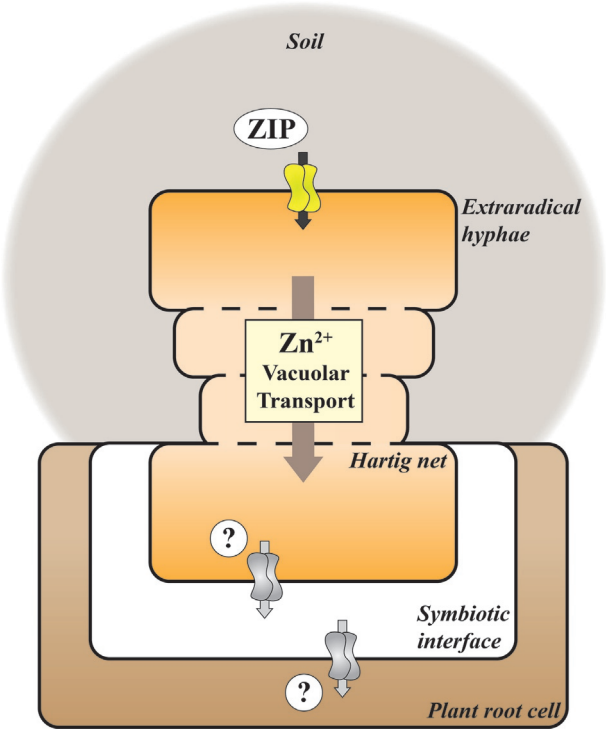

Fig. 6 Schematic representation of the molecular mechanisms that control the transport of zinc (Zn^{2+}) in ectomycorrhizal roots. Extra-radical hyphae take up Zn^{2+} from the soil by Zrt/Irt-like proteins (ZIP) transporters. Zn^{2+} is transported into vacuoles through cation diffusion facilitators (see main text) and then translocated from the extra-radical mycelium to the Hartig net. Unidentified fungal and plant proteins are responsible for the release of Zn^{2+} at the symbiotic interface and its uptake by cortical cells.

P. sylvestris (Duddridge, Malibari, & Read, 1980). Several tree species show an improved performance under water stress by the ECM symbiosis (Garbaye, 2000; Lehto & Zwiazek, 2011). Symplastic and apoplastic pathways for water transport in ectomycorrhizas have been discussed in relation to the hydrophilic or hydrophobic properties of fungal cell walls (Behrmann & Heyser, 1992; Bücking, Hans, & Heyser, 2007; Bücking et al., 2002; Lehto & Zwiazek, 2011).

5.5.1 Water uptake by fungal hyphae

In addition to the larger volume of soil that is explored by fungal hyphae, specific water channels, called aquaporins, have been attributed to the higher

water uptake of mycorrhizal plants. Aquaporins of the major intrinsic protein (MIP) family are found in all organisms and mediate water transport across membranes (Maurel, Verdoucq, Luu, & Santoni, 2008; Nehls & Dietz, 2014). They are divided into five subfamilies and among them plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs) that contribute the most to water transport.

In the genome of several ECM fungi, including *L. bicolor*, different aquaporin families were discovered (Fig. 7; Dietz et al., 2011), and it becomes increasingly clear that these aquaporins can play an essential role during ECM root development. For example, knockout mutants of *L. bicolor*, with a reduced expression of the aquaporin *LbAQP1*, showed

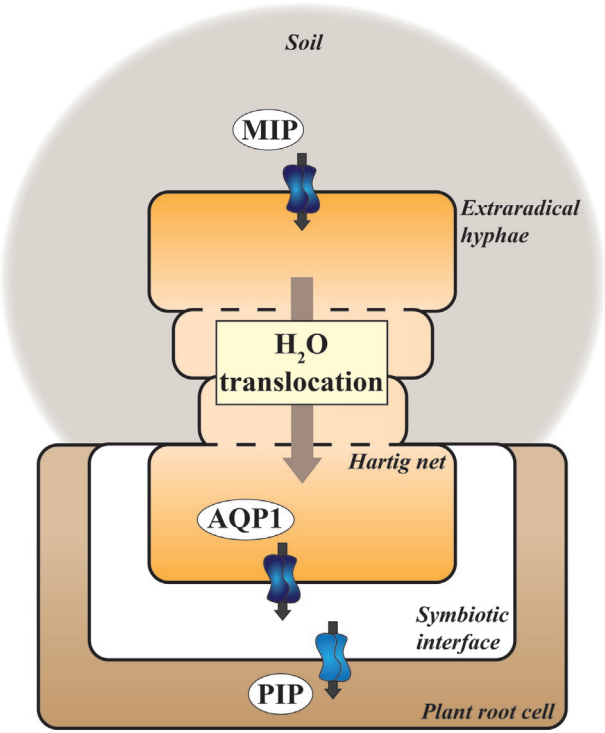

Fig. 7 Schematic representation of molecular mechanisms that control the transport of water (H_2O) in ectomycorrhizal roots. Extra-radical hyphae take up water from the soil through major intrinsic protein (MIP) aquaporins. Water is translocated from the extra-radical hyphae to the Hartig net where aquaporins (AQP1) are responsible for its release into the symbiotic interface. H_2O is transported from the symbiotic interface to plant cortical cells through plant aquaporins belonging to the plasma membrane intrinsic protein (PIP) family.

