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Expression analysis of *Clavata1*-like and *Nodulin21*-like genes from *Pinus sylvestris* during ectomycorrhiza formation

Gregory Heller · Karl Lundén · Roger D. Finlay · Frederick O. Asiegbu · Malin Elfstrand

Abstract The ecology and physiology of ectomycorrhizal (EcM) symbiosis with conifer trees are well documented. In comparison, however, very little is known about the molecular regulation of these associations. In an earlier study, we identified three EcM-regulated *Pinus* expressed sequence tags (EST), two of which were identified as homologous to the *Medicago truncatula* nodulin *MtN21*. The third EST was a homologue to the receptor-like kinase *Clavata1*. We have characterized the expression patterns of these genes and of auxin- and mycorrhiza-regulated genes after induction with indole-3-butyric acid in *Pinus sylvestris* and in a time course experiment during ectomycorrhizal initiation with the co-inoculation of 2,3,5-triiodobenzoic acid, an auxin transport inhibitor. Our results suggest that different *P. sylvestris* nodulin homologues are associated with diverse processes in the root. The results also suggest a potential role of the *Clv1-like* gene in lateral root initiation by the ectomycorrhizal fungus.

Keywords Auxin · IBA · TIBA · *Pinus sylvestris* · *Laccaria bicolor* · Ectomycorrhiza · Symbiosis · *MtN21* · *Clavata1*-like

G. Heller · K. Lundén · R. D. Finlay · M. Elfstrand (✉)
Uppsala BioCenter, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Box 7026, 75007 Uppsala, Sweden
e-mail: Malin.Elfstrand@slu.se

F. O. Asiegbu
Department of Forest Sciences, University of Helsinki, P.O. Box 27, 00014 Helsinki, Finland

Introduction

Symbiotic associations between tree roots and ectomycorrhizal fungi play important roles in boreal forests. Ectomycorrhizal fungi enhance the growth and fitness of trees by providing them with mineral and organic nutrients from the soil and by protecting the roots from pathogenic organisms in exchange for carbohydrates and organic compounds resulting from photosynthesis (Read 1991). In order to understand what characterizes the interaction between ectomycorrhizal (EcM) fungi and trees, a number of transcriptome studies has been carried out (Voiblet et al. 2001; Podila et al. 2002; Le Quere et al. 2005; Heller et al. 2008). Studies comparing the host responses to symbiotic, pathogenic, and saprotrophic interactions (Adomas et al. 2008) are also available. These studies have indicated that the number of mycorrhiza-specific genes in both partners may be relatively small. A characteristic of EcM interactions, which may explain these observations, is the morphogenetic differentiation in the plant root with the formation of a symbiotic organ in association with the fungal partner. As a result of colonization by EcM fungi, the tips of pine short roots may undergo dichotomous branching (Kaska et al. 1999). The molecular mechanisms regulating root development during interaction with the EcM fungus are still unclear (Felten et al. 2009, 2010). Phytohormones, especially auxins, play central role in EcM formation (Barker and Tagu 2000, and references therein; Jambois and Lapeyrie 2005; Felten et al. 2009). EcM fungi, e.g., *Laccaria bicolor* produce auxin (Ek et al. 1983) that can influence the root morphology and mycorrhization process (Gay et al. 1994). The interaction between auxin signaling and fungal volatile signals such as ethylene (Kaska et al. 1999; Splivallo et al. 2009; Felten et al. 2010) seems to be very important in this process.

In a previous study of the transcriptome in ectomycorrhizal development in *Pinus sylvestris* with *L. bicolor*, we have identified a set of expressed sequence tags (ESTs) representing Pine genes which were expressed at early stages in EcM formation (Heller et al. 2008). In the present study, we focused on three ESTs with a potential role in root morphogenesis. The first of these three ESTs showed similarity to *Clavata1* receptor-like kinase (RLK) genes of *Medicago truncatula* and *Arabidopsis* (*Clv1-like*). Several genes with homology to *Clavata1* RLK genes of *Arabidopsis* have been implicated in various types of organogenesis (Oka-Kira and Kawaguchi 2006; van Noorden et al. 2006). The remaining two are *MtN21-like-a* and *MtN21-like-b* clones showing similarity to an *MtN21* nodulin-like gene of *M. truncatula* and to *5NG4*, another member of the *Pinus* MtN21 gene family (Busov et al 2004). 5NG4 has been implicated in adventitious root formation (Busov et al 2004), suggesting that especially the *MtN21-like-a* and *MtN21-like-b* ESTs could be suitable marker genes for the onset of lateral root formation in the *P. sylvestris/L. bicolor* symbiosis. In the present work, we studied the expression of these candidate genes during rhizogenesis and EcM development. We also compared the transcription of the genes under indole-3-butyric acid (IBA) and auxin transport inhibitor treatments to elucidate the role of auxin and auxin signaling in their regulation.

