Transcription analysis of *Pinus sylvestris* during ectomycorrhizal development

Gregory Heller
Faculty of Natural Resources and Agricultural Sciences
Department of Forest Mycology and Pathology
Uppsala, Sweden

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Abstract

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Ectomycorrhizal associations of fungi with forest trees play an important role in the nutrition, growth and health of temperate and boreal forests. The ecology and physiology of ectomycorrhizal associations with *Pinus spp* are well documented but little is known about the molecular mechanisms behind these mutualistic interactions with gymnosperms compared to angiosperms. Through a complex and finely tuned molecular cross-talk between the two organisms, new lateral roots are initiated and new structures formed. The outcome is a symbiotic organ capable of exchanging nutrients essential for the survival of both partners. The main aim of this thesis was to identify the genes involved in the early stages of ectomycorrhizal development using *Pinus sylvestris* as a gymnosperm and *Laccaria bicolor* as the ectomycorrhizal fungus. Micro-array transcript profiling was used to ascertain the specificity in the response of conifer tissues to ectomycorrhizal fungi, using pathogenic (*Heterobasidion annosum*) and saprotrophic (*Trichoderma aureoviride*) fungi as non-mututalistic models. The profiles revealed differential responses of *P. sylvestris* roots to pathogenic, saprotrophic and ectomycorrhizal fungi within 24 h. We identified 236 differentially expressed genes potentially important for the initiation and regulation of biochemical, physiological and morphological changes during ectomycorrhizal establishment. The molecular regulation of ectomycorrhizal development appears largely comparable to that in angiosperm hosts, but differences exist in the timing and spatial scale of gene regulation. Of particular interest is the regulatory pattern of genes encoding stress related and also cell wall modification proteins whose down-regulation presumably enabled the development of the fungus within root tissues. Additionally, the expression and regulatory pattern of a subset of four symbiosis associated genes identified in the micro-array study was characterised in seedling roots; after exposure to exogenous auxin (indole-3-butyric acid) alone, *L. bicolor* alone, or *L. bicolor* in combination with the auxin transport inhibitor 2,3,5-triiodobenzoic acid. Knowledge of the genes regulated by auxin and involved in initiation of ectomycorrhizal symbiosis is scarce. The results identified a homolog to the receptor-like kinase *Clavata1-like* gene, two genes homologous to *nodulin-21* and a gene of unknown function named *Auxin-Induced1*. This study is the first attempt to characterize the plant transcriptome in the *Pinus sylvestris* - *Laccaria bicolor* model system.

Keywords: Pinus sylvestris, *Laccaria bicolor*, ectomycorrhiza, symbiosis, auxin, micro-array, qRT-PCR.

Author's address: Gregory Heller, Department of Forest Mycology and Pathology, SLU, P.O. Box 7026, SE-750 07 Uppsala, Sweden. E-mail: Gregory.Heller@mykopat.slu.se

Je dédie ce livre à ma mère bien aimée qui me manque énormément,
Brigitte Ducom Heller
(1945-2005)

Contents

Appendix

Papers I-III

The present thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Adomas A., Heller G., Olson Å., Osborne J., Karlsson M., Nahalkova J., van Zyl L., Sederoff R., Stenlid J., Finlay R.D. & Asiegbu F.O. (2008) Comparative analysis of transcript abundance in *Pinus sylvestris* roots after challenge with a saprotrophic, pathogenic or mutualistic fungus. *Tree Physiology*. In press.

II. Heller, G., Adomas, A., Li, G., Osborne, J., van Zyl, L., Sederoff, R., Finlay, R.D., Stenlid, J. & Asiegbu, F.O. (2008) Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus *Laccaria bicolor*. *BMC Plant Biology* 8:19.

III. Heller, G., Elfstrand, M., Asiegbu, F.O. & Finlay, R. Expression of symbiosis regulated genes after auxin treatment in *Pinus sylvestris* roots. Submitted.

Other articles not included in this thesis

Adomas, A., Heller, G., Li, G., Olson, A., Chu, T.-M., Osborne, J., Craig, D., van Zyl, L., Wolfinger, R., Sederoff, R., Dean, R.A., Stenlid, J., Finlay, R. & Asiegbu, F.O. (2007). Transcript profiling of a conifer pathosystem: response of Pinus sylvestris root tissues to pathogen (Heterobasidion annosum) invasion. *Tree Physiology* 27, 1441-1458.

Nygren, C.M.R., Edqvist, J., Elfstrand, M., Heller, G. & Taylor, A.F.S. (2007). Detection of extracellular protease activity in different species and genera of ectomycorrhizal fungi. *Mycorrhiza 17*, 241-248.

Introduction

The rhizosphere (from the Greek words "rhiza," meaning root and "sphere" field of action) represents the soil region subjected to the influence of plant roots. It is characterized by increased microbiological activity and is an area where complex biological interactions and ecological processes between symbiotic organisms occur (Bais *et al.*, 2006). The first definition of symbiosis was given 130 years ago by the German botanist Albert Bernhard Frank and was later expanded by Anton de Bary in 1887 as two different species that live together in close physical contact. However, this original definition encompasses in fact three kinds of interactions in nature: mutualistic, commensal or parasitic/pathogenic, depending on which partner benefits or does not benefit from the association. The environment in which plant roots develop represents a continuum from beneficial to potentially harmful associations. Therefore a key feature in biology of plant roots is the ability to recognize "friend from foe" (Staal & Dixelius, 2007; Singh *et al.*, 2004). This is necessary to respond adequately to surrounding microorganisms and develop associations that may increase fitness and/or even determine the survival of the plant.

Response of tree tissues to beneficial and non-beneficial interactions

Plants constantly monitor microbe associated molecular patterns (MAMPs). Typically MAMPs represent essential structures for microbial life (Altenbach & Robatzek, 2007) such as chitin and ergosterol from fungal cell walls and membranes or lipo-chito-ologosaccharides and flagellin from bacteria (Felix *et al.*, 1999; Melotto *et al.*, 2006; Meyer, Puhler & Niehaus, 2001; Zipfel *et al.*, 2006). It appears that chitin, lipo-chito-oligosaccharides and flagellin are recognized by a large number of plant families (Altenbach & Robatzek, 2007). Usually, plant responses to pathogenic infection consist of both constitutive and inducible defences (Lamb & Dixon, 1997; Pearce, 1996). The most commonly studied inducible defence is the hypersensitive response (HR). HR could play a central role during critical distinctions between pathogenic or beneficial microbes. Saprotrophic fungi, the common decomposers of dead organic matter, though not interacting directly with living plant tissues, may still play important roles e.g. in deterioration of natural products or in impeding the growth of pathogenic organisms and are seldom reported to cause disease. In contrast to pathogens and saprotrophs, beneficial mycorrhizal fungi form the mutualistic associations that are usually necessary for successful nutrient acquisition and growth (Smith & Read, 1997). Formation of mycorrhizal roots involves both structural and metabolic integration of the symbionts and modification of both plant and fungal patterns of gene expression. Usually, the plant shows a transient, uncoordinated, or weak defence response during both arbuscular mycorrhizal (AM) and ectomycorrhizal (ECM) colonization. Although the host responses have been shown to be weaker than towards a pathogen (Adomas *et al.*, 2007), the exact characteristic transcript profile of how these distinctions are made by the plant is still unknown.

Evolution and significance of mycorrhizal associations

Fossil evidence suggests that the associations between plant roots and soil fungi, also called mycorrhizal associations, existed more than 400 million years ago (Redecker, Kodner & Graham, 2000; Taylor *et al.*, 1995). This association is believed to be the oldest among terrestrial plants and probably originated at the early stage of land colonization by the first plant species (Fig.1). Consequently, both plant and fungal species have co-evolved to adapt to new environments. During the Carboniferous age (approximately 300 million years ago (mya)) fossil evidence shows the separation of angiosperms and gymnosperms from their common ancestor (Crane, Friis & Pedersen, 1995). It is also here that we find the oldest evidence of AM symbiosis on gymnosperm trees. During the Cretaceous age, in temperate and cold climates, trees developed associations with basidiomycetous and ascomyceteous fungi that can access nitrogen and phosphorus compounds in the soil organic matter through a wide range of enzymatic capabilities (Smith & Read, 1997; Selosse & Le Tacon, 1998) more efficiently than fungi forming AM symbiosis. The first fossil record of ECM association, the most characteristic association with plants in the Pinaceae, is 50 million years-old (LePage *et al.*, 1997), whereas sequence data based on the molecular clock concept suggest that the ectomycorrhizal habit may be over 100 million years old (Pirozynski & Malloch, 1975; Taylor & Osborn, 1996), coinciding with the appearance of the first members of the Pinaceae (Axelrod, 1986). Nowadays ECM symbiosis is the most prevalent symbiosis in temperate and boreal forests (Smith & Read, 1997). Although only 3% of all the vascular plant species on earth occupy these ecosytems, they cover a disproportionately large area and together form the largest terrestrial biome. The majority of woody plants with high economic value are dependent on ECM fungi. The artificial inoculation of tree roots with ECM fungi may improve the growth and resistance of tree seedlings in forest nurseries. This makes ECM research potentially important for practical forestry. The benefits gained from ECM symbiosis are numerous. The fungus increases plant growth by enabling better nitrogen and phosphate nutrition, increasing water supply and providing protection against pathogens. These functional properties originate from the special, intimate and complex structures observed in ECM root tips.

