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LbNrt RNA silencing in the mycorrhizal symbiont *Laccaria bicolor* reveals a nitrate-independent regulatory role for a eukaryotic NRT2-type nitrate transporter

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Summary

Fungal nitrogen metabolism plays a fundamental role in function of mycorrhizal symbiosis and consequently in nutrient cycling of terrestrial ecosystems. Despite its global ecological relevance the information on control and molecular regulation of nitrogen utilization in mycorrhizal fungi is very limited. We have extended the nitrate utilization RNA silencing studies of the model mycorrhizal basidiomycete, *Laccaria bicolor*, by altering the expression of *LbNrt*, the sole nitrate transporter-encoding gene of the fungus. Here we report the first nutrient transporter mutants for mycorrhizal fungi. Silencing of *LbNrt* results in fungal strains with minimal detectable *LbNrt* transcript levels, significantly reduced growth capacity on nitrate and altered symbiotic interaction with poplar. Transporter silencing also creates marked co-downregulation of whole *Laccaria* fHANT-AC (fungal high-affinity nitrate assimilation cluster). Most importantly, this effect on the nitrate utilization pathway appears independent of extracellular nitrate or nitrogen status of the fungus. Our results indicate a novel and central nitrate uptake-independent regulatory role for a eukaryotic nitrate transporter. The possible cellular mechanisms behind this regulation mode are discussed in the light of current knowledge on NRT2-type nitrate transporters in different eukaryotes.

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Introduction

Access to nitrogen is one of the fundamental growth-limiting factors in terrestrial and aquatic ecosystems. While the majority of soil nitrogen is organic, nitrate (NO_3^-) is the major inorganic nitrogen source assimilated by many eukaryotes such as fungi and plants. In the fungal kingdom the capacity to utilize nitrate is limited to the Dikarya (Ascomycota and Basidiomycota), and to some members of the mucorales (Sarbhoy, 1965; Slot and Hibbett, 2007). Temperate and boreal forest soil fungi, as their relatives from agricultural soils, show a widespread capacity to grow on nitrate (Yamanaka, 1999; Nygren *et al.*, 2008; Gorfer *et al.*, 2011). This strongly suggests that nitrate plays a relevant role, not only in agricultural systems where its role as the most dominant inorganic nitrogen source is well established, but in forest ecosystems as well (Booth *et al.*, 2005; Miller *et al.*, 2007; Myrold and Ritchie Posavatz, 2008; Dechorgnat *et al.*, 2011). Moreover, recent field studies on climate change effects have demonstrated that elevated CO_2 exposure and soil warming both induce increased nitrate concentration in temperate forest soils (Butler *et al.*, 2012; Schleppi *et al.*, 2012).

Many of the nitrate-utilizing fungi in boreal and temperate forests' soils are symbiotic and engaged in mycorrhizal interactions with plants. Ectomycorrhiza (ECM) is the dominant form of mycorrhiza of boreal and temperate tree species and this interaction is formed by a vast number of basidiomycete and a minor number of ascomycete species. In ECM the fungus supplies the host plant with different nutrients, of which nitrogen and phosphorus are the most important, while the fungus receives carbon as plant photosynthetic compounds (Smith and Read, 2008). While the majority of ECM fungi are reported to prefer ammonium as an inorganic nitrogen source the capacity to grow on nitrate is also widely distributed among them (Nygren *et al.*, 2008) and some ECM species even prefer nitrate over ammonium (Scheromm *et al.*, 1990; Aouadj *et al.*, 2000). Most importantly, the ECM interaction can dominate the whole nitrogen uptake of the host tree which now occurs via the extraradical mycelium of the fungal partner (Gobert and Plassard,

2002; 2007; Guescini *et al*., 2003; Bailly *et al*., 2007). This fungal dominance over the plant nutrient acquisition makes the uptake and control of nitrogen source utilization, such as nitrate, in ECM fungi of special interests as it can be expected to have a significant impact in nutrient acquisition of the symbiotic host plants and nutrient cycling in forest ecosystems.

Fungal utilization of nitrate requires its uptake by specific plasma membrane NRT2-type high-affinity nitrate transporters (NRT) and its sequential cytosolic reduction from nitrate to nitrite and further to ammonium by nitrate reductase (NR) and ferredoxin-independent nitrite reductase (NIR) respectively. These nitrate metabolism genes are often present as a cluster, forming the so-called fHANT-AC (fungal high-affinity nitrate assimilation cluster). The fHANT-AC can be found in the majority of nitrate-growing ascomycetes and basidiomycetes to which it was most likely horizontally transferred during the evolution from a lineage leading to oomycetes. While higher plants have multiple high- (HATS) and low-affinity (LATS) nitrate transporters (Dechorgnat *et al*., 2011), fungi show a very reduced nitrate transporter repertoire. Fungal transporters belong to the NRT2 type of nitrate transporters and the species studied harbour one or two transporter genes (Slot *et al*., 2007). Further differentiation to high- and low-affinity transporters and differential expression control has been demonstrated in some fungi which harbour the two transporter copies (Machín *et al*., 2000; Zhou *et al*., 2000; Unkles *et al*., 2001; Slot *et al*., 2010).

The fact that fungal nitrate growth relies on the activity of such a limited number of genes has made these organisms a target of intensive study of nitrate utilization in eukaryotes. While the basic regulatory circuits in fungi were identified more than 30 years ago, the precise molecular mechanisms responsible for control of fungal nitrate growth have been described only recently (Marzluf, 1997; Wong *et al*., 2008). The current information on nitrate utilization gene regulation in fungi comes almost entirely from studies on saprotrophic and pathogenic ascomycete species such as *Aspergillus nidulans*, *Neurospora crassa* and *Magnaporthe grisea*, and on the ascomycete yeast *Hansenula polymorpha* (Crawford and Arst, 1993; Marzluf, 1997; Tao and Marzluf, 1999; Siverio, 2002; Wong *et al*., 2008; Rossi and Berardi, 2009). Unfortunately, the information on genetic control of nitrate growth in other taxa, such as basidiomycetes, is minimal. This lack of information has special implications on ECM research.

Utilization of nitrate has a high energy cost and it is therefore shown to be under strict transcriptional feedback regulation in fungi, algae and plants (Crawford and Arst, 1993; Caddick *et al*., 1994; Berger *et al*., 2008; Fernandez and Galvan, 2008). The transcriptional activation of nitrate utilization genes, under sufficient carbon supply, requires in saprotrophic ascomycetes both the presence of nitrate as inducer and the absence of a preferred nitrogen source, ammonium or L-glutamine as repressors (Marzluf, 1997; Wong *et al*., 2008). The intracellular nitrate is believed to mediate the induction response via nitrate-specific activating transcription factors (i.e. NirA in *A. nidulans*, Nit4 in *N. crassa*) (Fu *et al*., 1995; Punt *et al*., 1995). Promoter binding of these transcription factors depends on the synergistic action of global nitrogen status-sensing positive acting GATA binding factors (AreA, *A. nidulans*; Nit2, *N. crassa*) (Fu and Marzluf, 1987; Kudla *et al*., 1990; Scazzocchio, 2000; Bernreiter *et al*., 2007). These GATA factors mediate nitrogen metabolite repression (NMR) of nitrate and other secondary nitrogen utilization pathways in filamentous ascomycetes in the presence of preferred nitrogen sources such as ammonium and L-glutamine (Narendja *et al*., 2002; Mo and Marzluf, 2003; Muro-Pastor *et al*., 2004; Berger *et al*., 2006; 2008). However, AreA-responsive genes can also become activated by nitrogen starvation as lack of nitrogen has been demonstrated to cause gradual accumulation of the GATA factor in nucleus and leads to NirA/Nit4-type inducer-independent activation of the nitrate pathway (Todd *et al*., 2005; Berger *et al*., 2008). The repressing effect of ammonium in NMR has been firmly linked to elevated cellular L-glutamine concentration in *A. nidulans* indicating that this molecule, and not ammonium, would be the real intracellular repressor of the nitrate metabolism pathway (Berger *et al*., 2008).