Materials and methods

Sequences and phylogenetic analysis

The conserved regions in the amino acid sequences of Pt_MtN21-like (BG485740.1) and Pt_Clv1-like (BF517399.1) and orthologous protein sequences (Shiu et al. 2004; Schnabel et al. 2005; Morillo and Tax 2006) were aligned using ClustalW. Thereafter, neighbor-joining trees were constructed. All the sequences used in this study can be found in Electronic supplementary material S1. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al. 2007). Statistical support was estimated with a bootstrap analysis of 1,000 replicates.

Biological material

Four fungal isolates were used in the study: *L. bicolor* (Maire) Orton 238A (Finlay et al. 1992) and *Paxillus involutus* Maj (Gafur et al. 2004), which are capable of forming EcM *in vitro* with pine and birch, respectively, *Heterobasidion parviporum* FS6 (Karlsson et al. 2007), and *Phanerochete chrysosporium* RP78 (Stewart et al. 2000). *P. sylvestris* (Scots pine) seeds from Svenska

Skogsplantor (Saleby FP-45) were surface-sterilized with 33% hydrogen peroxide (H_2O_2) for 15 min, rinsed in several changes of distilled water, sown on 1% water agar Petri plates, and left to germinate under a photoperiod of 16 h light at a temperature of 18°C.

Preparation of the co-cultivation study

All fungi were cultivated on modified Melin-Norkrans (MMN) medium (Marx 1969) solidified with 1.5% agar. A 5-mm diameter agar plug was added to 15 mL of MMN medium in a 50-mL Falcon tube. After 14 days at 19°C, 14-day-old pine seedlings were transferred to tubes containing actively growing fungal colonies. Mock inoculations were prepared by transferring seedlings to 15 mL of MMN medium.

The co-cultivation experiment was incubated with a photoperiod of 16 h light at a temperature of 20°C. Three biological replicates were harvested at 0, 3, 6, 10 24, 48, and 72 h post initiation of the experiment (hpi), flash-frozen in liquid nitrogen, and stored at −80°C until processed. All manipulations in the co-cultivation experiment were performed under axenic conditions.

EcM, auxin treatment, and spatial analysis of gene expression in *P. sylvestris*

Two-week-old *P. sylvestris* seedlings were transferred under axenic conditions to a hydroponic box system and were grown for 14 days in MMN medium devoid of glucose. Systems were kept at 21°C under a 16-h photoperiod.

To quantify the basal expression of the candidate genes, 1-month-old seedlings were harvested with RNA-later (Ambion). To facilitate the infiltration of RNAlater into the tissues, a light vacuum was applied for 2 min; thereafter, seedlings were dissected in RNAlater and the fractions of root primordia, rot tips, hypocotyl, cotyledon, epicotyl, elongated root, intact shoot, and intact root were collected in two pooled representatives for each fraction.

For analysis of the effect of IBA on root morphogenesis and gene expression, *P. sylvestris* seedlings were treated with 100 μM IBA (Saveen Werner). Three biological replicates, each of 100 seedlings, were harvested after 2, 8, 16, and 24 hpi.

In the EcM experiment, EcM fungal inoculum (*L. bicolor*) was grown for 21 days in liquid Hagem medium (Stenlid 1985), washed with sterile-distilled water, and homogenized for 60 s in a sterile blender. The homogenized mycelium was then added to the box systems. For treatments with *L. bicolor* and the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA; Saveen Werner), 10 μM of TIBA was added to the box system in combination with

L. bicolor. Control plants were grown with sterile MMN medium alone. Three biological replicates, each of 100 seedlings, were harvested for each treatment at 1, 5, 15, and 30 days post inoculation (dpi), flash-frozen in liquid nitrogen at harvest, and stored at −80°C until use.