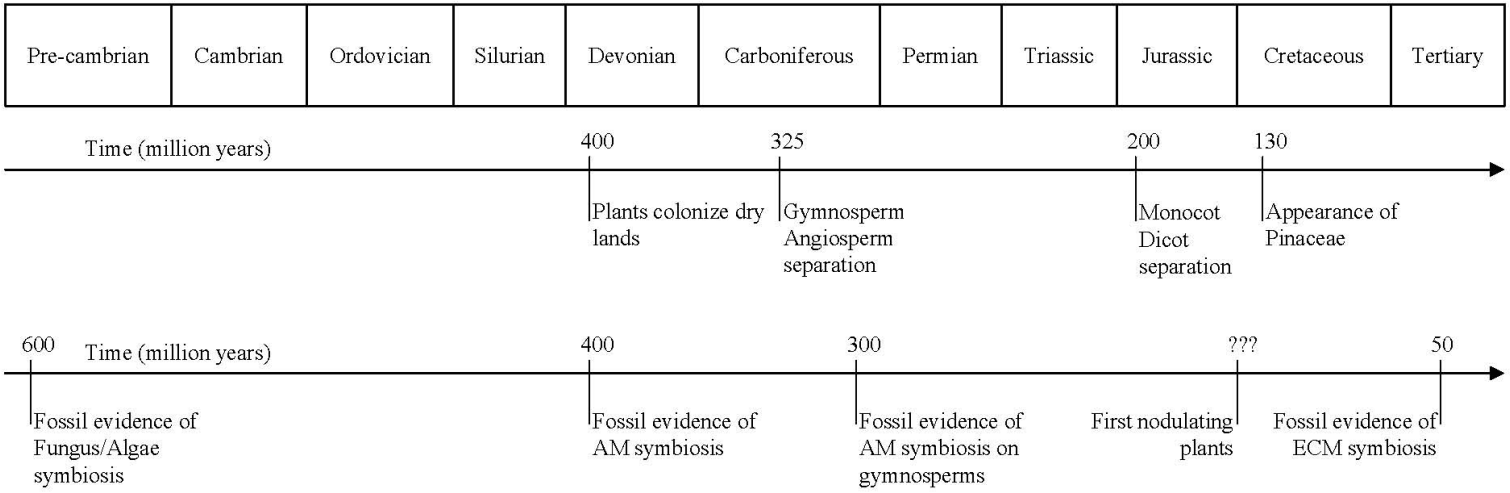

Fig 1. Evolutionary development of vascular plants together with fossil evidence of different types of symbiosis.

Morphology and function

The ECM fungal hyphae surround the entire surface of newly formed root tips and aggregate to form a multi-cellular sheath or mantle. In some cases this can represent 40% of the total weight of the rootlet. The fungal mantle functions as a protective barrier against environmental stresses e.g. drought, pollutants. It also acts as a physical barrier preventing pathogenic infections of the roots. As a physiological barrier it prevents from degrading toxins and enzymes secreted by the pathogens from degrading root tissues. The mantle is a connecting zone; hyphae can grow from the mantle inward between the epidermal and cortical cells, forming a fine network called the Hartig net; and outward to the surrounding soil, forming the extra-radical mycelium, which reaches resources in the soil beyond the rhizosphere. The ECM fungi can also produce antibiotic compounds and deplete the rhizosphere from the rich exudates usually released by the roots. This has the effect of significantly decreasing the free resources and suitable living conditions for pathogenic micro organisms. In addition, ECM fungi have also been shown to increase bacterial diversity and influence community structure (Johansson, Paul & Finlay, 2004)

Unlike AM fungi, ECM fungi do not normally penetrate the cell walls of their host. Hyphae forming the Hartig net can be observed between the cortical cells of the roots but not in endodermic tissues. Intercellular penetration induces profound morphogenetic changes in the root cells. Typically, hyphae from the mantle start growing in between the root cells of the root cap immediately behind the apex and then deeper between the epidermal cells. The epidermal cells undergo considerable radial enlargement as the colonization progresses in an acropetal direction. The surface contact between the two organisms is greatly increased, facilitating the exchange of nutrients and carbon between the root and fungus in the mature symbiosis. The root tips and associated mycelium represent important carbohydrate sinks for the tree and up to 18 times more photosynthetic products are relocated to the mycorrhizal roots compared to non mycorrhizal ones (Cairney, Ashford & Allaway, 1989). Most of the nutrients acquired by fungus are initially stored in the mantle (Lopez *et al.*, 2007). Some of these nutrients, which may originate from distant areas, are often transported through the extraradical mycelium to the root tip and exchanged against photosynthetic carbohydrates.

Molecular mechanisms and developmental stages

The cloning of several key genes in the signal transduction pathway leading to nodulation in *Medicago truncatula* (Ane *et al.*, 2004; Levy *et al.*, 2004) suggested strongly that the symbiotic bacterium *Sinorhizobium meliloti* may have recruited a pre-existing symbiosis pathway initially evolved in the context of AM symbiosis (Kistner *et al.*, 2005). It is reasonable to assume that ECM fungi may also share common signalling components with AM fungi although no proof of this has been shown. The molecular mechanisms involved in the pre-mycorrhizal stages like the adaptation of the plant roots to the forming mantle, the penetration of invasive hyphae between epidermal cells or the subsequent intercellular growth of the Hartig net are still unclear. To date, there are still only a limited number of large-

scale gene profiling experiments about symbiosis related genes in plant model systems such as *Betula pendula*, *Eucalyptus globulus*, *Quercus robur* and *Tilia platyphyllos* (Duplessis*, et al.*, 2005; Johansson*, et al.*, 2004; Kruger *et al.*, 2004; Le Quere*, et al.*, 2005; Polidori *et al.*, 2001; Voiblet*, et al.*, 2001). These studies show that the morphological and physiological changes observed throughout ECM development are concomitant with changes in gene expression in both partners and take place at the onset of physical interaction (Duplessis*, et al.*, 2005; Le Quere*, et al.*, 2005). The number of genes and the amplitude of their expression vary in time and seem to be important at the very early stages of development, soon after the initial contact.

In the pre-infection stage, the intracellular signalling in plant cells is triggered by G-proteins, which are responsible for effluxes of anions and cations causing membrane depolarizations, extracellular alkalinization, phosphorylation or dephosphorylation of unknown target proteins and finally the release of reactive oxygen species (Baptista *et al.*, 2007; Hebe, Hager & Salzer, 1999). Hydrogen peroxide (H_2O_2) has been shown to play a crucial role in the establishment of ECM symbiosis (Asiegbu, Johansson & Stenlid, 1999; Salzer, Hebe & Hager, 1997; Salzer *et al.*, 1996). Calcium spiking has been demonstrated to be an important intracellular signal involved in the initiation of plant-symbiotic gene programming (Harrison, 2005; Martin, Kohler & Duplessis, 2007) and different calcium binding proteins such as calmodulins and calreticulins have been identified in *Eucalyptus* and *Betula sp.* during the pre-infection stage. Profound cytoskeletal rearrangements and intense vacuolar activity have also been described (Duplessis*, et al.*, 2005; Le Quere*, et al.*, 2005).

After the fungal colonization of the root tip, morphological changes, such as loss of root hairs, start to take place in the tree roots. These typical traits involve a coordinated change in gene expression from both partners (Le Quere*, et al.*, 2005; Podila *et al.*, 2002; Slankis, 1973; Voiblet*, et al.*, 2001). Very little is known about the communication between the fungus and the plant during ECM formation. Several studies, however, report the production of plant hormones such as absissic acid, ethylene and auxin by ECM fungi (Barker & Tagu, 2000; Jambois & Lapeyrie, 2005; Scagel & Linderman, 1998). These phytohormones are known to act endogenously as signalling molecules for resistance mechanisms against microorganisms or for triggering developmental programs in plants. The role of auxin in symbiosis was investigated by Slankis (1973) in non-ectomycorrhizal pine roots, when treated with exogenous auxins, developed similarly to ECM roots. These results suggest that the plant, which maintains a hormonal balance within its tissues, triggers developmental programs when the concentration of auxin is changed. The use of auxin transport inhibitors (2,3,5-triiodobenzoic acid, TIBA) corroborated these findings but also suggested that other components are important in root formation (Karabaghli-Degron *et al.*, 1998; Niemi *et al.*, 2002; Rincon *et al.*, 2003). For instance, ethylene is shown to be able to regulate dichotomous branching in *Pinus sylvestris* (Kaska, Myllyla & Cooper, 1999). Modifying root morphology by promoting fine root development seems to be a strategy that appears recurrently in plant-microbe interactions. This is true for pathogenic relationships (A*grobacterium rhizogenesis*), neutral bacteria (Lopez-

Bucio *et al.*, 2007) to beneficial mycorrhizal symbioses (Olah *et al.*, 2005). Identifying symbiosis related genes involved in the formation of new organs will allow a better understanding of the fundamental mechanisms behind plant-microbe interactions.

The first cellular structures that undergo drastic changes during ECM formation are cell walls and extracellular matrices. Several studies show the transcription of genes coding for enzymes such as chitinases and proteases (Duplessis*, et al.*, 2005; Le Quere*, et al.*, 2005; Martin *et al.*, 1999) and suggest also the release of peroxidases into the extracellular interface in early stages of mycorrhizal development (Charvet-Candela *et al.*, 2002b). The presumed function of these enzymes is to modify plant and fungal cell wall structures but also to produce secondary signal molecules that are the by-products of their enzymatic activity. Cell walls are connected to plasma membranes where receptors are anchored. At this stage a large number of genes related to stress signalling, defence and cell rescue are found to be induced in different systems, notably pathogenesis related (PR) proteins. A typical gene expressed during this defensive response shared homology to the *Bet V1* allergenic protein (Duplessis*, et al.*, 2005; Le Quere*, et al.*, 2005; Martin *et al.*, 1999). Their expression is quickly modulated, probably by the fungus. Overcoming these general defence responses is a pre-requisite for the two next stages of ECM development in which the mycelium ensheaths the root during mantle formation. Following mantle formation, the hyphae penetrate between the epidermal and cortical cells, increasing the surface area of contact between the symbionts and enabling exchange of metabolites between the fungus and the root cells (Feugey*, et al.*, 1999). This process provokes a defence response with up-regulation of stress-related genes at the early stages of the interaction, probably to limit the fungal invasion of root tissues (Martin, Kohler & Duplessis, 2007). Many of these studies have been performed exclusively with angiosperm host plant species while very few have been carried out using gymnosperm hosts, in particular conifers.