In the last few years genome sequencing has resulted in major advances in ECM research (Martin *et al*., 2008; 2010; Plett and Martin, 2011) making possible to assess the full nitrogen transport gene repertoire of these symbionts (Lucic *et al*., 2008). Despite these advances the understanding of nitrate utilization regulation in symbiotic ECM fungi is still very limited. Furthermore, the information originating from saprotrophic ascomycetes is apparently not directly applicable to these symbiotic fungi. This has become strikingly clear as the expression analyses of nitrate utilization genes in the few ECM fungi studied this far (i.e. ascomycete *Tuber borchii*, and basidiomycetes *Hebeloma cylindrosporum* and *Laccaria bicolor*) have revealed some unique regulation characteristics clearly separating the mycorrhizal lifestyle from saprotrofism (Jargeat *et al*., 2000; 2003; Montanini *et al*., 2006a,b; Rékangalt *et al*., 2009; Kemppainen *et al*., 2010). The most outstanding about the nitrate gene regulation in these ECM fungi is that they show no induction (by nitrate, or any other identified compound), which affects the whole (*H. cylindrosporum* and *L. bicolor*) or part (*T. borchii*) of the nitrate metabolism pathway.

The fungal nitrate utilization genes have also been shown to be upregulated in the ECM interaction (Guescini

et al., 2003; 2007; Bailly *et al*., 2007; Rékangalt *et al*., 2009) and uncompromised fungal nitrate metabolism has been related to successful establishment of the ECM interaction between *L. bicolor* and poplar (Kemppainen *et al*., 2009). ECM has also been shown to cause down-regulation of plant nitrate utilization genes which reveals the dominance of the fungal nitrate metabolism pathway over the host (Bailly *et al*., 2007). All these data support the relevance of the nitrate utilization pathway for functioning of the ECM interaction and consequently for nutrient cycling in temperate and boreal forest soils.

Laccaria is susceptible to *Agrobacterium*-mediated transformation (AMT) and RNA silencing making it the first ECM fungus whose gene expression can efficiently be manipulated (Kemppainen *et al*., 2005; 2009; Plett *et al*., 2011). The fungus can utilize nitrate and its nitrate assimilation genes consist of one nitrate transporter (*LbNrt*), nitrate reductase (*LbNr*) and nitrite reductase (*LbNir*) which are present as a fHANT-AC. While the whole cluster is clearly under NMR in presence of ammonium, also relative strong basal transcript levels can be detected under repressive conditions suggesting a simultaneous capacity to utilize both ammonium and nitrate. While transcript accumulation of the cluster genes can be observed under nitrate feeding, most striking is, however, the strong nitrate-independent activation of the whole cluster detected under nitrogen limitation (starvation) or when the fungus is growing on organic nitrogen. One of these non-repressive organic N sources is L-glutamine, molecule which in ascomycete systems has been considered responsible for NMR (Marzluf, 1997; Berger *et al*., 2008; Wong *et al*., 2008). The *Laccaria* fHANT-AC regulation seems perfectly optimized for maximal nitrate uptake in soils with spatial and temporal gradients of this anion and rich in organic N. This high energy cost regulation pattern fits perfectly with its habitat, which is the upper temperate and boreal forest soil horizon rich in organic nitrogen, and its lifestyle as symbiont obtaining carbon from the ECM host plant. These postulations are further supported by recent data from *H. cylindrosporum* nitrogen utilization (Avolio *et al*., 2012). The potential of RNA silencing in resolving regulatory circuits of *Laccaria* nitrate metabolism and ECM interaction was previously demonstrated by altering the expression of the nitrate reductase gene (*LbNr*). These studies provided the first genetic confirmation of the importance of efficient fungal nitrogen metabolism in establishment of the ECM interaction (Kemppainen *et al*., 2009). They also demonstrated that *LbNr* plays a minimal role in regulation of other *Laccaria* fHANT-AC genes (Kemppainen *et al*., 2010). Here we have extended the RNAi-based studies of *Laccaria* fHANT-AC activity and control by targeting the nitrate transporter gene (*LbNrt*). *Laccaria* offers a unique opportunity to study the role of a fungal nitrate transporter in a symbiotic system which shows very different, highly relaxed and nitrate-independent fHANT-AC regulation pattern. Here we describe the structural characteristics of *LbNrt* and its gene product, and report the phenotype of the first RNAi transporter mutants in mycorrhizal fungi reconfirming the fundamental role of efficient fungal nitrogen metabolism in establishment of the symbiosis. RNA silencing of *LbNrt* reveals a central role of its gene product in upregulation of the rest of the fHANT-AC. Such a nitrate-independent regulatory role has not been described in eukaryotic nitrate transporters before.

Results

LbNrt DNA and protein sequence analysis

Laccaria bicolor nitrate transporter gene *LbNrt* (JGI protein ID 254042) forms part of a fHANT-AC in the genome of the fungus (Fig. S1A). The *LbNrt* and the nitrate reductase gene *LbNr* (protein ID 254066) are closely clustered and separated only by 593 bp, but the nitrite reductase gene *LbNir* (protein ID 291348) is located almost 14 kb upstream. Such an open fHANT-AC is not present in other studied nitrate-utilizing basidiomycetes (Kemppainen *et al*., 2010).

The predicted genomic coding sequence of *LbNrt* stretches over 1902 bp and it consists of eight exonic and seven intronic sequences (Fig. S1B). The promoter sequence prediction with NNPP (version 2.2) identifies a putative minimal promoter (score 0.93) at 21–70 nucleotides upstream of the *LbNrt* coding sequence with the proposed transcription start site (TSS) at 30 nt upstream of the start codon. The analysis of the *LbNrt* 5′-flanking region (700 bp upstream from ATG) for transcription factor binding sites with TESS search reveals three sequence motifs with similarity to DNA binding sites of AreA/NIT2-type global positive acting nitrogen regulator GATA factor from *Aspergillus nidulans* and *Neurospora crassa*. Gene transcription studies have demonstrated that nitrate utilization by basidiomycete fungi is under global ammonium repression. This resembles the AreA/NIT2-dependent regulation mode of ascomycete fungi and searches of different basidiomycete genomic sequences generally result in detection of putative basidiomycete AreA/NIT2 homologues. The transcription of *Laccaria* fHANT-AC genes is also controlled via dominant ammonium repression (Kemppainen *et al*., 2010) and *Laccaria* genome encodes for at least one putative AreA/Nit2 homologue (protein ID 488576). Moreover, this precise gene appears to be participating in control of nitrate utilization in the fungus (M.J. Kemppainen and A.G. Pardo, unpubl. results). On the other hand, TESS search did not detect any nitrate induction-specific regulator of the NirA/NIT4-type binding sites (Fu *et al*., 1995; Strauss *et al*., 1998) in

the upstream region of *LbNrt*. This reflects the specific regulation mode of fHANT-AC genes in *Laccaria* as the activity of the cluster shows no induction (Kemppainen *et al*., 2010). The same behaviour has been observed in fHANT-AC genes of another ectomycorrhizal basidiomycete, *Hebeloma cylindrosporum* (Jargeat *et al*., 2000; 2003; Avolio *et al*., 2012), and in the nitrate transporter (*TbNrt2*) of the ectomycorrhizal ascomycete *Tuber borchii* (Montanini *et al*., 2006a). This lack of induction is an interesting feature of phylogenetically distant species which share the same functional niche, the ectomycorrhizal symbiosis. Such a regulation mode thus may represent an important evolutive adaptation to the symbiotic lifestyle.