In order to follow the physiological changes occurring after treatment with IBA, *L. bicolor* and the combination of *L. bicolor* and TIBA, the numbers of lateral roots, and the size of the main root were recorded for 100 seedlings per treatment over 30 days spanning the experiment and pictures were taken throughout the course of the experiment.

qRT-PCR analysis of gene transcription

Total RNA was extracted according to the methods of Chang et al. (1993) but samples less than 100 mg were homogenized directly in the extraction buffer. The final pellet was dissolved in RNAse free water. One microgram of total RNA was digested with deoxyribonuclease I (Sigma-Aldrich). The RNA samples were reverse-transcribed with MMLV reverse transcriptase (Invitrogen) or the MMLV-derived enzyme iScript (Bio-Rad). All specific primer pairs were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Electronic supplementary material S2). The relative transcript abundance was detected using SYBR Green PCR Master Mix (Applied Biosystems) with 1 μL of template (concentration of template about 10 ng/μL) and 0.5 or 0.75 μM of each primer depending on the optimization results. The program used was as follows: initial denaturation for 1 min at 95°C, followed by a cycle of 15 s at 95°C, 1 min at 60°C, repeated 40 times with a 5 min final extension at 60°C. After the completion of the protocol, samples were subjected to a melting curve analysis.

Relative expression levels were calculated using REST software (Pfaffl et al. 2002; Vandesompele et al. 2002) using two internal reference genes: *alpha-tubulin* (Karlsson et al. 2007) and *elongation factor 1a* (Electronic supplementary material S2). The amount of product generated by qRT-PCR from control roots at each time point was used as a reference sample. In the spatial distribution experiment, the intact root and shoot were used as well to establish the relative expression levels among the fractions in the shoot and the root. The results were expressed as the fold change of messenger RNA level over the reference. The effect of time points and treatments within gene studies were tested by one-way ANOVA, and the results were confirmed with non-parametrical Wilcoxon test. The differences between the time points for every studied gene were determined by Tukey–Kramer test. The confidence level of all tests was set to 0.05. Data analysis was performed with STATISTICA 8.0 (StatSoft Inc. USA).

Results

In *P. sylvestris*, lateral roots emerge about 5 to 6 weeks following seed germination without the addition of any exogenous factor. We observed that 4-week-old seedlings inoculated for 15 days with a homogenate of *L. bicolor* displayed lateral root formation.

At 30 dpi, the roots treated with *L. bicolor*, *L. bicolor* and TIBA, or IBA displayed distinctive features. Extension growth of the main root axis was reduced in the presence of the EcM fungus, and the number of lateral roots increased compared to the control (Supplementary Material S4). At 30 dpi, these lateral roots displayed the typical dichotomous branching of EcM pine roots and possessed a mantle of fungal mycelium (Electronic supplementary material S4). When *L. bicolor* was inoculated together with the addition of 10 μM of TIBA, an auxin transport inhibitor, roots were shortened but no lateral roots could be observed even though the fungus was growing near the roots (Electronic supplementary material S4). *P. sylvestris* roots treated with IBA had excessive numbers of lateral roots, which were much shorter in length compared to control roots (Electronic supplementary material S4).

Expression of the auxin-induced genes *GH3* and *iaa88*

The effect of IBA on the transcription levels of the genes coding for *P. sylvestris* *GH3* and *iaa88* genes led to 18- and 5-fold changes, respectively, when compared to the basal expression. Although the transcription levels were elevated throughout the experiment, they reached a significant peak at 8 h of treatment ($p > 0.05$; Table 1). In the presence of the EcM fungus, the patterns observed were very different. The transcript levels for *GH3* were comparable to control at 5 dpi and decreased at 15 and 30 days ($p < 0.05$). Transcript levels for *iaa88* decreased initially at day 1, and then gradually increased until day 15 (Fig. 2), after which they dropped to levels comparative to 1 dpi. Treatment with TIBA led to a transient increase of *GH3* at day 1 of the interaction with *L. bicolor*, but did not affect the *iaa88* transcription pattern ($p > 0.05$; Fig. 2).

Expression of *Nodulin*-like genes (*MtN21-like-a/b* and *5NG4*)

The *Pinus taeda* *MtN21-like-a* and *MtN21-like-b* sequences showed a certain sequence overlap (seven amino acids, Supplementary Information S1 and S2) and they align within the same *P. taeda* EST contig (TC86303, DFCI Pinus Gene Indices; http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=pine). This indicates that they originated from the same *P. taeda* gene and consequently the *PtMtN21-like* gene is referred to as *MtN21-like-a/b* unless

Table 1 Relative gene expression of *Pinus sylvestris* roots treated with indole-3-butyric acid (IBA)

Gene	2 h Fold change	8 h Fold change	16 h Fold change	24 h Fold change
GH3	4.2 (0.02)a	17.7 (0.06)b	13.3 (0.05)c	9.1 (0.19)c
iaa88-like	2.3 (0.04)a	5.0 (0.18)b	4.7 (0.11)bc	2.5 (0.14)ac
MtN21-like-a	1.7 (0.02)a	1.1 (0.01)a	1.6 (0.01)a	0.3 (0.01)b
MtN21-like-b	1.6 (0.02)a	0.6 (0.01)b	0.8 (0.03)b	0.2 (0.01)c
5NG4	1.0 (0.12)a	1.6 (0.23)b	2.5 (0.28)c	1.4 (0.17)b
Clv1-like	1.6 (0.02)a	2.2 (0.00)b	2.9 (0.07)b	1.8 (0.01)ab