Aims of the study

The aim of this study was to improve our knowledge on the molecular regulation of the early stages of ectomycorrhizal development by assessing gene expression and transcript profiling of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus *Laccaria bicolor* as experimental model. More specifically, we investigated the following:

- Comparative analysis of the transcript profiles of *P. sylvestris* roots when challenged with either ectomycorrhizal, pathogenic or saprotrophic fungi. **Paper I**
- Identification and molecular characterisation of genes involved in the regulation of ectomycorrhizal association in a conifer system. **Paper II**
- Molecular characterization of the role of auxin and auxin-inhibitors in different stages of root development and mycorrhiza synthesis. **Paper III**

Material and Methods

Plant material and fungal inoculum.

Scots pine (*P. sylvestris*) seeds (provenance Eksjö, Sweden) 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 hr light at a temperature of 21°C. The ECM fungal inoculum (*L. bicolor* (Maire) Orton, strain S238 (Crater Lake National Park, Oregon)) was obtained after 21 days of growth in liquid Hagem medium (Stenlid, 1985). The mycelium was washed with sterile, distilled water and homogenized for 60 seconds in a sterile blender.

Petri-plate system

For the inoculation, the 14 day old Scots pine seedlings were transferred under sterile conditions onto a wet filter paper on a new 1% water agar Petri plate (**Paper I** and **II**). The seedling roots were inoculated with 1mL of homogenized mycelium and covered with another wet, sterile filter paper (Fig. 2a). Control plants were inoculated with 1mL of sterile distilled water. Each Petri plate was sealed with parafilm and the lower half wrapped with an aluminium foil to protect the roots from light. This method is adapted from the paper sandwich technique designed for synthesis of ECM (Chilvers, Douglass & Lapeyrie, 1986). Prior to sampling the seedlings were then incubated with a photoperiod of 16 hr light at a temperature of 21^{o}C. After 1, 5 and 15 days post inoculation (d.p.i.), the roots were frozen in liquid nitrogen, ground and stored at -80°C. Three biological replicates (100 seedlings/replicate) were harvested for control and inoculated plants at each time point.

Hydroponic box system

In the study described in **Paper III,** Pine seedlings were grown in a new inoculation system that was developed to study root development under auxin treatment (Fig. 2b). This new system permitted the plants to reach a more advanced stage of ECM development more efficiently than the Petri plate system, and also maintained the axenic conditions necessary for molecular studies. The 14-day old Scots pine seedlings were transferred under sterile conditions into the small hydroponic culture boxes containing modified Melin-Norkrans medium (Marx, 1969). The biological material was obtained by growing pine seedlings under axenic conditions in liquid medium supplemented either i) with the auxin indole-3-butyric acid (IBA) alone, ii) with *L. bicolor* alone, or iii) with *L. bicolor* in combination with the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA). The inoculation was carried out with a final concentration in the hydroponic culture box of 100µM of IBA and 10µM of TIBA. The control plants were grown with only sterile medium. The time points studied in i) were at 2, 8, 16 and 24 hours post inoculation (h.p.i.); and for ii) and iii) were at 1, 5, 15 and 30 d.p.i.. Three biological replicates as above were harvested.

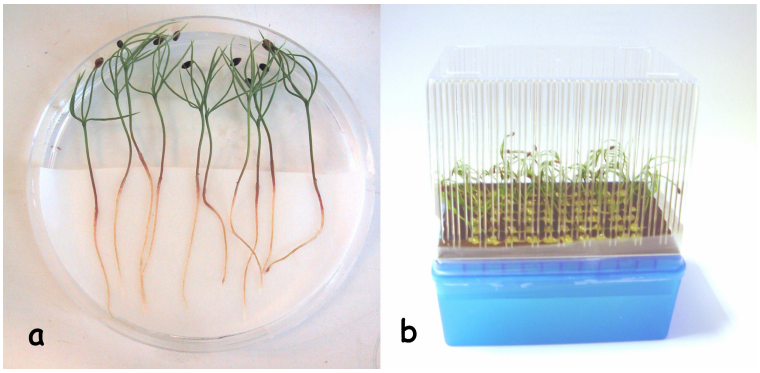

Fig. 2a-b. Petri plate system and Hydroponic box system with Scots pine seedlings growing in axenic conditions.

Reverse transcription and normalization

In studies **I**, **II** and **III**, the RNA samples were reverse transcribed with M-MLV reverse transcriptase (Invitrogen, Sweden) and as a positive control of the reverse transcription, 5000 copies of kanamycin mRNA (gene NTP11) (Promega) were added to the reaction mixture. The correction for reverse transcription reaction based on kanamycin amplification was included in the calculations. In studies **I** and **II**, the absolute quantity of the product in each sample was calculated from the standard curves and was normalized against the total amount of RNA (Hashimoto, Beadles-Bohling & Wiren, 2004). In study **III**, the results were normalized with two internal reference genes named beta-tubulin (Karlsson *et al.*, 2007) and elongation factor 1a.

Micro-array procedure

Micro-array expression analysis is the most widely used method for profiling mRNA expression at a large scale. DNA segments representing the collection of genes to be assayed are amplified by PCR and mechanically spotted at high density onto glass microscope slides using robotic systems, creating a micro-array containing thousands of elements. Micro-array technology assays differential gene expression by co-hybridization of fluorescently labelled probes prepared from different RNA sources. The kinetics of hybridization allows relative expression levels to be determined based on the ratio with which each probe hybridizes to an individual array element. Hybridization is assayed using a confocal laser scanner to measure fluorescence intensities, allowing simultaneous determination of the relative expression levels of all the genes represented in the array (Duggan *et al.*, 1999; Morrison, Weis & Wittwer, 1998).

The micro-arrays used in **Paper I** and **II** to assess the transcriptional changes in *P. sylvestris* roots challenged by the ECM fungus *L. bicolor* contained 2109 expressed sequence tags (ESTs) from *Pinus taeda*. The 2109 ESTs used for micro-array construction were obtained from six cDNA libraries of *P. taeda*. The DNA

from each EST was printed onto amino-silane coated slides in four replications for hybridizations performed at 1 d.p.i. and in two replications at 5 and 15 d.p.i. The experimental design involved pair wise comparisons between inoculated and control samples at each time point: 1, 5 or 15 d.p.i. Taking into consideration dye-swaps and technical replicates, each sample was hybridized six times and there were a total of 72 data points for each gene on the array at 1 d.p.i. and 36 at 5 and 15 d.p.i..

Micro-arrays are typically hybridized with cDNA prepared from two samples to be compared (in this case pine roots challenged by different fungi) and that are labelled with two different fluorophores. The fluorescent dyes used for our labelling were Cy3, which has a fluorescence emission wavelength of 570 nm (corresponding to the green part of the light spectrum), and Cy5 with a fluorescence emission wavelength of 670 nm (corresponding to the red part of the light spectrum). The two Cy-labelled cDNA samples are mixed and hybridized to a single micro-array that is then scanned in a micro-array scanner to visualize fluorescence of the two fluorophores after excitation with a laser beam of a defined wavelength. Relative intensities of each fluorophore may then be used in ratio-based analysis to identify up-regulated and down-regulated genes. In studies **I** and **II**, the cDNA generated from inoculated and control roots at each point was reciprocally labelled with Cy3 and Cy5-dUTP (Perkin Elmer, USA) using a Klenow method. Labelling, hybridization, and stringency washes followed the protocol from the Forest Biotechnology group, North Carolina State University, Raleigh (USA).

Although the arrays used in this study contain cDNAs from *P. taeda*, we hybridized the arrays with RNA harvested from the roots of its close relative *P. sylvestris*. The high degree of cross hybridization between *P. taeda* arrays and *P. sylvestris* has earlier been demonstrated by van Zyl *et al.* (2002). The number of studies using *P. taeda* micro-arrays proved these arrays to be a common and acknowledged tool for assessing differential gene expression in several species belonging to the Pinaceae (Brinker *et al.*, 2004; Stasolla *et al.*, 2003).

For probe preparation, total RNA was isolated from inoculated and control roots of *P. sylvestris* seedlings. The cDNA was synthesised from the same amount of RNA (1 µg) using SMART™ PCR cDNA synthesis kit (Clontech, USA). In our micro-array studies, it was necessary, for technical reasons, to use SMART™ PCR to amplify all the RNA samples isolated from *P. sylvestris*. This method is efficient for amplifying RNA exponentially but this non linear amplification could result in a target in which sequence representation is skewed when compared to the original mRNA pool (Puskas *et al.*, 2002; Wadenback *et al.*, 2005). This might have been the case in our studies where fold changes indicated by the array data were generally lower than those revealed by qRT-PCR. Nevertheless, the stringency and power of the statistical methodology employed for the 2-mixed model analysis (Wolfinger *et al.*, 2001) allowed us to detect statistically significant changes in transcript abundance with fold changes as low as 1.3 in **Paper I**. In **Paper II**, the genes were considered to be differentially expressed if

the following criteria: i) an average fold change of 1.4 in two out of three biological replicates or in all three biological replicates ii) at a p value <0.01.