The *LbNrt* encodes a 503-amino-acid protein with an estimated molecular mass of 54.16 kDa. LbNRT shares the characteristics of the NNP (nitrate–nitrite porter) family of NO_3^- and NO_2^- porters (TC 2.A.1.8). The NNPs belong to the major facilitator superfamily (MFS) proteins (TC 2.A.1) which are membrane transporters involved in symport, antiport and uniport of various substrates (Pao *et al*., 1998).

The nitrate transport via NNPs is shown to be energy-demanding proton-coupled symport (McClure *et al*., 1990a,b; Miller *et al*., 2007) and the nitrate transporters of the NNP family in algae, higher plants and fungi are referred to as NRT2 (Forde, 2000; Galván and Fernández, 2001). The sequence analysis of LbNRT and a set of fungal and plant NRT2 proteins together with bacterial NNP sequences places LbNRT within a strongly supported clade of fungal NRT2 together with other basidiomycete nitrate transporters while the ascomycete transporters form their own clade (Fig. S2). Some ascomycete fungi, such as *Aspergillus* species, harbour several NRT2 isoforms and their sequence similarity indicates that these have originated from gene duplication events. This NRT2 duplication is apparently a sporadic event in fungi and also differential gene losses have happened within fungal lineages. A second NRT2 gene paralogue can be found in some mushroom-forming basidiomycete fungi such as *Hebeloma edurum*, but this duplication was probably later lost in the ECM fungus *H. cylindrosporum*. Interestingly, a single nitrate transporter is not a fully dominant feature of *Laccaria* genus either as one *Laccaria* species is demonstrated to have undergone a recent NRT2 gene duplication (Slot *et al*., 2010). An important exception among the analysed fungal protein sequences is the NRT2 of the ascomycete *Trichoderma reesei*. This transporter clusters together with the basidiomycete proteins and a horizontal gene transfer from basidiomycetes has been proposed to be behind the nitrate utilization capacity of this ascomycete species (Slot *et al*., 2007).

The NRT2 transporters share the general 12 helical MFS structure and the fungal NRT2 proteins (TC 2.A.1.8.5) belong to the NNP subfamily II. It is characterized by relatively short N- and C-terminal sequences outside the 12 transmembrane helices, a long hydrophilic central loop separating the sixth and the seventh helices with a conserved protein kinase C recognition motif S/T-x-R/K (Forde, 2000). The secondary structure topology models of the LbNRT were created by using three different prediction methods (TMHMM2, TopPred and TMpred). All three methods used proposed for LbNRT a conserved fungal-type NRT2 structure of 12 transmembrane helices, separated by an extended cytosolic sequence loop (71–72 aa, depending on the prediction program used), extended cytosolic N-terminal sequence (44–47 aa) and a very short cytosolic C-terminal end (0–3 aa) (Fig. S3A). The analysis of the N-terminal sequence with SignalP-HMM prediction also suggests that this part of the protein could function as a signal anchor (probability score 0.853) and participate in association of the protein with the cell membrane via posttranslational modifications. These secondary structure predictions obtained for LbNRT closely resemble the predicted membrane topology of NRT2 in another ectomycorrhizal basidiomycete, *H. cylindrosporum* (Jargeat *et al*., 2003). The 3D model of the LbNRT was constructed via homology modelling using the crystal structure of the *E. coli* glycerol-3-phosphate transporter (PBD ID 1PW4; Huang *et al*., 2003) as a folding template. The model covering nearly the full length of LbNRT sequence (20–499 aa) was obtained and in this model the 12 transmembrane helices group to form a pore-like structure. Also the N-terminal tail, the long inter-helical loop and the C-terminus all face the same side, this being most probably the cytosolic side of the protein (Fig. S3D).

Major facilitator superfamily proteins are known to carry repeated signature sequences (G-x-x-x-D/N-R/K-x-G-R-R/K), referred to as MFS I and MFS II, and these are located between the transmembrane helices II and III, and VIII and IX respectively (Forde, 2000). In LbNRT the MFS I signature is detected at amino acid positions 101–110 between the helices II and III (GPLVDQYGPR). The less conserved MFS II motif carries only the conserved G and D residues (GYVGDLLYRS) and is located at position 367–376, between the transmembrane domains VIII and IV (Fig. S3C).

The NNP family of MSF proteins is also reported to carry another conserved signature motif within their transmembrane helix V (F/Y/K-x-x-x-I/L/Q/R/K-x-G/A-x-V/A/S/K-x-G/A/S/N-L/I/V/F/Q-x1,2,G-x-N/I/M-x-G-G/V/T/A) and this signature is abbreviated as NNP I. Another related sequence, NNP II, is also generally repeated in the transmembrane helix XI (Trueman *et al*., 1996; Pao *et al*., 1998; Unkles *et al*., 2012). The NNP I signature is present also in LbNRT in a slightly modified form between sites 161 and 182 (FDKNCVGTANALVGGWGMCPGG) and it runs from the cytosolic loop between the helices IV and V

to the transmembrane part of the helix V (Fig. S3C). On the other hand, only a partial sequence related to the NNP II signature (GVMSGLAGSFGTLGG) could be detected within the predicted transmembrane helix XI of LbNRT (Fig. S3C).

Two conserved arginine residues present in the TM helices II and VIII of NRT2 transporters have been shown to be needed for substrate binding and high-affinity nitrate transport via NrtA in *A. nidulans* (Unkles *et al.*, 2004). Consistently with these results LbNRT also carries such conserved arginine residues in the given helices at amino acid positions 97 and 363.

The large cytosolic transhelical loop is a specific feature of fungal NRT2 proteins. This sequence typically carries protein kinase C recognition motifs S/T-x-R/K, which suggests that the loop plays an important role in posttranslational regulation of the transporters via phosphorylations and dephosphorylations (Forde, 2000). Also LbNRT carries two conserved kinase C recognition motifs in its cytosolic loop sequence at sites 238–240 and 294–296 (Fig. S3B). In addition to the protein kinase C motif, the analysis of the LbNRT sequence with the NetPhos 2. phosphorylation prediction program detected two potential serine (scores 0.988 and 0.992) and two threonine phosphorylation sites (scores 0.915 and 0.982) within the loop (Fig. S3B). Interestingly, the phosphorylation of serine residues in the cytosolic loop sequence of *Hansenula polymorpha* nitrate transporter YNT1 has recently been demonstrated to play a fundamental role in posttranslational control of this protein by sorting it to the plasma membrane in respect to the quality of nitrogen and availability of the carbon sources (Martín *et al.*, 2011). The presence of a potential protein kinase C recognition motif and Ser/Thr phosphorylation sites in LbNRT loop similarly to YNT1 strongly suggests that this part of the protein is important for posttranslational regulation of nitrate transporter in *Laccaria*.