Transcript levels were assessed by qPCR at 2, 8, 16, and 24 h of 100 µM IBA treatment. The numbers represent the mean fold change of transcript levels compared to control roots. Values within brackets represent the standard deviation. Different letters indicate significant difference between the time points for each analyzed gene (Tukey–Kramer test, $p < 0.05$)

otherwise specified. The gene is a new member of the MtN21 gene family in *P. taeda*. Transcript levels of the *5NG4* and *MtN21-like-a/b* genes were higher in roots than the seedling as a whole in 1-month-old roots *P. sylvestris* seedlings (*MtN21-like-a/b* 6.6 times higher, $p = 0.05$; *5NG4* 3.2 times higher, $p = 0.066$). There is also a higher accumulation of *5NG4* and *MtN21-like-a/b* transcripts in regions with protruding root primordia (45- and 85-fold increase, respectively) compared to the intact plants. Co-cultivation with a 21-day-old *L. bicolor* colony led to a pronounced increase in *MtN21-like-a/b* transcript levels (about 28 times the control) after 24 h of contact with the colony ($p < 0.05$; Fig. 1). *P. involutus* induced a small increase in *MtN21-like-a/b* transcript levels at 12 and 24 hpi. In contrast, the orthologous gene with similarity to Pt*5NG4*, responded to all fungal isolates with elevated transcript levels at 12 hpi and throughout the experiment (Fig. 1).

In the presence of homogenized *L. bicolor*, the expression of the *MtN21-like-a/b* gene was repressed compared to mock-inoculated controls (Fig. 2). The addition of TIBA resulted in an induction of the *MtN21-like-a/b* gene at 5 and 30 dpi compared to the treatment with *L. bicolor* alone ($p < 0.05$). The patterns of gene expression observed for the *MtN21-like-a/b* and *5NG4* differed after treatment with the plant growth regulator IBA (Table 1). *MtN21-like-a/b* showed a small upregulation at 2 h, and at 24 h, a downregulation was seen while *5NG4* showed a 2.5 fold upregulation at 16 h ($p < 0.05$). Transcript levels of *5NG4* seem to increase after 5 days of co-cultivation with homogenized *L. bicolor* and decreased slightly at 15 and 30 dpi compared to the mock-inoculated control. Treatment with TIBA inhibits this regulation (Fig. 2).

Expression of the *Clavata-like* gene (*Clv1-like*)

The *Clv1-like* gene appears to be a member of the LRR-XI superfamily of RLKs in plants. However, the sequence was positioned basally with respect to angiosperm LRR-XI

(Electronic supplementary material S1). The gene was expressed at very low levels in *P. sylvestris*. In the experiment aiming to analyze the spatial distribution, the *Clv1-like* gene was close to the detection limit at about 10 copies at the start of the qPCR in all tissues except the root primordial, where 200–500 copies could be detected. The levels of *Clv1-like* gene expression remained under the
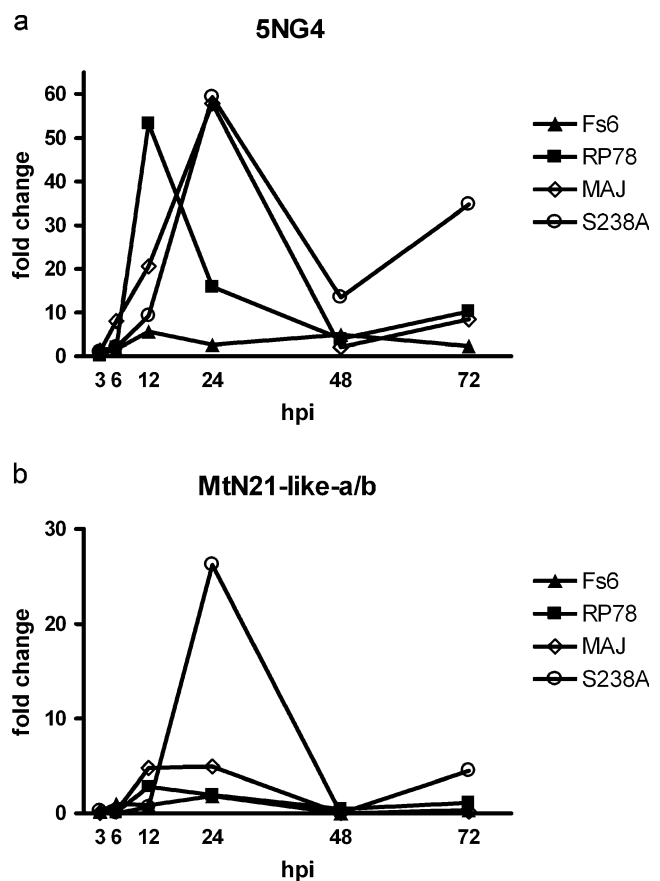