The data discussed in **Paper I** and **II** have also been deposited at NCBI Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO platform GPL4039, series accession numbers: GSE5407, GSE5408 and GSE5410.

qRT-PCR analysis of gene transcription

In studies **I** and **II** a subset of genes was used to verify the micro-array results. In study **III** the expression of an additional subset chosen due to their functional relevance was followed after an auxin induction and also at different stages of ECM development.

The principle for quantitative PCR, also called real time PCR, is based on the ability to monitor the quantity of DNA present in a sample at each cycle of amplification throughout the reaction and not only at the end of the reaction as in conventional PCR. Every PCR reaction occurs in three phases, first a stationary phase where DNA is amplified slowly because the template available for PCR is only present in limited amounts, second an exponential phase without limiting factor and third a plateau that is reached when the reagents in solution run out (e.g. no more free dNTP available). The exponential increase of the product is used to determine the threshold cycle, CT, i.e. the number of PCR cycles at which a significant exponential increase in fluorescence is detected, and which is directly correlated with the number of copies of DNA template present in the reaction. Several strategies exist to measure the amount of amplicons formed at each cycle during the exponential phase. In our experiment we used the fluorescent dye Sybr green that binds only to double stranded DNA. However molecular beacons and Taqman probes, that are sequence specific, also exist. Their fluorescence appears when their corresponding quencher is no longer in proximity due to the PCR enzymatic reaction (Arya *et al.*, 2005; Nolan, Hands & Bustin, 2006; Wong & Medrano, 2005). In **Paper III**, the different CT values obtained for the genes of interest in our treated and control samples were used in the equations in the Relative Expression Software Tool (REST) (Pfaffl, Horgan & Dempfle, 2002; Vandesompele *et al.*, 2002). The transcript levels were calculated using the Pfaffl method (Pfaffl, 2001) based on the calculations described below:

$$\text{ratio} = (E_{\text{target}})^{\Delta \text{CT target (control-sample)}} / (E_{\text{reference}})^{\Delta \text{ CT reference (control-sample)}}$$

In this equation "*ratio"* is the relative expression between control and sample; E the efficiency of the reaction; and the ratio is the relative expression of the gene of interest normalized against the relative expression of a reference gene.

Results and Discussion

Comparative transcript profiling of *Pinus sylvestris* roots response to fungi with different living modes

In study **I** *P. sylvestris* was challenged with three fungi with different trophic strategies in order to investigate functional differences in the recognition and response mechanisms of conifer roots to fungi at 1, 5 and 15 d.p.i. In the study, the ECM fungus *L. bicolor*, the saprotrophic fungus *Trichoderma aureoviride* and the pathogenic fungus *Heterobasidion annosum* were used as models. Mycorrhizal symbioses have been alternatively viewed as stable derivatives of ancestral antagonistic interactions or as inherently unstable, reciprocal parasitisms. Recent phylogenetic analyses of free living and mycorrhizal homobasidiomycetes (Hibbett, Gilbert & Donoghue, 2000) suggest that mycorrhizal symbionts have evolved repeatedly from saprotrophic precursors and vice versa. Moreover, there may also have been multiple reversals to a free living condition, supporting the view that mycorrhiza are unstable, evolutionarily dynamic associations. In this context it is therefore interesting to know whether *P. sylvestris* responds to a symbiotic fungus fundamentally differently to a saprotrophic fungus, or how similarly Scots pine roots respond to a pathogenic fungus compared with their reaction to symbiotic or saprotrophic fungi.

Using threshold fold values of +1.3 and -1.3, the number of genes differentially expressed by pine roots in response to challenge with the different fungi varied. The results show that for the pathogen and the ECM symbiont the number differentially expressed genes were overall similar to each other (*ca* 18% and 22% of the genes on the array) whereas for the saprotrophic fungus the number of genes was considerably lower (3% of the genes on the array). In Figures 4 and 5 the gene expression is represented by functional categories as a percentage of all the genes belonging to the given category present on the array. At 1 d.p.i. the most prevalent genes up-regulated in response to the mycorrhizal fungus belonged to the functional categories of protein with binding function and protein activity regulation (Fig. 4a). The highest number of genes down-regulated in response to the mycorrhizal fungus was related to protein activity regulation (Fig. 5b). The pathogen infection at 1 d.p.i. led to up-regulation of genes related to signal transduction and down-regulation of those responsible for cell cycle and DNA processing (Fig. 4a and 5a). At 5 d.p.i. the interaction involving *L. bicolor* resulted mostly in an increase of the transcript levels of genes belonging to the functional class of proteins with binding functions, cell fate and energy acquisition. The genes down-regulated in response to *L. bicolor* at 5 d.p.i. were mostly related to protein activity regulation (Fig. 5b). Similarly, pine roots responded to the pathogen at 5 d.p.i. with increased transcription of genes with functions important in metabolism and regulation of interaction with environment (Fig. 4b). Two of the most abundant groups of transcripts that decreased after pathogen challenge coded for proteins related to regulation of interaction with environment and protein activity regulation (Fig. 5b).

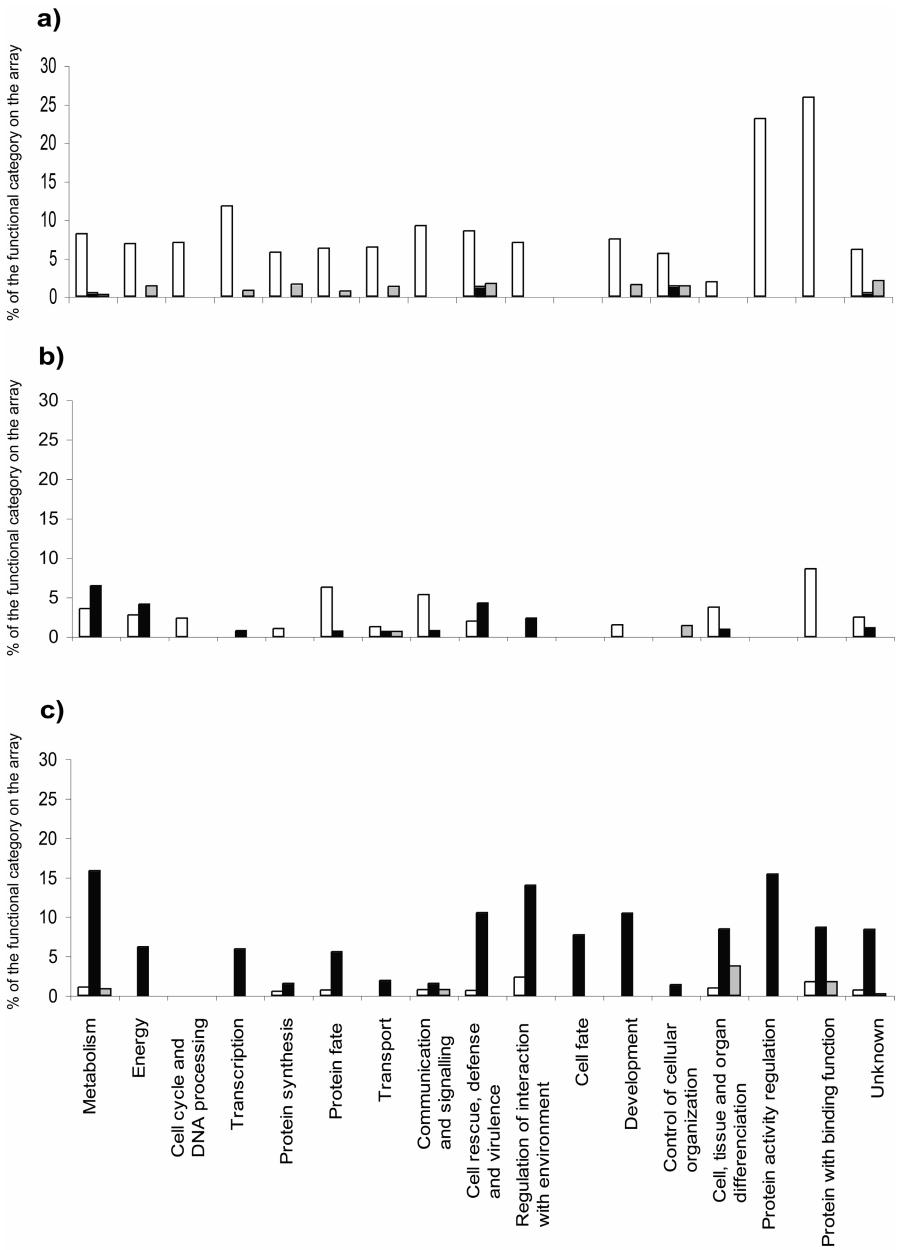

Fig. 4. The percentage of genes in different functional categories on the array up-regulated by *Pinus sylvestris* in response to challenge with an ectomycorrhizal symbiont (*Laccaria bicolor*) [white], a pathogen (*Heterobasidion annosum*) [black] or a saprotroph (*Trichoderma aureoviride*) [grey] at a) 1; b) 5 and c) 15 d.p.i