The long cytosolic loop sequences of ascomycete NRT2s are also reported to contain regions resembling PEST sequences. The PEST sequences act as a signal for polyubiquitinylation which has been shown to initiate proteolytic cleavage and degradation. The PESTfind algorithm indicates the presence of a poor PEST sequence also within the loop of LbNRT (Fig. S3B). Similar poor PEST sequences have been reported for NRT2 loop sequences of several mushroom-forming basidiomycete species (Slot *et al.*, 2010).

Creation and characterization of LbNrt-silenced strains of Laccaria

We wanted to study the *LbNrt* expression, its relevance for the symbiotic interaction and its possible role in *Laccaria* fHANT-AC regulation under variable nitrogen regimens via RNA silencing. Intron spacer hairpin RNA (ihpRNA) expression can efficiently launch gene silencing in *L. bicolor* (Kemppainen *et al.*, 2009; Plett *et al.*, 2011). *Agrobacterium*-mediated transformation in combination with the pHg/pSILBAγ vector system (Kemppainen and Pardo, 2010) was used to create transgenic dikaryotic *Laccaria* strains carrying an ihpRNA expression cassette targeting *LbNrt*. This cassette consisted of 716 bp long inverted repeated sequence fragments of *LbNrt* cDNA (Fig. 1A). Altogether 86 hygromycin B-resistant strains were created with the pHg/pSγ/*LbNrt*Loop construct of which 43 randomly selected strains were evaluated for their growth on nitrate as N source in liquid medium. Of the screened strains 65% showed severely reduced growth capacity on nitrate when compared with the wild type fungus. This growth phenotype was further confirmed for 10 randomly selected strains for their growth on solid nitrate medium with and without hygromycin B pressure. Independently of the presence of the selection antibiotic the affected strains had a thin superficial growth pattern and minimal hyphal penetration in the medium (Fig. 1B). A similar growth phenotype has previously been linked to efficient *LbNr* silencing in *Laccaria* (Kemppainen *et al.*, 2009).

Two transformed strains (st. 2 and st. 4) with severely reduced capacity to grow on nitrate were used for further molecular analyses to demonstrate the affected expression status of the RNAi target, the presence of transgenes and to identify the T-DNA integration site in the genome of the fungus. The initial semi-quantitative RT-PCR studies on nitrate-grown strains confirmed that the *LbNrt* transcript levels in the tested strains were minimal in comparison with the wild type fungus (Fig. 1C). The PCR testing for the presence of several sequence components of pHg/pSγ/*LbNrt*Loop T-DNA (Fig. 1D) further demonstrated that both of the studied RNAi strains carried an intact *hph* resistance and ihpRNA expression cassettes. However, lack of amplification for the ampicilline resistance gene, which belongs to the T-DNA plasmid rescue component of pHg/pSILBAγ, in the strain 2 indicated that it carried a RB-truncated T-DNA integration. Consequently, the genomic T-DNA integration sites were recovered from the RNAi strain 4 by plasmid rescue and from the strain 2 by inverse PCR. The results revealed that in both cases the T-DNA had integrated within ORFs with EST support. Such integration pattern in active genomic sites has been demonstrated before in *Laccaria* AMT under hygromycin B selection (Kemppainen *et al.*, 2008). The RNAi strain 2 had the T-DNA integrated in a gene encoding a predicted protein of unknown function (ID 307960) and the strain 4 in a gene encoding a predicted protein (ID 479891). Neither of these integration sites was the *LbNrt* locus, or physically close to genes of *Laccaria* fHANT-AC. This demonstrated that the altered *LbNrt* expression was not