Fig. 1 Gene expression measured by qPCR of the **a** *5NG4* and **b** *MtN21-like-a/b* genes in *P. sylvestris* roots during the interaction with 21-day-old growing colonies of *H. annosum* (FS6, *triangles*), *P. chrysosporium* (RP78, *squares*), *P. involutus* (MAJ, *diamonds*), and with *L. bicolor* (S238A, *circles*)
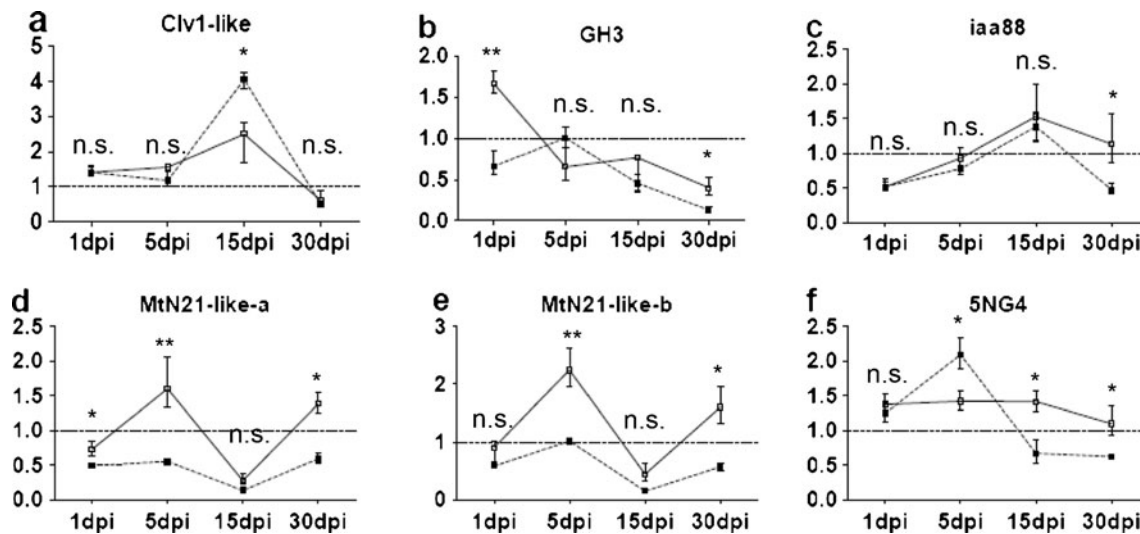
Fig. 2 Relative gene expression of **a** *Clv1-like*, **b** *GH3*, **c** *iaa88*, **d** *MtN21-like-a*, **e** *MtN21-like-b*, and **f** *5NG4* in *P. sylvestris* roots during interaction with the EcM fungus, *L. bicolor*. The relative fold change of the genes was assessed by qPCR at 1, 5, 15, and 30 days post inoculation in the presence (full connectors) or absence (dashed connectors) of 2,3,5-triibenzoic acid (TIBA). The average of fold changes of three biological replicates are presented for every time point, and *error bars* demonstrate standard errors. Statistical difference between the treatments within every time point is shown as follows: *n.s.* not significant, *$p < 0.05$, **$p < 0.01$

detection limit in the co-cultivation experiment (data not shown). Treatment with IBA led to a relative increase in transcript level of the *Clv1-like* gene compared to the control at all time points during the 24-h monitoring period ($p < 0.05$; Table 1). In the presence of *L. bicolor*, the transcript levels of the *Clv1-like* gene rose to about 4-fold compared to the control from 5 to 15 dpi (Fig. 2). Addition of TIBA led to attenuated transcript accumulation levels at 15 dpi ($p < 0.05$; Fig. 2).