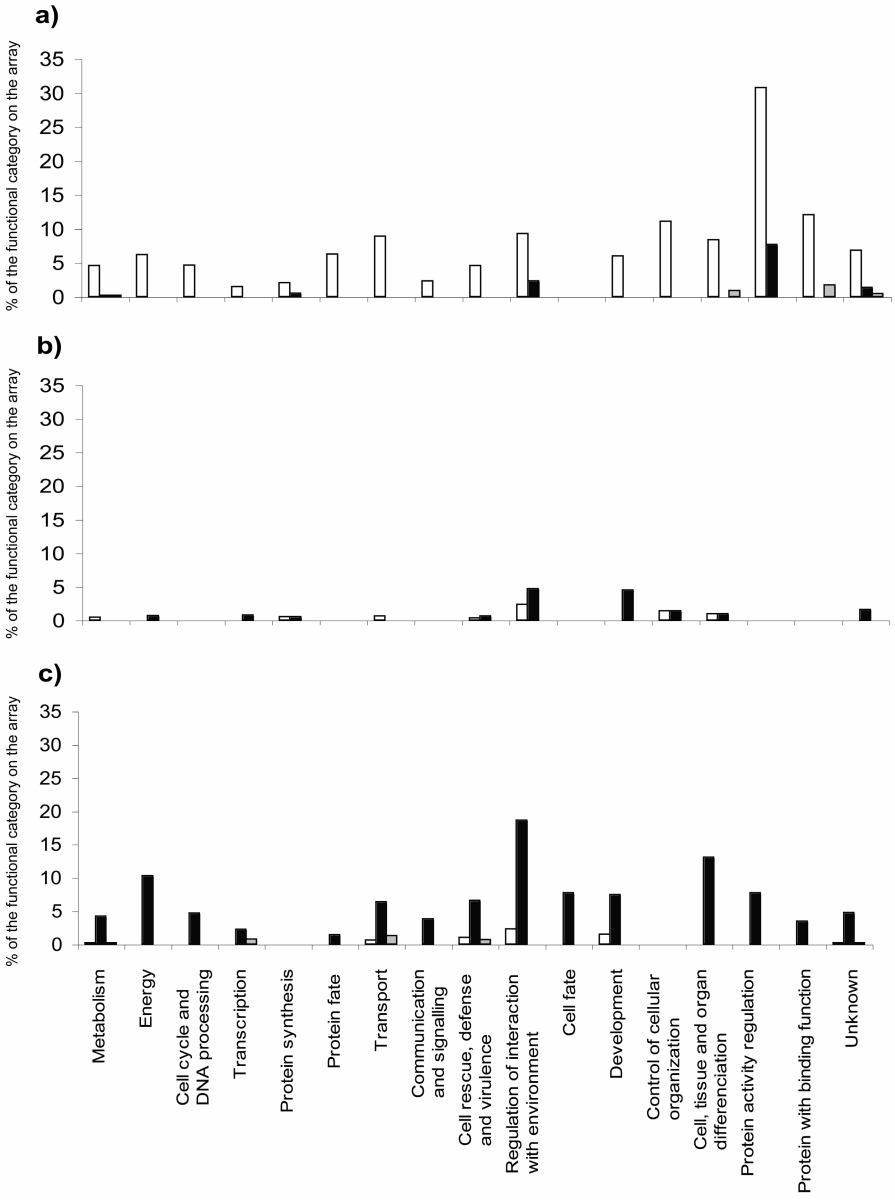

Fig. 5. The percentage of genes in different functional categories on the array down-regulated by *Pinus sylvestris* in response to challenge with an ectomycorrhizal symbiont (*Laccaria bicolor*) [white], a pathogen (*Heterobasidion annosum*) [black] or a saprotroph (*Trichoderma aureoviride*) [grey] at a) 1; b) 5 and c) 15 d.p.i..

At 15 d.p.i. the *P. sylvestris* response was specific only to the pathogen; mycorrhiza did not cause a significant change in the gene expression (Fig. 4c and 5c). Among the genes with transcript levels that increased after *H. annosum* infection, most belonged to the functional groups associated with metabolism, regulation of interaction with environment, protein activity regulation, and development (Fig. 4c). Most of the genes down-regulated in response to the pathogen were related to regulation of interaction with environment, protein activity regulation and energy (Fig. 5c).

Of the 2109 genes represented on the array, there was only one single gene expressed in common between all three treatments at just 1 d.p.i. (nuclear RNA binding protein). There were very few genes with common expression patterns between two treatments at various time points. At 1 d.p.i. seven genes were found to be differentially regulated in the same manner in roots exposed to the pathogen or the saprotroph (e.g. antimicrobial peptide and thaumatin) and at 15 d.p.i. only two genes were downregulated in the same manner (two non-specific lipid transfer proteins). Six genes were differentially expressed in the same way in response to the ECM fungus and the saprotroph at 1 d.p.i. (e.g. catalase and zinc finger proteins), three genes at 5 d.p.i. (e.g. pectate lyase and two at 15 d.p.i. (calcium binding protein and glycine-rich protein homolog) in *P. sylvestris* roots. The overlap in response to the pathogenic fungus and the ECM fungus in pine roots was more evident. At 1 d.p.i. there were two genes up-regulated (hypothetical proteins) and eight genes down-regulated (tonoplast intrinsic protein, gibberelin regulated protein and unknown proteins); at 5 d.p.i. one gene (biphosphoglycerate independent phosphotase) was up-regulated and twelve down-regulated (e.g. aquaporin, chitinase); and at 15 d.p.i. six genes presented increased transcript levels (e.g. peroxidase, heat shock protein 70, early response to drought), and three genes presented decreased transcript levels (aquaporin, abscisic acid/water stress/ripening inducible and unknown protein).

In spite of their suggested common evolutionary origin (Hibbett, Gilbert & Donoghue, 2000), the saprotrophic and ECM symbiotic interactions studied in our study displayed very little overlap in terms of differential gene expression. Furthermore, there were nine genes regulated in the same way after challenge with the pathogen or the saprotroph; interestingly, three of them had defence related functions. In response to both the pathogen and the ECM fungus, one of the genes upregulated by pine roots coded for peroxidase. Peroxidases have been associated with plant defence and resistance, particularly with lignin and suberin synthesis, but also with crosslinking phenolic compounds into papillae and production of toxic compounds (Fossdal *et al.*, 2003). Peroxidases have also been shown to be transiently upregulated in the mycorrhizal associations (Blilou*, et al.*, 2000; Munzenberger*, et al.*, 1997; Spanu & Bonfantefasolo, 1988).

The results also show that despite a general defence reaction occurring in the presence of all three fungi, the genes involved with root responses to the three different fungi were mostly different (**Paper I, II** and Adomas *et al.*, 2007). The results suggest that there are specific transcriptional responses of conifer trees to colonisation by either mutualists, saprotrophs or pathogens. The genes specific for

only one kind of the interaction may be vital for mutualism or disease resistance as opposed to the responses that were shared between pathogen, saprotroph or mutualist that could possibly play a role in compatibility.

Transcriptional analysis of *Pinus sylvestris* roots challenged with the ectomycorrhizal fungus *Laccaria bicolor*

The genetic programmes regulating ECM formation have so far only been studied in angiosperms. Both from an economic and an ecological perspective, gymnosperms are the major tree species in boreal forest ecosystems and understanding the molecular basis of mutualistic associations that are beneficial for their growth is an important research priority. In **Paper II**, we used an *in vitro* system to conduct transcript profiling at different stages of mycorrhizal development of *P. sylvestris* with *L. bicolor* involving hyphal associations with both primary and lateral roots.

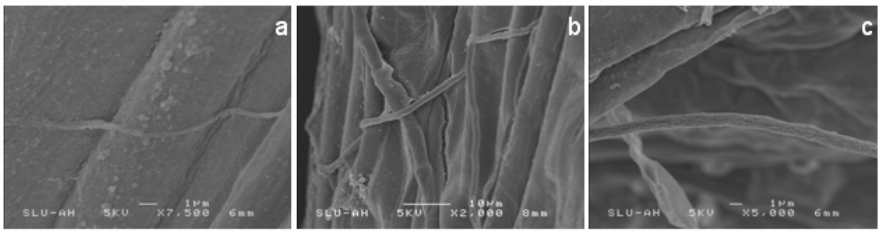

Fig. 6. Scanning Electron Microscopy of the *Pinus sylvestris – Laccaria bicolor system*
SEM pictures of *P. sylvestris* roots interacting with the ectomycorrhizal fungus *L. bicolor* after 1 day (a), 5 days (b) and 15 days (c).

Active adhesion of the homogenized *L. bicolor* hyphae to the Scots pine roots was visible within a few hours post inoculation. At 24 h.p.i. the adhering hyphae (Fig. 6a) did not show any growth, but at 5 and 15 days p.i. active growth of the hyphae (Fig. 6b-c) was observed, indicating recovery from homogenization. Despite active growth of the hyphae and increasing physical contact of the hyphae with the pine root, no statistically significant differences in numbers of living cells were detected between the control and mycorrhizal roots at 5 or 15 d.p.i.. This suggests that the normal physiology and active meristematic cell division was not perturbed due to the presence of the ECM fungus. At 15 d.p.i., emergence of several lateral roots was observed, accompanied by extensive colonization by the ECM fungus (Fig. 7a-b).

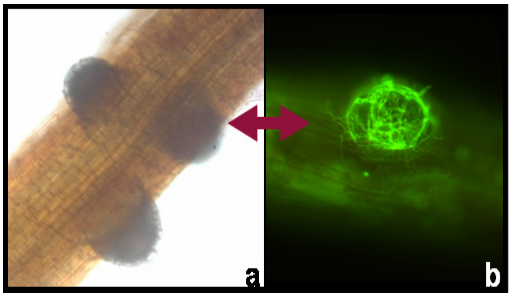

Fig. 7. Light and fluorescence micrographs of *Pinus sylvestris* emerging lateral root. a) Light microscopy of emerging *Pinus sylvestris* lateral root at 15 d.p.i. under normal light. b) Fluorescence micrograph of the same lateral root stained with acridine orange dye under UV light. *Laccaria bicolor* mycelium is dense and active around this new lateral root.