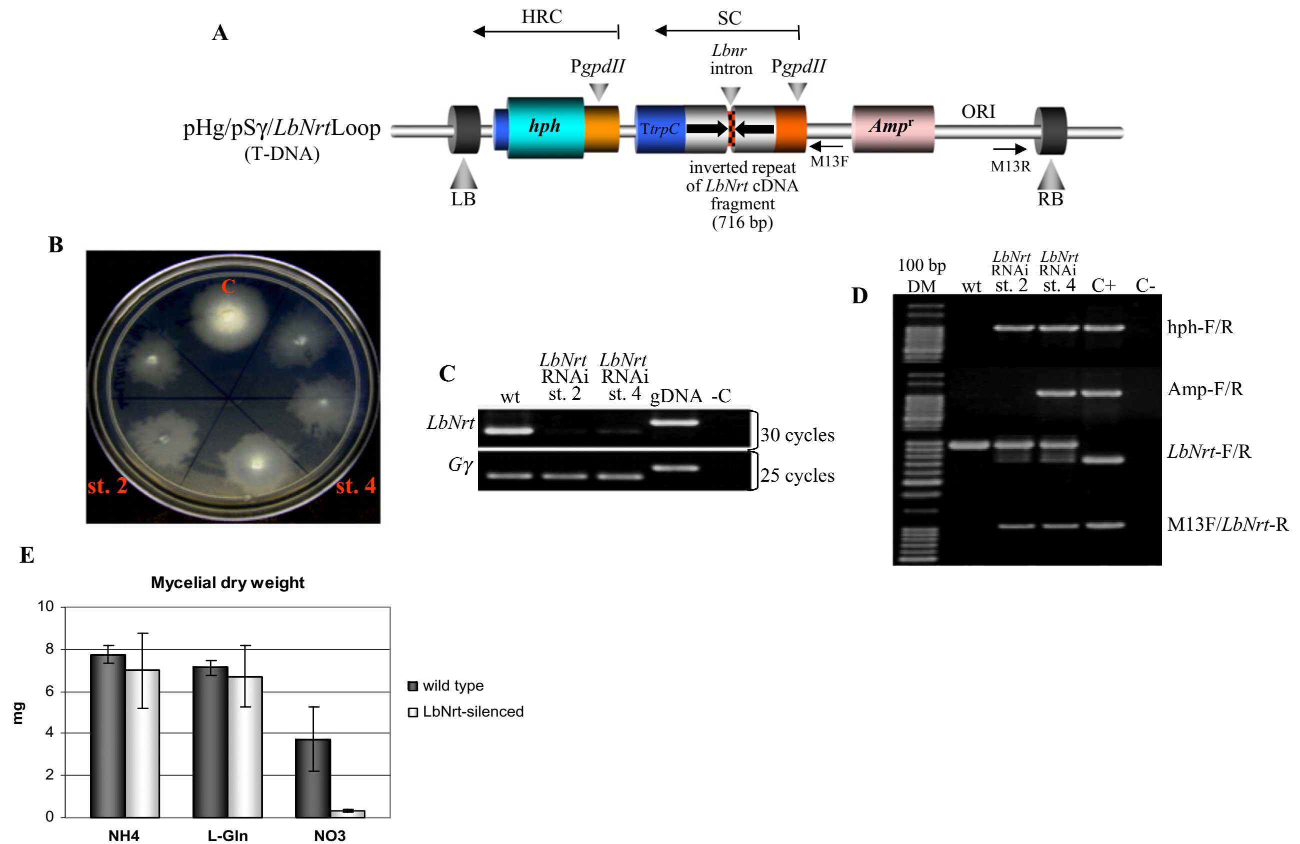


Fig. 1. A. Schematic representation of the transformation/silencing binary vector pHg/pSγ/*LbNrt*Loop T-DNA. P*gpdII*, *A. bisporus* glyceraldehyde-3-phosphate dehydrogenase promoter; *hph*, *E. coli* aminocyclitol phosphotransferase gene which confers resistance to hygromycin B; T*trpC*, tryptophan synthetase terminator of *A. nidulans*; *Amp*^{r}, ampicilline resistance gene of *E. coli*; HRC, hygromycin B resistance cassette; SC, silencing cassette, LB/RB, T-DNA left and right border repeats; M13R, M13/pUC-reverse sequencing primer site; M13F, M13/pUC sequencing primer site.
B. Growth of five pHg/pSγ/*LbNrt*Loop transformed *Laccaria* strains and a mock transformant strain (C) on solid medium with 4 mM KNO_3 as nitrogen source. Strongly affected strains 2 and 4 used in further molecular analyses are marked.
C. RT-PCR results on *LbNrt* expression with respect to heterotrimeric G-protein gamma subunit (JGI protein ID 181979) expression levels (Gγ) in the wild type fungus and in two pHg/pSγ/*LbNrt*Loop transformed, weak nitrate growth phenotype strains 2 and 4. gDNA, amplicons produced from genomic DNA template; −C, no template PCR control.
D. PCR detection of various pHg/pSγ/*LbNrt*Loop T-DNA elements in gDNA of *LbNrt* RNAi strains 2 and 4. DM, DNA marker; C+, pHg/pSγ/*LbNrt*Loop as positive PCR control; C−, no template PCR control. *LbNrt*-F/R amplification shows both the gDNA and ihpRNA cDNA fragment amplification in the transformants but not in the wild type strain. M13F/*LbNrt*-R amplicon produced from the pHg/pSγ/*LbNrt*Loop T-DNA in RNAi strains (but not in the wild type strain) harbours both the ihpRNA cDNA arm and the *A. bisporus* gpdII promoter sequence. Lack of Amp^r amplification in the *LbNrt* RNAi strain 2 indicates a truncated T-DNA integration.
E. Biomass production of the wild type and the *LbNrt*-silenced strain 2 in liquid growth medium supplemented with ammonium (NH_4^+), L-glutamine (L-Gln) or nitrate (NO_3^-) as nitrogen source (4 mM N).

due to know nitrate utilization gene interruption and the ihpRNA expression launched silencing was responsible for the minimal *LbNrt* transcript levels and consequently for the weak growth of the strains on nitrate.

In order to still confirm the connection between *LbNrt* silencing and the reduced growth capacity specifically on nitrate, the biomass production of RNAi strain 2 was evaluated in liquid cultures under ammonium (*LbNrt* repressing N source), L-glutamine (non-repressive N source in *Laccaria*; Kemppainen *et al.*, 2010) and nitrate feeding. The effect of *LbNrt* silencing was clearly specific for nitrate growth as the capacity of the fungus to grow on ammonium or L-glutamine was unaltered but the biomass production on nitrate was less than 10% of the wild type strain (Fig. 1E).

Silencing of LbNrt affects the symbiotic interaction

Silencing of *Laccaria* nitrate reductase (*LbNr*) was previously shown to inhibit mycorrhization with poplar (Kemppainen *et al.*, 2009). This result showed the fundamental role of uncompromised nitrogen metabolism of the fungal symbiont in formation of the ectomycorrhizal interaction. The two molecularly analysed strongly *LbNrt*-silenced

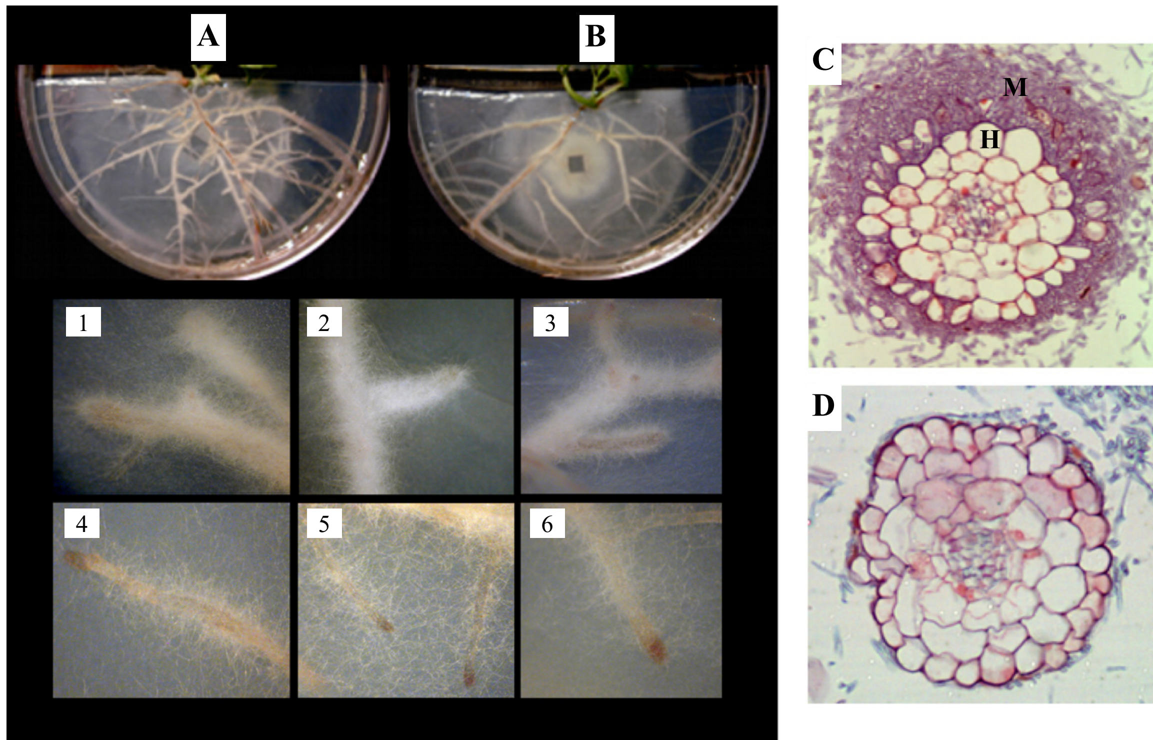


Fig. 2. Summary of the mycorrhization assays with the wild type (A: 1–3) and *LbNrt*-silenced strains (B: 4–6) of *Laccaria* with poplar. The wild type fungus efficiently formed the symbiotic mantle covering the lateral roots of poplar while the interaction between the plant and silenced strains was limited to loose mycelial coverage on some of the lateral roots. Microscopic sections of the symbiotic organs formed by the wild type fungus (C) had fully developed mantle (M) and Hartig net (H) but no such structures were observed in lateral roots of poplar confronted with the silenced *Laccaria* strains (D).

strains were tested for their symbiotic capacity with poplar. The mycorrhization assay was run on a growth medium with minimal carbon availability for the fungus (10 g l^{-1} sucrose; a carbon source not utilizable by *Laccaria* as such) and combined nitrogen source (simultaneous high nitrate 10 mM, and low L-glutamine 1.4 mM). This addition of the organic N source was used for compensating the weak biomass production of the silenced strains during co-cultivation with the plant. *Laccaria* fHANT-AC shows the unusual feature of not being repressed by L-glutamine and the fungus has capacity to simultaneously utilize both nitrate and organic nitrogen sources (Kemppainen *et al*., 2010). The mycorrhization assay confirmed the results previously observed by *LbNr* silencing. While the wild type fungus vigorously interacted with the plant forming a symbiotic mantle covering the lateral roots of poplar (Fig. 2A, panels 1–3), the interaction of *LbNrt*-silenced strains with the plant was minimal and reduced to loose hyphal growth on the surface of the roots (Fig. 2B, panels 4–6). The microscopic sections showed that no hyphal penetration between the epidermal and cortical cells had happened and consequently no Hartig net was formed by the *LbNrt*-silenced strains (Fig. 2D) compared with the wild type (Fig. 2C). These results from *LbNrt* silencing confirm the impaired symbiotic capacity of *Laccaria* when its nitrogen metabolism is compromised and consequently no efficient nitrogen delivery from the fungus to the plant partner can be established.