Discussion

We studied the expression of the candidate genes during rhizogenesis and EcM development under normal conditions as well as the influence of the auxin transport inhibitor TIBA. The application of auxin transport inhibitors has been reported to lead to local increase in auxin concentration in plant tissues through a blockage of the PIN1 cycling (Geldner et al. 2001). It has been shown that *L. bicolor* can induce lateral root formation in *Populus tremula × Populus tremuloides* and *Arabidopsis thaliana* even without physical contact. This induction can be completely inhibited by the auxin transport inhibitor 1-naphthylthalamic acid, showing that polar auxin transport is involved in lateral root formation in the early *L. bicolor*–plant interaction (Felten et al. 2009). We also observed an almost complete inhibition of *L. bicolor*-induced lateral root formation in our material after treatment with TIBA (Electronic supplementary material S4).

MtN21-like-a/b was identified through its upregulation during EcM formation (Heller et al. 2008), and just like the *P. taeda* gene *5NG4*, it shared homology with the *nodulin 21* gene from *M. truncatula* (Gamas et al. 1996). The two genes have a similar expression pattern in the untreated plant, with higher basal expression levels in roots than in shoots. However, in our system, the *P. sylvestris* homologues *5NG4* and *MtN21-like-a/b* did not respond similarly to fungal contact and colonization (Figs. 1 and 2). The *5NG4* gene is associated with adventitious root formation in *P. taeda* (Busov et al. 2004). Accordingly, the *P. sylvestris* *5NG4* transcript accumulated in root primordia. The *P. taeda* *5NG4* expression is induced by auxin in roots, stems, and hypocotyls (Busov et al. 2004) and the *P. sylvestris* homologue also responded to IBA treatment. Our results seem to emphasize the association of *5NG4* with local increases of auxin levels in developing tissues and a possible role in (adventitious) root formation as the gene responded to colonization by *L. bicolor* at 5 dpi, but application of TIBA inhibited the accumulation of *5NG4* transcripts at 5 dpi. The *MtN21-a* and *MtN21-b* clones correspond to a previously not studied member of the MtN21 family in *Pinus*. It has recently been shown that MtN21 homologues encode members of a transmembrane drug/metabolite exporter family and that the *Arabidopsis* mutant *Walls are thin1* encoding an MtN21 exhibit defect cell elongation (Ranocha et al 2010). In *Pinus,* EcM colonization occurs primarily at short roots that have limited capacity to elongate, possibly because of peroxidase-mediated auxin degradation (Tarkka et al.

2001). We found that 24 h of IBA treatment and colonization with *L. bicolor* lead to lower steady-state levels of *MtN21-like-a/b* compared to untreated roots. Treatment with TIBA led to higher levels of *MtN21-like-a/b* expression compared to *L. bicolor* colonization alone. This indicates that inhibited auxin transport in roots may activate the expression of *MtN21-like-a/b* and that *5NG4* and *MtN21-like-a/b* are involved in different processes in the root, making *MtN21-like-a/b* and *5NG4* interesting candidates for more detailed functional and localization studies.

To validate the comparison of *5NG4* and *MtN21-like-a/b* expression patterns in response to *L. bicolor* and auxin treatment of *P. sylvestris* roots, the expression of two known auxin-regulated genes was also tested in our material. The upregulation of the auxin homeostasis gene *GH3* in *P. sylvestris* in the presence of IBA and the gradual down-regulation with *L. bicolor* colonization agree with the findings in *Pinus pinaster* where the transcript levels of *Pp-GH3.16* decreased gradually in the presence of the EcM fungus *Hebeloma cylindrosporum*, but increased in auxin treatments (Reddy et al. 2006). Our results confirm that the expression of plant genes associated with EcM formation is similar between closely related plant species. This is further underlined by the observation on the expression of *iaa88-like* genes in EcM colonization. We could detect expression of a *P. sylvestris iaa88-like* sequence with primers designed on the basis of two homologous genes, *Pp-iaa88* in *P. pinaster* and *PTIAA2* in *P. taeda* (Charvet-Candela et al. 2002; Goldfarb et al. 2003). The *Pp-iaa88* gene was reported to be highly expressed during fungal sheath formation (Charvet-Candela et al. 2002). Similarly, we found that the *P. sylvestris iaa88-like* sequence is upregulated at 15 dpi (Fig. 2), which also coincides with fungal sheath formation. TIBA application had no influence on the expression levels of *P. sylvestris iaa88-like* gene up to 15 dpi; however, it repressed the *P. sylvestris* gene significantly at 30 days, indicating that the induction of the *P. sylvestris iaa88-like* gene at 15 dpi is associated with the development of the symbiotic structures, supporting the concept of general EcM-associated transcription programs within the genus *Pinus*, possibly associated with organogenesis.