In this study, 236 transcripts were found to be differentially abundant during the early stages of ECM development. The numbers of differentially abundant transcripts increased during the initial phase of the interaction with a decline at 15 days, coinciding with the first stages of physical contact and lateral root formation respectively. Such periodical and transient changes in gene expression indicate a non-specific nature of host response during the early stages of interaction. The down regulation of several defence and cell wall related genes at later stages of mycorrhiza development could represent an attempt by the host to accommodate the symbiont. Interestingly, many of the initial changes in the transcript profile during the early stages of interaction represent transcripts involved in metabolism, cell rescue/stress related responses and protein fate. The over representation of transcripts belonging to the functional categories metabolism, cell rescue/stress related responses and protein fate are in line with data reported in other studies using *B. pendula* (Johansson *et al.*, 2004) or *E. globulus* (Duplessis *et al.*, 2005; Voiblet *et al.*, 2001). On the other hand, some genes commonly documented in these studies of mycorrhiza development, such as those encoding metallothioneins (Duplessis *et al.*, 2005) and chitinases (Johansson *et al.*, 2004; Le Quere*, et al.*, 2005) were less frequently represented in our system. These represent broad spectrum responses to the presence of the ECM fungus and are believed to be expressed in order to limit invasive growth of the symbiont (Feugey*, et al.*, 1999). Evidence for fatty acid change or transport is a striking feature of the early stages of interactions between *P. sylvestris* and the ECM fungus *Pisolithus tinctorius* (Laczko, Boller & Wiemken, 2004) but was missing in the present study. These differences may partly reflect the numbers of gene families within these groups that are present on the array used in this study.

In addition, differences between our results and those reported by other authors may be related to the different tree species, the experimental microcosm conditions (including the methods of inoculation) and different temporal programmes required for functional ECM development in the various tissues. For example in the *Betula pendula – Paxillus involutus* system, the mantle starts to form within two days and by eight days the Hartig net is already visible in cross sections of the root tip and active mycorrhiza are formed after 21 days (Johansson*, et al.*, 2004). This contrasts with our system where colonization of lateral roots was visible within 15 days but intercellular growth within the cortex was first observed at 30 days. A common feature described in all systems, also documented in our study, is the transient expression of diverse range of stress-related genes during the

interaction process. The cyclical changes in expression of genes encoding enzymes involved in cell wall modification suggests that they play a significant role in the control of hyphal penetration inside the roots.

About twenty ESTs spanning different functional groups were used for further transcript profiling analysis in our system during the later stages of mycorrhiza development. A number of interesting patterns emerged from abundant changes in a number of ESTs encoding proteins involved in cell wall development or cell rescue, defence and stress related functions.

One interesting feature is the regulation pattern of the transcript encoding an antimicrobial peptide. The transcript was increased during initial contact of *L. bicolor* hyphae with pine roots at 1 d.p.i. but decreased at the other time-points. At 30 d.p.i. the antimicrobial peptide (AMP) transcript abundance was again strongly decreased. The initial increase suggests a non-specific response by host tissue but it is possible that upon recognition of this fungus as a beneficial partner the gene is turned off. In pathogenic systems, over-expression of an AMP has been shown to reduce invasive growth by fungal pathogens (Kazan *et al.*, 2002). In the present study down-regulation of the gene encoding AMP following recognition of the symbiotic fungus *L. bicolor* may be a prerequisite for the penetration of the hyphae and subsequent accommodation of the invading hyphae within the pine root tissues.

Other ESTs within the above category code for PR10 and PR5 and have similar profiles on the array as the AMP. They are both slightly increased at 1 d.p.i. and then decreased at 5 and 15 d.p.i. with abundant changes at 30 d.p.i.. PR10, which has homology to the *BetV1* family which was similarly highlighted in other ECM systems, continued to be decreased at 30 d.p.i.. In contrast PR5, which is a thaumatin-like protein with anti-fungal properties (Selitrennikoff, 2001), was found to be increased at 30 d.p.i. coinciding with the period of intercellular hyphal penetration inside epidermal and cortical tissues. It is difficult however to provide an explanation for the differences in regulation pattern of these two PR-protein transcripts, but, as documented in other studies, the increases in PR5 could be transient.

Furthermore, we also documented cyclical regulation of abundance of another stress related transcript, glutathione-S-transferase. The transcript was found to be decreased at 1 d.p.i., increased at 5 d.p.i., then decreased again at 15 d.p.i. and increased at 30 d.p.i.. In contrast, the thioredoxin transcript assessed in this experiment was constantly decreased at 1, 5 and 15 d.p.i. but slightly increased at 30 d.p.i.. Thioredoxins are involved in responses to pathogens and oxidative stresses (Laloi, Apel & Danon, 2004).

Such up and down regulation of several genes belonging to the same functional group underlines the complex nature of the interaction. It is possible that such genes possess dual functions apart from involvement in host defences. It is also most probable that the expression of many of these stress or defence related genes is induced each time the hyphae attempt to enter into new cellular tissues.

Apart from genes with defence-related functions, the regulation pattern of transcripts involved in cell wall modification was also interesting. One of the transcripts in this category encodes glycine rich protein (GLP) which was found to be considerably decreased at 30 d.p.i. whereas it was increased at all time points in the array results. GLPs represent the third group of structural protein components for cell walls. They can be exported to neighbouring cells where they contribute to cell wall strengthening (Ringli, Keller & Ryser, 2001).

A similar observation was made for another cell wall modification EST (xyloglucan endo transglycosylase (XET)), which was decreased at 30 d.p.i.. XET may function in modifying cell walls to allow reinforcement of regions under mechanical stress (Antosiewicz *et al.*, 1997). The concomitant decrease in transcripts encoding GLP and XET suggests a cell wall softening which may be a preparative step for intensive transport mechanisms observed in mature mutualistic associations.

Equally interesting is the transcript profiling pattern of genes encoding important enzymes in lignin biosynthesis (Chabannes *et al.*, 2001) (cinnamoyl alcohol dehydrogenase (CAD), cinnamoyl CoA reductase (CCR), peroxidase). CCR has been characterized as an effector in defence signalling in rice (Kawasaki *et al.*, 2006). The decrease in abundance of its transcript at 30 d.p.i. in our study can be interpreted as a cell wall softening but also an attenuation of a defensive reaction in *P. sylvestris* allowing the presence of fungal hyphae between plant root cells. Peroxidase transcript, unlike CCR, was increased through all the developmental stages assessed. Peroxidase is one of the last enzymes involved in lignin biosynthesis. Peroxidases have also been implicated in plant defence reactions where they play an active role in strengthening plant cell walls at the site of fungal invasion (Asiegbu, Daniel & Johansson, 1994; Johansson, Lundgren & Asiegbu, 2004).

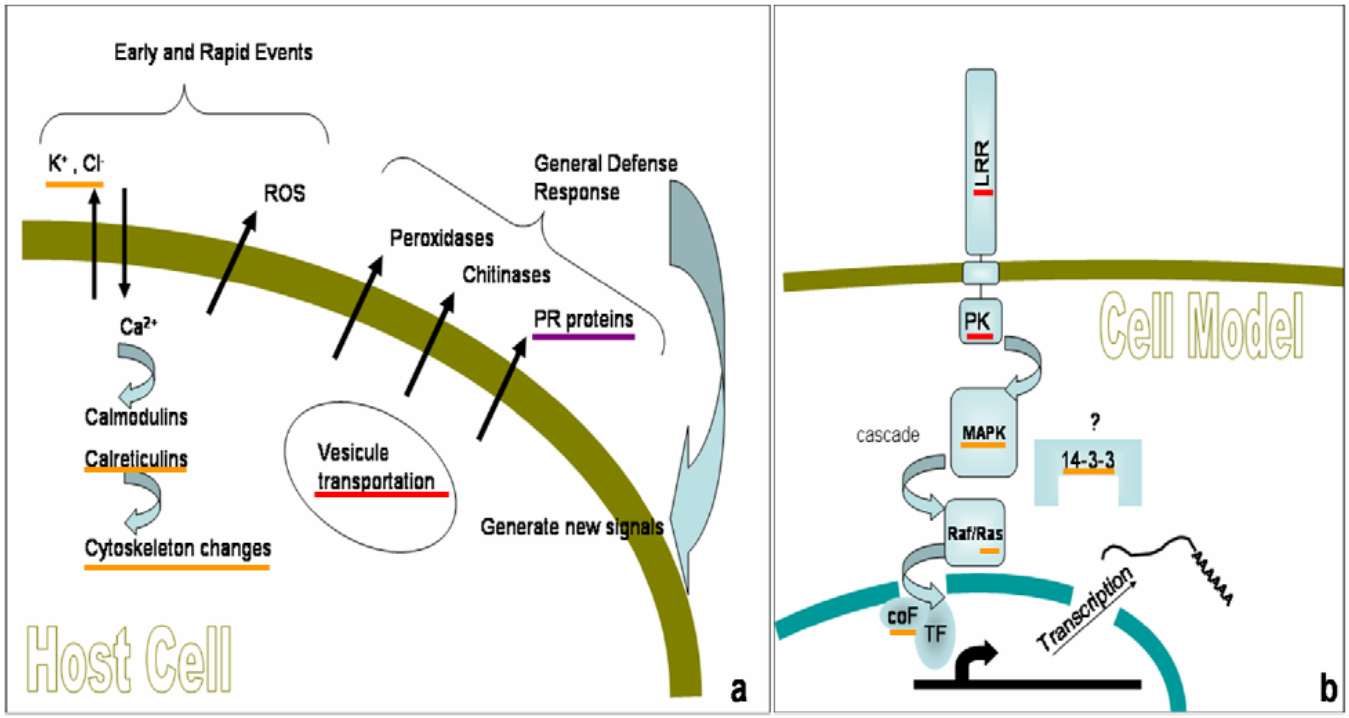

Fig 8. Schematic representation of early events during ectomycorrhizal initiation. Color codes beneath each gene name indicate differential regulation in *Pinus sylvestris* roots at specified time points 1 (red line), 5 (orange) and 15 (purple) d.p.i. with *Laccaria bicolor*.