Silencing of LbNrt causes nitrate-independent downregulation of LbNr and LbNir

Silencing of *LbNr* was previously shown not to affect the expression of other fHANT-AC genes (Kemppainen *et al*., 2010). This unaffected transcript status of *LbNrt* in a *LbNr*-silenced fungus was still confirmed by RT-PCR and Northern blotting (Fig. S4) in two nitrate reductase-silenced strains generated with the pHg/pSILBAγ vector system (Kemppainen and Pardo, 2010). However, RT-PCR detection of lower amounts of *LbNr* and *LbNir* transcripts in nitrate-grown *LbNrt*-silenced strain suggested that the silencing of the transporter causes a dramatic reduction of nitrate reductase transcripts and alters the nitrite reductase expression as well (Fig. 3A). Identical semi-quantitative RT-PCR results were repeatedly obtained from three different pHg/pSγ/*LbNrt*Loop-transformed strains (st. 2, st. 4 and st. 13) which shared the strongly affected nitrate growth phenotype (Fig. 4A). The relative fHANT-AC gene expression levels were calculated in two

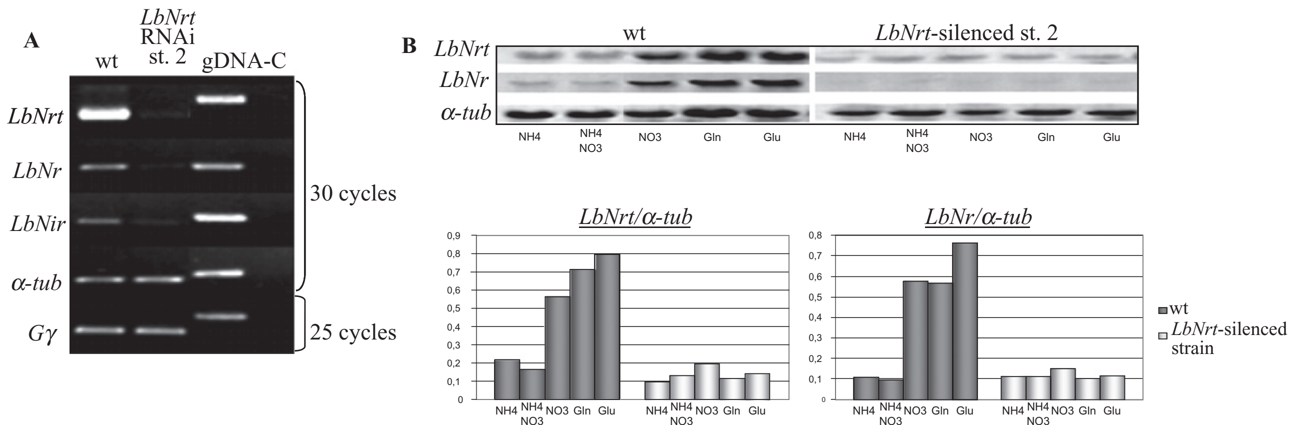
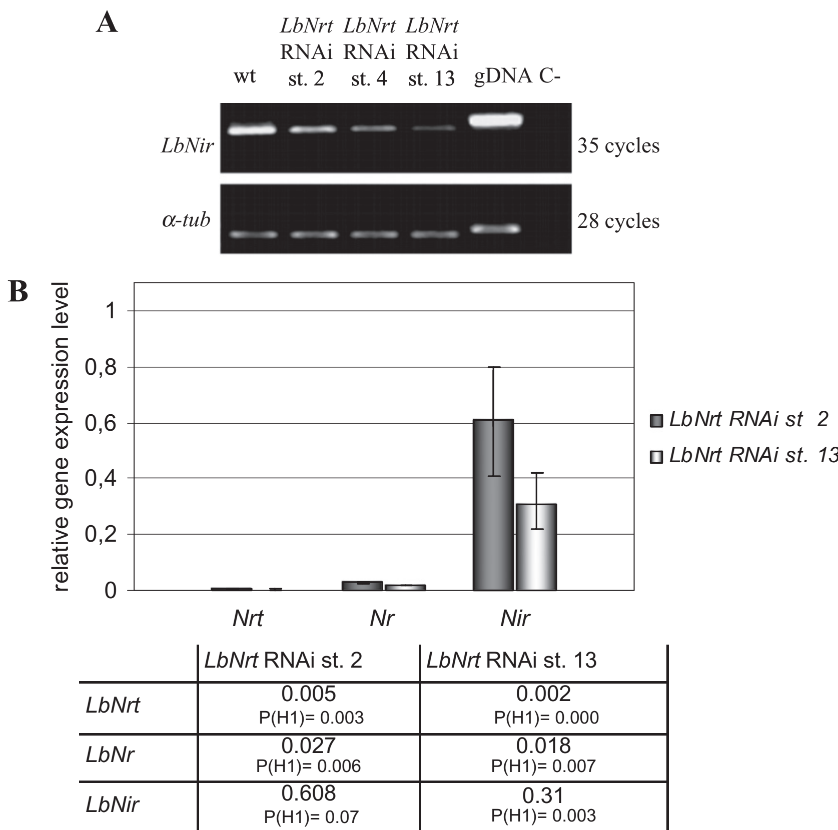


Fig. 3. A. RT-PCR assay showing the *LbNrt*, *LbNr* and *LbNir* transcript levels of nitrate-grown (4 mM N) wild type and strongly *LbNrt*-silenced strain 2 with respect to alpha tubulin (α-*tub*) and G-protein gamma subunit (Gγ) expression levels. gDNA, PCR amplicons produced from genomic DNA template; –C, no template PCR control.
B. Northern blot detection of the relative *LbNrt* and *LbNr* transcript levels in wild type and *LbNrt*-silenced strain 2 under variable nitrogen-feeding conditions. NH_4, ammonium (4 mM N); NO_3, nitrate (4 mM N); NH_4NO_3, simultaneous ammonium and nitrate feeding (8 mM N); Gln, L-glutamine (4 mM N); Glu, L-glutamate (4 mM N).