There are at least 38 ESTs with similarities to the LRR-XI superfamily of RLKs in the Pine EST databases. However, the expression pattern has been described for only one gene up to now (*Ps*RLK, Avila et al. 2006). The LRR-XI RLK superfamily includes the well-known *Clavata1* gene of *Arabidopsis* (Shiu and Bleecker 2001) and the *M. truncatula* SUNN gene involved in autoregulation of nodulation (Oka-Kira and Kawaguchi 2006). The *Clv1-like* amino acid sequence clusters basally among the LRR-XI RLK super-family (Electronic supplementary material Fig. 1). In

rhizobia symbiosis, SUNN controls the symbiosis, although it acts in the shoot (Oka-Kira and Kawaguchi 2006; van Noorden et al. 2006). On the basis of sequence similarity, it is tempting to suggest the possibility of an LRR-RLK-mediated autoregulatory mechanism in EcM formation as well, but the expression pattern of the *Clv1-like* gene suggests that the gene product is involved in other processes compared to other LRR-RLK members, which are active in aerial tissues. The *Clv1-like* gene showed higher expression levels in roots and root primordia compared to aerial tissues. The *Clv1-like* gene was induced by *L. bicolor* at 15 dpi in the EcM experiment, coinciding with lateral root and fungal sheath formation (Heller et al. 2008). The role of the *Clv1-like* gene in lateral root formation and fungal sheath formation deserves further exploration in future studies.

References

Adomas A, Heller G, Olson Å, Osborne J, Karlsson M, Nahalkova J, Van Zyl L, Sederoff R, Stenlid J, Finlay R, Asiegbu FO (2008) Comparative analysis of transcript abundance in *Pinus sylvestris* after challenge with a saprotrophic, pathogenic or mutualistic fungus. Tree Physiol 28:885–897

Avila C, Perez-Rodriguez J, Canovas FM (2006) Molecular characterization of a receptor kinasae gene from pine (*Pinus sylvestris* L.). Planta 224:12–19

Barker SJ, Tagu D (2000) The roles of auxins and cytokinins in mycorrhizal symbioses. J Plant Growth Regul 19:144–154

Busov VB, Johannes E, Whetten RW, Sederoff RR, Spiker SL, Lanz-Garcia C, Goldfarb B (2004) An auxin-inducible gene from loblolly pine (*Pinus taeda* L.) is differentially expressed in mature and juvenile-phase shoots and encodes a putative transmembrane protein. Planta 218:916–927

Chang S, Puryear J, Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. Plant Mol Biol Rep 11:113–116

Charvet-Candela V, Hitchin S, Ernst D, Sandermann H, Marmeisse R, Gay G (2002) Characterization of an Aux/IAA cDNA upregulated in *Pinus pinaster* roots in response to colonization by the ectomycorrhizal fungus *Hebeloma cylindrosporum*. New Phytol 154:769–777

Ek M, Ljungquist PO, Stenström E (1983) Indole-3-acetic acid production by mycorrhizal fungi determined by gas chromatography–mass spectrometry. New Phytol 94:401–407

Felten J, Kohler A, Morin E, Bhalerao RP, Palme K, Martin F, Ditengou FA, Legué V (2009) The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signalling. Plant Physiol 151:1991–2005

Felten J, Legué V, Ditengou FA (2010) Lateral root stimulation in the early interaction between *Arabidopsis thaliana* and the ectomycorrhizal fungus *Laccaria bicolor*. Is fungal auxin the trigger? Plant Signalling and Behavior 5(7):864–867

Finlay RD, Frostegård Å, Sonnerfelt A-M (1992) Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi of different successional stages grown in pure culture and in symbiosis with *Pinus contorta* (Dougl. ex Loud). New Phytol 120:105–115

Gamas P, De Carvalho NF, Lescure N, Cullimore JV (1996) Use of a subtractive hybridization approach to identify new *Medicago truncatula* genes induced during root nodule development. Mol Plant Microbe Interact 9:233–242

Gafur A, Schützendübel A, Langenfeld-Heyser R, Fritz E, Polle A (2004) Compatible and incompetent *Paxillus involutus* isolates for Ectomycorrhiza formation *in vitro* with poplar (*Populus × canescens*) Differ in H_2O_2. Plant Biol 6:91–99

Gay G, Normand L, Marmeisse R, Sotta B, Debaud JC (1994) Auxin overproducer mutants of *Hebeloma cylindrosporum* Romagnesi have increased mycorrhizal activity. New Phytol 128:645–657

Geldner N, Friml J, Stierhof Y-D, Jürgens G, Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature 413:425–428

Goldfarb B, Lanz-Garcia C, Lian Z, Whetten R (2003) Aux/IAA gene family is conserved in the gymnosperm, loblolly pine (*Pinus taeda* L.). Tree Physiol 23:1181–1192