Expression of symbiosis regulated genes after auxin treatment in *Pinus sylvestris* roots

In **Paper III,** we investigated the effect of auxin in the regulatory patterns of a subset of selected genes identified in **Paper II.** Four ESTs were found in **Paper II** to be involved in the initial stages of ECM development in *P. sylvestris* with *L. bicolor*. These genes shared homology to a Clavata-like gene (*Clv1-like*), an Auxin Induced gene (*AI1*), and two *MtN21-like* nodulin sequences (named in this study as *MtN21-like-a* and *MtN21-like-b*). However it is not known how these four genes are influenced by auxin. The main aim of this project was to determine whether exogenous auxin plays a role in the regulation of these candidate genes and to compare their expression profiles during ECM development in a system where the auxin transport inhibitor TIBA was applied simultaneously with the inoculation of the ECM fungus *Laccaria bicolor*. The use of auxin molecules (e.g. indole-3-acetic acid, IAA, and indole-3-butyric acid, IBA) to mimic the presence of an ECM fungus has led to the isolation of several genes which have the dual property of being triggered by auxin as well as being involved in ECM development. In this study we also compared the expression of the *P. sylvestris* homologs of the genes coding for: the peroxidase *Pp-prx75* (Charvet-Candela *et al.*, 2002b), the gene *Pp-GH3.16* (Reddy *et al.*, 2006), the cell wall protein *Pp-C61* (Reddy *et al.*, 2003) and an *Aux/IAA* cDNA isolated from *Pinus pinaster* (Charvet-Candela *et al.*, 2002a) and henceforth named *prx75*, *GH3*, *C61* and *Aux1* in this particular study.

At 30 d.p.i. the roots treated with either *L. bicolor*, TIBA and *L. bicolor* 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 was increased compared to the control. At 30 d.p.i. these lateral roots displayed the typical dichotomous branching of ECM pine roots and possessed a mantle of fungal mycelium (Fig. 9). In the presence of the auxin transport inhibitor TIBA, drastic morphological changes occurred. Roots were shortened and no lateral roots were observed although the fungus was growing in the vicinity of the roots. Scots pine roots treated with IBA looked abnormal compared to the control with further shortening of the length of the main root axis. IBA treated roots had an excessive number of lateral roots which were much shorter in length compared to control roots.

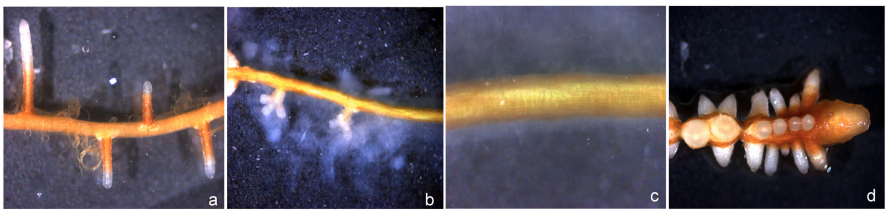

Fig. 9. Light micrographs of *Pinus sylvestris* roots after 30 days of treatment. a) control, b) with *Laccaria bicolor*, c) with *L. bicolor* and TIBA and d) with the auxin IBA.

The relative gene expression of *P. sylvestris* roots treated with the hormone auxin IBA led to the identification of two groups among the genes studied in our experiment. The first group is composed of *C61*, *GH3*, *iaa88*, *5NG4* and *Clv1-like*. These genes display a large increase in transcript levels from 2 to 24 h.p.i. as a result of the IBA induction. The second group is composed of genes displaying a small increase or no differential levels of transcription from 2 to 16 h.p.i. and a strong decrease at 24 h.p.i. compared to control. This group is represented by *MtN21-like-a*, *MtN21-like-b*, *Aux1*, *prx75* and *AI1* (Fig. 10 and 11).

In all cases, with the exception of *C61*, the detected transcription levels were decreased at 24 h.p.i.. It is possible that in our study, the first group of genes, with high levels of expression, could play a role in auxin perception and potentially be involved in the initiation of developmental changes within the root. In IBA treated *Quercus robur* shoots, mitotic and de-differentiating cells, eventually leading to the formation of adventitious roots, can be observed around 24 h.p.i. (Vidal *et al.*, 2003). Furthermore, in a related conifer *P. radiata,* IBA treatment elevates the expression of *Scarecrow-like* genes, reaching a peak expression between 8 and 36 h.p.i.. These genes encode GRAS proteins, a conserved gene family among plant species, which is pivotal for root patterning and triggering periclinal cell divisions (Sanchez *et al.*, 2007). In *P. contorta*, the gene expression analysis of micro-array data during adventitious root development following a pulse of auxin showed that the early stages of the root initials are laid down during the first 3 days before there is any sign of root primordia (Brinker *et al.*, 2004). The second group of genes in our study is down regulated after 24 h.p.i. of IBA treatment. It cannot be excluded that this apparent repression in transcript levels is a result of an increased transcriptional activity in the roots upon initiation of new lateral roots.

The *Pp-C61* gene was first characterized in the *P. pinaster / Hebeloma cylindrosporum* symbiosis (Reddy *et al.*, 2003). The authors restricted its eventual role in ECM symbiosis to a general defence mechanism against micro-organisms. In our system there was a strong increase in transcript levels in *P. sylvestris* roots under IBA treatment between 1 and 5 days (9 fold change) followed by a decline from 15 to 30 d.p.i.. We can only hypothesize that in our system, *C61* responds to the external presence of *L. bicolor*. An unknown mechanism may modulate the defence reaction during the process of ECM recognition. This adaptation to fungal presence has also been observed in pine roots in previous studies with the ECM fungus *L. bicolor* (**Paper II**) and the saprotrophic fungus *T. aureoviride*.

The up-regulation of the auxin responsive gene *GH3* in *P. sylvestris* in the presence of IBA and down-regulation with *L. bicolor* in our system is consistent with the findings of other authors (Reddy *et al.*, 2006). Reddy *et al.* (2006) reported that in *P. pinaster* roots the transcript levels of *Pp-GH3.16* are gradually decreased in the presence of the ECM fungus *H. cylindrosporum* but increased in the presence of auxin. *Pp-GH3.16* can be considered to be a non-specific fungal species marker for ECM establishment. These results show that the expression of this plant gene is similar between closely related plant species and not specific to the fungal partner. This also suggests the existence of a conserved molecular response among conifer species, triggering ECM formation and the possibility of a

common genetic program leading to the development of the symbiotic organ among conifers.

MtN21-like-a and *MtN21-like-b* are two other ESTs that were identified to be up-regulated during ECM development. They share homology with the nodulin 21 first found in *Medicago truncatula* with a high level of expression in mature nodules (Gamas*, et al.*, 1996). In our ECM system, the three *P. sylvestris* ESTs *5NG4*, *MtN21-like-a* and *MtN21-like-b*, share homology to the *nodulin 21* found in *Medicago* but do not respond similarly either to auxin or to *L. bicolor*. *MtN21-like-a/b* are transiently expressed over time and have very similar profiles, whereas the transcript levels of *5NG4* remain unchanged in the presence of *L. bicolor*. The application of the auxin transport inhibitor TIBA results in a strong decrease in transcript levels of *MtN21-like-a/b* but a 2-fold increase of *5NG4* transcripts was observed at 5 days. The similarities in transcript profiles of *MtN21-like-a* and *MtN21-like-b* in this experiment suggest that these two ESTs may belong to the same gene family or parts of the same gene if they span different regions of *MtN21*. On the other hand their different expression pattern compared to *5NG4*, another homolog of *MtN21*, show that they do not have the same function as *5NG4*. In *P. taeda 5NG4* seems to be involved in adventitious root formation (Busov *et al.*, 2004). The authors concluded that the high conservation between *Medicago, Arabidopsis and Pinus sp* sequences suggests that MtN21 carries out essential functions in plant growth and development.

Interestingly, a common feature of plant nodulation and ECM symbiosis is the microorganism's ability to alter plant cell identity and developmental patterns resulting in the formation of a new organ or modification of its structure. In our study another gene involved in plant development, the *Clv1-like* gene, displayed a similar induction pattern to *5NG4* after the application of exogenous auxin.

Increased transcript levels of *Clavata1-like* receptors have been documented to be involved in the autoregulation of nodulation in rhizobial symbiosis (Searle *et al.*, 2003). Additionally, root flavonoids are necessary for nodule initiation in *M. truncatula* by acting as auxin transport regulators (Wasson, Pellerone & Mathesius, 2006). The mechanism of action is still unknown, but several genes encoding a Clavata1-like-receptor-like-kinase in several legumes were found to mediate the autoregulation of nodulation (Oka-Kira & Kawaguchi, 2006). Autoregulatory mechanisms also exist for AM symbiosis (Catford *et al.*, 2003). The study of a supernodulating mutant suggests that the autoregulation occurring in mycorrhizal and rhizobial symbiosis is controlled in a similar manner and that IAA plays a crucial role (Meixner *et al.*, 2005b). In the *P. sylvestris* system, *Clv1-like* transcripts were highly increased in the presence of auxin and also in the presence of the ECM fungus *L. bicolor*. The application of the auxin transport inhibitor TIBA with *L. bicolor* also led to a higher increase in transcript levels at 15 days of interaction compared to *L. bicolor* without TIBA. The application of TIBA is believed to result in a local increase in auxin concentration in plant tissues. Whether a member of the *Clavata1* family of genes could control the number of lateral root initiations during ECM development needs to be

investigated further and no conclusions can presently be drawn for *Clv1-like* in our system.