nitrate-grown *LbNrt*-RNAi strains by quantitative real-time PCR (Fig. 4B). Both of the studied strains had statistically very significant downregulation of both *LbNrt* and *LbNr* transcripts (0.2–0.5% and 1.8–2.7% respectively) when compared with the wild type fungus. Also the *LbNir* transcript status was clearly affected in the RNAi strains but showed some variation in its degree. In the *LbNrt*-silenced strain 13, in which the residual *LbNrt* transcript level was also minor, the silencing co-downregulation effect on *LbNir* was statistically significant and the strain had only 31% of the wild type expression level. On the other hand, in the slightly less *LbNrt*-silenced strain 2 the relative



	LbNrt RNAi st. 2	*LbNrt* RNAi st. 13
LbNrt	0.005 P(H1)= 0.003	0.002 P(H1)= 0.000
LbNr	0.027 P(H1)= 0.006	0.018 P(H1)= 0.007
LbNir	0.608 P(H1)= 0.07	0.31 P(H1)= 0.003

Fig. 4. A. RT-PCR of *LbNir* transcript levels in nitrate-grown (4 mM N) wild type and strongly *LbNrt*-silenced strains 2, 4 and 13 in respect to alpha tubulin (α-*tub*) expression. gDNA, PCR amplicons produced from genomic DNA template; C–, no template PCR control.
B. Relative expression of fHANT-AC genes in two *LbNrt*-silenced strains (2 and 13) in respect to nitrate-grown wild type fungus determined by qPCR. The transcript levels were normalized with two *Laccaria* endogenous reference genes, β-actin (JGI protein ID 192701) and elongation factor 1 (ID 6073790). Hypothesis test $P(H1) < 0.05$ indicates statistically significant difference between the sample and the control.

expression of the *LbNir* was 60%. This weaker silencing co-downregulation of *LbNir* compared with *LbNr* and its variability between the strains were already observed in semi-quantitative RT-PCR assays. When correlated with the qPCR calculated *LbNrt*-silencing strength levels indications for a dose effect can be observed (i.e. more complete silencing of *LbNrt* initiates more profound co-downregulation of *LbNir* explaining the variation observed between the RNAi strains).

The semi-quantitative RT-PCR and qPCR results were further confirmed for *LbNrt* and *LbNr* by Northern blotting twice (Fig. 3B). Most importantly, the *LbNrt* silencing-linked co-downregulation of the nitrate utilization pathway was not detected only in presence of nitrate but clearly also in absence of it. Minimal *LbNr* transcript levels were detected equally when the fungus was feeding on L-glutamine or L-glutamate even though these two organic N sources do not cause repression of *Laccaria* fHANT-AC. Identical results were obtained in nitrogen-free growth conditions as well (data not shown). The Northern blot data finally revealed that the detected co-downregulatory effects of *LbNrt* silencing on *LbNr* and *LbNir* could not be simply explained by reduced nitrate transporting activity of the *LbNrt*, and consequently by lower intracellular concentration of the substrate of the given metabolic pathway.

Discussion

The herein presented *LbNrt*-silenced *Laccaria* strains are the first nutrient transporter mutants of ECM fungi to date. The silencing gene targeting was shown to be highly efficient reducing the *LbNrt* mRNA level under fHANT-AC gene expression conditions to less than 1% of the wild type expression level. The gene silencing was also specific to nitrate metabolism as the biomass production on nitrate, but not on other nitrogen sources, such as ammonium and L-glutamine, was affected. The silencing-altered nitrate metabolism status of *Laccaria* had also direct effect on the symbiotic capacity with poplar under predominant nitrate-feeding conditions. This reconfirms our previous results obtained with *LbNr*-silenced strains (Kemppainen *et al*., 2009). The same lack of normal mantle development and Hartig net formation was observed with *LbNrt*-silenced strains when growing on nitrate and confronted with the host tree.

Our previous studies (Kemppainen *et al*., 2010) demonstrated that the nitrate metabolism pathway in *Laccaria* shows a novel nitrate-independent expression pattern (i.e. no induction, ammonium repression but not glutamine repression). Also the *LbNr*-silencing results proved that *Laccaria* nitrate reductase is not a key regulator of the two other nitrate pathway genes as wild type transcript levels of *LbNrt* and *LbNir* were detected in *LbNr*-silenced strains (Kemppainen *et al*., 2010, and results presented here). In the light of the previous data and especially considering the nitrate-independent expression pattern of *Laccaria* fHANT-AC genes, the detected co-downregulation effect of *LbNrt* silencing on *LbNr* and *LbNir* was unexpected. The reduced *LbNr* transcript levels were detected in *LbNrt*-silenced strains not only when the fungus was growing on nitrate but also in its absence.

RNA silencing, even though demonstrated to lead to very significant reduction of the target transcript level, in contrast to knockout mutants, does not lead to complete elimination of the target gene activity (as detected by RT-PCR, Northern blotting and qPCR for *LbNrt*). The basal *LbNrt* transcript levels were also detected under nitrate feeding and in accordance with that the *LbNrt*-silenced strains can grow on nitrate, even though this capacity is severely reduced (~ 10% of wild type biomass). If intracellular nitrate, taken up by the transporter, would influence the activation of fHANT-AC genes one would expect that at least when growing on nitrate the residual *LbNrt* expression would be enough for carrying out nitrate uptake sufficient for induction of the rest of the nitrate pathway genes. This is not the case and neither does the silencing-linked co-downregulation appear to be a result of a possible nitrogen starvation response in *LbNrt*-silenced strains as the same fHANT-AC transcript reduction was observed when the mutants were grown on a highly preferred, not fHANT-AC expression-repressive nitrogen source, such as L-glutamine. Therefore, our results reveal a nitrate-independent and direct role of the nitrate transporter protein in activation of other nitrate utilization genes. In *Laccaria* this happens under any fHANT-AC non-repressive growth condition (i.e. absence of ammonium) while in the nitrate induction-responsive fungus *H. polymorpha* apparently only during nitrogen starvation. Interestingly, all of these conditions represent potentially sole positive nitrogen regulator GATA factor-dependent gene activation modes. To our knowledge this is the first time that an altered fungal nitrate transporter expression has been linked to nitrate-independent regulation of other nitrate metabolism genes under non-nitrogen starvation growth conditions.

The information on basidiomycete nitrate assimilation and its control is minimal and to which extend data originating from ascomycete fungi are applicable to this group is not known. Therefore, how such nitrate-independent and transporter-linked control of fHANT-AC genes is exploited in *Laccaria* is at this point only speculative. LbNRT protein has a predicted structure of 12 transmembrane helices forming the membrane pore and a relative long cytosolic N-terminal tail with a putative signal anchor sequence. Similarly to other fungal nitrate transporters of the subfamily NRT2 II, LbNRT also carries a long cytosolic loop between the helices VI and VII. The presence of

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several posttranslational regulation-liked sequence motives in this region strongly suggests that protein level regulation of LbNRT may play a role in *Laccaria* nitrogen utilization control. Posttranslational modifications have been shown to be fundamental in control of several eukaryotic nitrate transporters. Transporter phosphorylation and ubiquitinylation has been demonstrated to play a central role in regulation of *H. polymorpha* YNT1 cellular localization (Navarro *et al*., 2006; Martín *et al*., 2011). Also in *Aspergillus* NR mutants the nitrate transporter mRNA and protein levels are significantly higher than in the wild type. Despite this elevated protein level, the nitrate uptake is severely compromised, and relative cell nitrate efflux is increased and the lack of uptake is probably controlled via posttranslational modification of the transporter protein (Wang *et al*., 2007).