Heller G, Adomas A, Li G, Osborne J, van Zyl L, Sederoff R, Finlay RD, Stenlid J, Asiegbu FO (2008) Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus *Laccaria bicolor*. BMC Plant Biol 8:19. doi:10.1186/1471-2229-8-19

Jambois A, Lapeyrie F (2005) Jasmonates, together with zeatin, induce hypaphorine accumulation by the ectomycorrhizal fungus *Pisolithus microcarpus*. Symbiosis 39:137–141

Karlsson M, Hietala AM, Kvaalen H, Solheim H, Olson Å, Stenlid J, Fossdal CG (2007) Quantification of host and pathogen DNA and RNA transcripts in the interaction of Norway spruce with *Heterobasidion annosum*. Physiol Mol Plant Pathol 70:99–109

Kaska DD, Myllyla R, Cooper JB (1999) Auxin transport inhibitors act through ethylene to regulate dichotomous branching of lateral root meristems in pine. New Phytol 142:49–58

Le Quere A, Wright DP, Soderstrom B, Tunlid A, Johansson T (2005) Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) fr. Mol Plant Microbe Interact 18:659–673

Marx D (1969) The influence of ectotrophic fungi on the resistance of pine roots to pathogenic infection. Phytopathology 59:153–163

Morillo SA, Tax FE (2006) Functional analysis of receptor-like kinases in monocots and dicots. Curr Opin Plant Biol 9:460–469

Oka-Kira E, Kawaguchi M (2006) Long-distance signaling to control root nodule number. Curr Opin Plant Biol 9:496–502

Pfaffl MW, Horgan GW, Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30:e36

Podila GK, Zheng J, Balasubramanian S, Sundaram S, Hiremath S, Brand JH, Hymes MJ (2002) Fungal gene expression in early symbiotic interactions between *Laccaria bicolor* and red pine. Plant and Soil 244:117–128

Ranocha P, Denancé N, Vanholme R, Freydier A, Martinez Y, Hoffmann L, Köhler L, Pouzet C, Renou JP, Sundberg B, Boerjan W, Goffner D (2010) *Walls are thin 1* (WAT1), an *Arabidopsis* homolog of *Medicago truncatula* NODULIN21, is a tonoplast-localized protein required for secondary wall formation in fibers. Plant J 63:469–483

Read DJ (1991) Mycorrhizas in ecosystems. Experientia 47:376–391

Reddy SM, Hitchin S, Melayah D, Pandey AK, Raffier C, Henderson J, Marmeisse R, Gay G (2006) The auxin-inducible GH3 homologue *Pp-GH3.16* is downregulated in *Pinus pinaster* root systems on ectomycorrhizal symbiosis establishment. New Phytol 170:391–400

Schnabel E, Journet EP, De Carvalho-Niebel F, Duc G, Frugoli J (2005) The *Medicago truncatula* SUNN gene encodes a *Clv1-like* leucine-rich repeat receptor kinase that regulates nodule number and root length. Plant Mol Biol 58:809–822

Shiu SH, Bleecker AB (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci U S A 98(19):10763–10768. doi:10.1073 /pnas.181141598

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KFX, Li WH (2004) Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. Plant Cell 16:1220–1234

Splivallo R, Fischer U, Göbel C, Feussner I, Karlovsky P (2009) Truffles regulate plant root morphogenesis via the production of auxin and ethylene. Plant Physiol 150:2018–2029

Stenlid J (1985) Population structure of *Heterobasidion parviporum* as determined by somatic incompatibility, sexual incompatibility and isozyme patterns. Can J Bot 63:2268–2273

Stewart P, Gaskell J, Cullen D (2000) A homokaryotic derivative of a *Phanerochaete chrysosporium* strain and its use in genomic analysis of repetitive elements. Appl Environ Microbiol 66:1629–1633

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tarkka MT, Nyman TA, Kalkkinen N, Raudaskoski M (2001) Scots pine expresses short-root-specific peroxidases during development. Eur J Biochem 268:86–92

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3(7):research0034.1–0034.11

van Noorden G, Ross JJ, Reid JB, Rolfe BG, Mathesius U (2006) Defective long-distance auxin transport regulation in the *Medicago truncatula* super numeric nodules mutant. Plant Physiol 140:1494–1506

Voiblet C, Duplessis S, Encelot N, Martin F (2001) Identification of symbiosis-regulated genes in *Eucalyptus globules–Pisolithus tinctorius* ectomycorrhiza by differential hybridization of arrayed cDNAs. Plant J 25:181–191