no Hartig net development in *Populus tremuloides* seedlings and a reduced expression of the mycorrhizal effector protein MiSSP7 (Navarro-Ródenas, Xu, Kemppainen, Pardo, & Zwiazek, 2015). Overexpression of JQ585595, another aquaporin of *L. bicolor*, led to higher hydraulic conductivities in roots of mycorrhizal *Picea glauca* plants, and revealed the importance of fungal aquaporins for the transport of water from the fungus to the roots (Xu et al., 2015). Recently, transcriptional profiling of *C. geophilum* demonstrated that one aquaglyceroporin and one classical aquaporin were highly up-regulated in ECM *P. sylvestris* roots (Peter et al., 2016). Under drought conditions, these two aquaporins were down-regulated in both ectomycorrhizal root and free-living mycelia, whereas another classical aquaporin was highly up-regulated (Peter et al., 2016). These observations support the hypothesis that fungal aquaporins are required for symbiotic transport of water and that their expression is regulated by the water demand of the host plant.

5.5.2 Water transfer towards colonised roots

The use of tracer techniques with isotopes and dyes allowed to follow the water exchange between the roots of woody plants and ECM fungi (Plamboeck et al., 2007). Increase of root hydraulic conductance in ECM roots of *Ulmus americana* and *Pinus banksiana* has been attributed to a decrease of the apoplastic water flow resistance and to an increase of aquaporin-mediated water transport (Lee, Calvo-Polanco, Chung, & Zwiazek, 2010; Muhsin & Zwiazek, 2002). Interestingly, the increase in water transport capacity of ECM poplar roots was correlated with the up-regulation of aquaporin-encoding PIP genes (Marjanović et al., 2005). A link between fungal water transport and expression of root aquaporins was established in *P. glauca* roots colonised by transgenic *L. bicolor* strains with an overexpression of JQ585595 (Xu et al., 2015). This overexpression resulted in the up-regulation of a plant root aquaporin, suggesting a positive correlation between fungal and plant water transport. However, there are also reports in which the root colonisation with *P. involutus* led to a downregulation of two plant aquaporins (*BpPIP1* and *BpPIP2*) of birch (Le Quéré, Wright, Söderström, Tunlid, & Johansson, 2005). Although there are results suggesting that a tight regulation of aquaporins from both partners is needed for water fluxes in ECM symbiosis, the water transport regulation may still depend on the plant and fungal species involved, and the water demand from the host (Fig. 7).

6. Future challenges and concluding remarks

Identifying the molecular mechanisms controlling the transport of water and nutrients from the soil to tree roots through ECM fungi is crucial to understand resource dynamics in forest ecosystems. Several proteins of the "ECM transportome" have already been described in both plant and fungal partners, but many others particularly at the symbiotic interface are still not identified and their regulation unknown.

Although ECM associations are the dominant mycorrhizal form in temperate and boreal forests, other forms of mycorrhizal symbioses co-exist in these ecosystems. For example, the arbuscular mycorrhizal symbiosis is one of the most important associations in terms of its economic impact and its ecology due to the high number of plant species that form this symbiosis, including many agronomically important crops (Lee, Eo, Ka, & Eom, 2013; Wang & Qiu, 2006). Interestingly, some woody plants like poplar can simultaneously be colonised by ECM and arbuscular mycorrhizal fungi. Some transporters are up-regulated in roots colonised by both root symbionts, whereas others are only specifically expressed during one symbiosis (Loth-Pereda et al., 2011). However, the actual involvement of these transporters in tree nutrition remains unclear. Although far less abundant than the two other associations mentioned above, ectendomycorrhizal mycorrhizas can also be found in shrub species and tree roots that are colonised by ECM fungi. This symbiosis is defined by the presence of a fungal mantle, and a Hartig net, but in contrast to ECM fungi, in the ectendomycorrhizal symbiosis an intracellular penetration of the root cortex and a different interface is involved in the hydro-mineral nutrition of woody plants (Smith & Read, 2008). However, no molecular data of this symbiosis form are yet available.

A remaining challenge in the study of mycorrhizal interactions is that our current understanding of many transport processes is only based on the symbiosis of one host plant with one fungal species. However, plants are simultaneously colonised by multiple fungi, and tree nutrition is therefore determined by communities of fungi, that may more or less overlap in their functionality of the host, and also compete for carbon supply from the host. The same is true for the fungal partner that can simultaneously colonise multiple hosts from one or more species and can choose to which host certain nutrients are delivered. Developing projects that investigate tripartite interactions with more than one symbiosis form will allow the identification of shared and specific allocation pathways for nutrients and water in very different associations.

Another impediment for the identification of molecular processes involved in ECM associations is that currently only a handful of transformable fungal and plant species, and only a limited number of tools for species transformation, are available. The development of more transformable fungal and plant models, particularly in the group of gymnosperms, will allow the assessment of both molecular diversity and common features driving the formation and functioning of ECM symbiosis. Although the use of reverse genetics has been successful in some species and provided with some insights into the effects of an over-expression or RNA-silencing of candidate genes, the lack of knock-out mutants limits the description of new and specific phenotypes. Interestingly, the increasing application of CRISPR/Cas9-based genome editing in many organisms, including trees (Fan et al., 2015) or filamentous fungi (Shi et al., 2017), opens a path towards its use in ECM species. Subsequent efforts in the development of original molecular tools, and additional fungal and plant models are necessary to unravel the development, functioning and evolution of ECM symbiosis.

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