Our findings strongly suggest that the unaffected cellular level of the *LbNrt* gene product is a crucial regulator of transcriptional activation of rest of the nitrate utilization pathway in *Laccaria*. No similar regulatory role has been reported for any other eukaryotic nitrate transporter under nitrogen-sufficient conditions this far. However, increasing amount of experimental data is now pointing towards a nitrate uptake-independent signal transducer role of *Arabidopsis* NRT2.1 protein (major HATS, NRT2 nitrate transporter family) as well (Malamy and Ryan, 2001; Little *et al*., 2005; Remans *et al*., 2006; Gojon *et al*., 2011). This signalling role of the transporter, which affects lateral root development, acts under low-nitrate conditions or in complete absence of nitrate varying its outcome in respect to carbon availability. The plasma membrane localization of this transporter and nitrate uptake requires also the partner protein NAR2.1 (Okamoto *et al*., 2006; Orsel *et al*., 2006). Both of these protein components are under posttranslational regulation even though the precise nature of it is not yet clear (Laugier *et al*., 2012). These findings on NRT2.1 closely resemble what is currently known from *H. polymorpha* YNT1 protein (NRT2 nitrate transporter family) regulation. Phosphorylation in the long cytosolic loop of this fungal transporter is required for its membrane sorting. This phosphorylation happens under carbon-sufficient conditions in presence of nitrate, but also in absence of it. Interestingly, membrane localization of YNT1 is required precisely for rapid induction of other nitrate utilization genes (Navarro *et al*., 2008; Martín *et al*., 2011). When joining the recent information from the two NRT2 class transporters, NRT2.1 from *Arabidopsis* and YNT1 from *H. polymorpha*, the picture of NTR2-type nitrate uptake-independent signalling role of these transporters starts to merge. In both of these eukaryotes correct membrane trafficking of the transporter protein under carbon-sufficient conditions and absence of a repressive nitrogen source are fundamental prerequirements for their nitrate uptake-independent signal transducer activities. The control of this membrane localization is carried out via either direct phosphorylation of the transporter and/or posttranslational modifications of its interactive proteins. Our results suggest that a threshold level of the *Laccaria* transporter is equally required for triggering the rest of the nitrate utilization pathway. Moreover, this regulatory role is independent of the presence of nitrate. Taking into account the novel data on nitrate uptake-independent signalling function of NRT2-type transporters, the co-downregulation effects of *LbNrt* silencing could be explained by action of a similar cellular mechanism. As non-inducible, capable of simultaneous uptake of nitrate and organic nitrogen and apparently only controlled by the presence/absence of the repressive nitrogen source ammonium, a predominant membrane localization of the LbNRT would be expected under carbon-sufficient and fHANT-AC non-repressive growth conditions. Also multiple posttranslational modification motifs in the protein suggest that besides regulated at transcriptional level, the membrane trafficking of the LbNRT could play a significant role in fHANT-AC fine-tune control. Therefore, we postulate that insufficient membrane allocation of the transporter due to efficient RNAi silencing could be behind initiating an intracellular LbNRT-linked but nitrate-independent signalling cascade. This would lead to significant reduction of *LbNr* and to a lesser extent of *LbNir* transcripts in the cell via either modifying the transcriptional activity and/or affecting the mRNA stability of these fHANT-AC genes. Such transporter level-dependent regulation may be important for flexible activation of the nitrate utilization pathway and consequently result in maximal cellular metabolization of nitrate under temporally and spatially highly variable nitrogen growth condition as is forest soil.

Since *L. bicolor* counts with only one nitrate transporter protein (versus multiple transporters in plants and two in *H. polymorpha* and *A. nidulans*) it offers a unique system for further studies on the regulatory role of this protein and nitrate metabolism in ECM fungi and in eukaryotes in general. Our results also strongly support the use of RNAi in manipulation of other fungal nutrient transporters, specially the ones predicted to be fundamental in nutrient transfer between the fungus and the host plant.

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Supporting information

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

Fig. S1. A. Schematic representation of *L. bicolor* fHANT-AC structure. *LbNr*, nitrate reductase; *LbNrt*, nitrate transporter; *LbNir*, nitrite reductase genes. White arrows between the *LbNrt* and *LbNir* refer to detected ORFs separating the fHANT-AC genes. ID numbers presented are *L. bicolor* genome v2.0 assembly JGI protein ID numbers.
B. A schematic representation of *LbNrt* 1902 bp coding sequence and its upstream region. The black bars represent the location of seven introns and the yellow bar the minimal promoter detected with NNPP version 2.2. The putative transcription start site, TSS (+1), is marked with an arrow. The red bars in the *LbNrt* upstream sequence represent sites with similarity to NIT2/AreA-type global positive acting GATA factor binding sites detected by TESS search. The binding sites of primers used for constructing the ihpRNA expression silencing vector for *LbNrt* (*LbNrt*-R/*LbNrt*-F), and the amplicons' sizes produced from both gDNA and mRNA are also indicated.

Fig. S2. Phylogenetic analysis of *Laccaria* nitrate transporter with a set of fungal, algal, higher plant NRT2 and bacterial NNP proteins. The aligning of the predicted polypeptides (ClustalW), the neighbour-joining analysis (using Poisson correction and 1000 bootstrap replicas) and the visualization of the generated phylogenetic trees were performed with the MEGA 4.0 software package. For sequence sources, see experimental procedures (Appendix S1).

Fig. S3. A. Secondary structure predictions of LbNRT by using TMHH2, TopPred and TMpred. The numbers (1–503) refer to amino acid positions and the roman numbers (I–XII) to predicted transmembrane α-helices. The numbers within the N- and C-terminal tails and the trans-helical loops in the scheme of LbNRT refer to the range of amino acids proposed to be involved in each structure by the three secondary structure detection programs used.
B. Amino acid sequence of the long cytosolic loop between the VI and VII transmembrane helices and the putative post-translational regulatory sequences in it. The amino acids in bold represent the conserved S/T-x-R/K protein kinase C recognition motives. The amino acids in bold with asterisks

mark the potentially phosphorylated serine and threonine residues detected by NetPhos2. The underlined amino acids represent a weak PEST sequence detected by PEST-find.

C. MFS I, MFS II, NNPI and NNPII sequence signatures in LbNRT. The numbers refer to amino acids in the protein. The fully conserved amino acids of MFS and NNP signature sequences are marked in bold and underlined. The amino acids not present in the consensus sequence or the potentially additional ones are presented with small letters. The absence of an amino acid is marked with a dash.

D. A 3D model of LbNRT, amino acids 20–499, obtained via homology modelling based on *E. coli* glycerol-3-phosphate transporter crystal structure at the SWISS-MODEL server. N- and C-terminal ends of the protein are marked in the model.

Fig. S4. A. RT-PCR assay showing the relative *LbNrt* and *LbNr* transcript levels of nitrate-grown wild type and two strongly *LbNr*-silenced strains, 21 and 30 (for the description of the strains, see Kemppainen and Pardo, 2010). gDNA, amplicons produced from genomic DNA template, C–, no template PCR control.

B. Northern blot detection and confirmation of the RT-PCR results for the unaffected *LbNrt* transcript status in the *LbNr*-silenced fungus.

Appendix S1. Experimental procedures.

Appendix S2. pHg/pSγ/L*bNrt*Loop T-DNA sequence (LB→RB 7680 bp).