The peroxidase *Pp-prx75*, one of the genes we investigated, is considered to be involved in symbiosis formation (Charvet-Candela *et al.*, 2002b). Peroxidases are a large family of genes whose members have different functions, including defence-related mechanisms in plant-microbe interactions (Asiegbu, Johansson & Stenlid, 1999). The role of peroxidases in mycorrhizal symbiosis remains unclear but this particular peroxidase was identified from a cDNA library constructed from roots of *P. pinaster* treated with auxin (Charvet-Candela *et al.*, 2002b). The authors hypothesized that *Pp-prx75* could be up-regulated as a primary response to fungal IAA. Furthermore, studies based on the auxin overproducing mutant of *H. cylindrosporum* demonstrated that their increased mycorrhizal activity and hypertrophied Hartig net were mediated by IAA. The fungal auxin also induced modifications in the host cortical cell wall that favoured Hartig net formation (Gay *et al.*, 1994; Gea *et al.*, 1994; Tranvan *et al.*, 2000). In our experiment, the *P. sylvestris* homolog *prx75* showed a strong decrease in transcript levels compared to uninoculated roots until 30 d.p.i. when an almost 2-fold increase was detected. This change is probably concomitant with the penetration of fungal mycelium between the plant cells after mantle formation and is a result of a general defence mechanism.

The transcript levels of Auxin-Induced 1 (*AI1*) were unchanged at 2, 8 and 16 h.p.i. but strongly decreased at 24 hours after application of IBA. *AI1* belongs to the group of genes that were not highly induced by the auxin treatment and were down-regulated after 24 h.p.i.. However, *AI1* showed a unique response to the presence of TIBA in this experiment. The levels of transcription were higher after TIBA treatment than with the fungus alone. A possible explanation can be found in the difference in sensitivity to IBA and IAA in *Arabidopsis thaliana* (Ludwig-Muller, 2000; Ludwig-Muller, Vertocnik & Town, 2005). Gymnosperms possess homologous sequences to the *A. thaliana* IBA synthase which may account for difference in sensitivity but it is unclear if this enzyme plays a similar role in conifer roots.

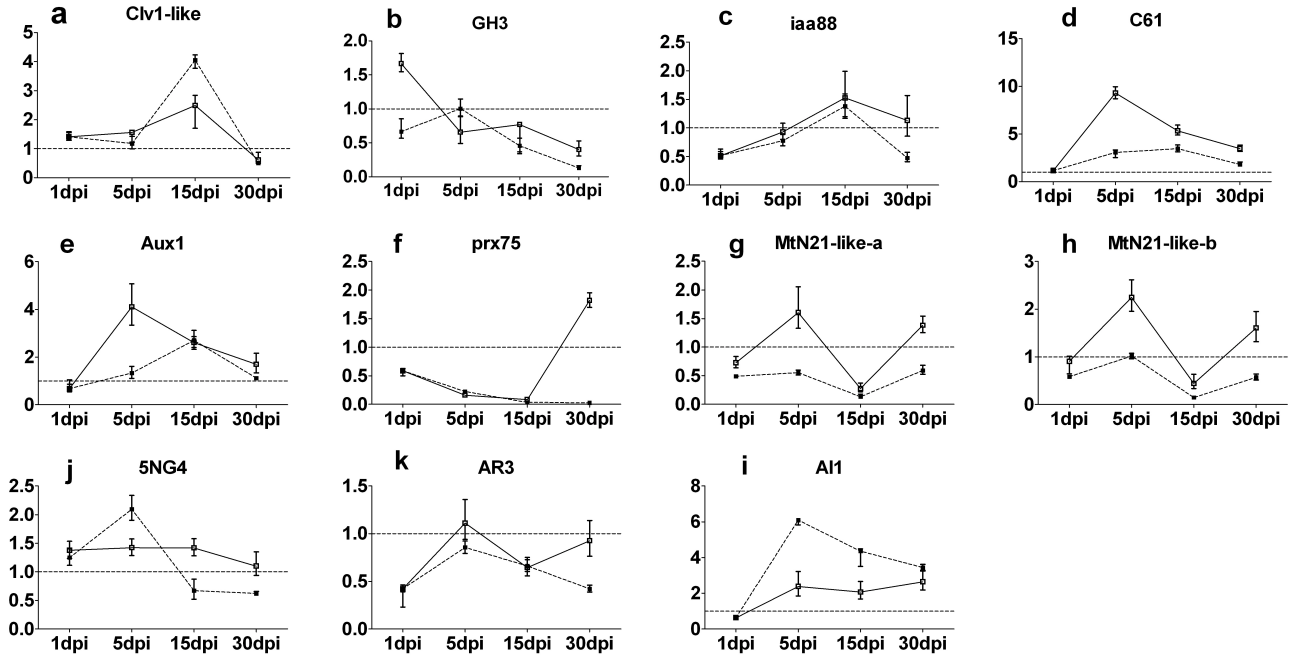

Fig. 10. Relative gene expression of *Pinus sylvestris* roots during early interactions with the ectomycorrhizal fungus *Laccaria bicolor* . The transcript levels of 11 genes (*Clv1-like, GH3, iaa88, C61, Aux1, prx75, MtN21-like-a, MtN21-like-b, 5NG4, AR3, AI1*) at four time points (1, 5, 15 and 30 days post inoculation) were assessed by qRT-PCR in the presence (full connectors) or absence (dashed connectors) of the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA). Each dot represents an average fold change of 3 biological replicates and the bars the standard error.

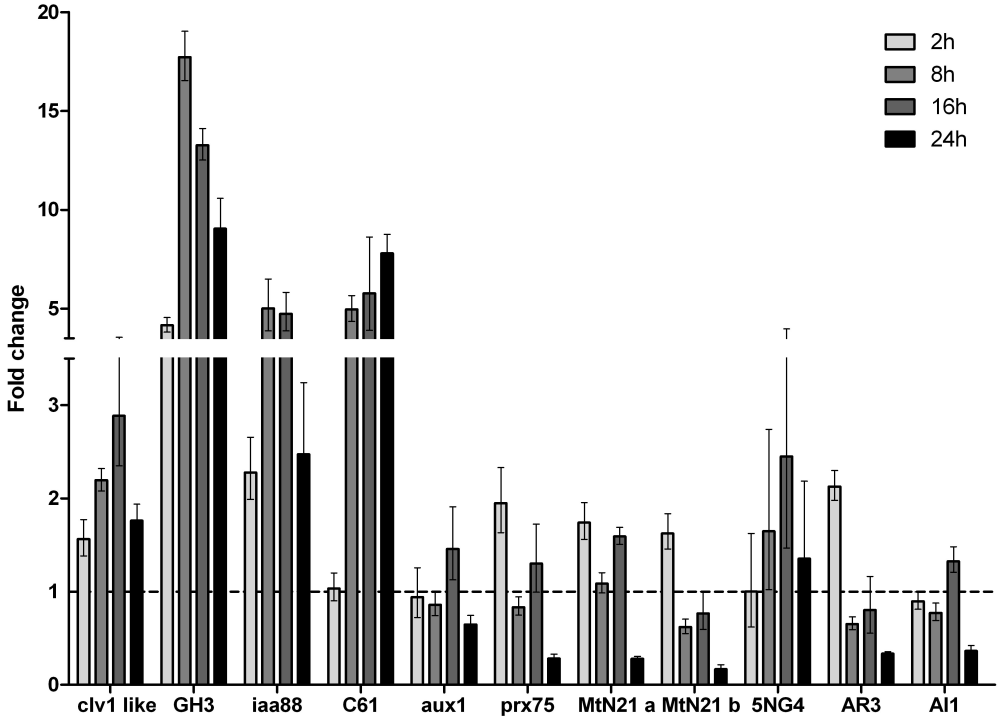

Fig. 11. Relative gene expression of *Pinus sylvestris* roots treated with the hormone auxin indole-3-butyric acid (IBA)**.** Transcript levels for 11 genes were assessed at 2, 8, 16 and 24 hours after inoculation of IBA by qRT-PCR. The graph represents the mean fold change of transcript levels compared to control roots. The bars represent the upper and lower limits of the standard error over 3 biological replicates.

Conclusions

In **Paper I**, micro-array transcript profiling was used to ascertain specificity in the response of conifer tissues to ECM fungi using a pathogenic and a saprotrophic fungus as non-mutualistic models. The transcript profile of *P. sylvestris* genes expressed during interaction with a mutualistic fungus was compared to those observed following challenge with saprotrophic or pathogenic fungal species. The results indicate that pine was able to recognize all three organisms and specifically distinguish whether they were pathogenic, neutral or beneficial microbial agents.

In **Paper II**, we conducted further detailed analysis of the expression profile with particular focus on the early, intermediate and late stages of ECM development in *P. sylvestris* - *L. bicolor* system. According to our results, the molecular regulation of the development of ECM symbiosis in this gymnosperm host appears largely comparable to that in angiosperm hosts. Nevertheless some differences were found in the timing and spatial scale of gene regulation during ECM development in conifer host. Of particular interest is the regulatory pattern of a number of stress related genes, as well as down regulation of several cell wall modification genes that enabled the development of the fungus within root tissues. The study additionally identified a number of potentially important molecular events responsible for the initiation and regulation of biochemical, physiological and morphological changes during the development of a fully functional symbiosis that are relevant for gymnosperm hosts.

In **Paper III**, some of the results are consistent with earlier published work, particularly for expression patterns of *GH3* and *prx75*. Our results show however, that even if the genes studied here demonstrate apparently similar patterns in response to exogenous auxin, this is no longer the case in a more complex system involving an ECM fungus. The unique patterns obtained with the co-inoculation of the auxin transport inhibitor suggest in the same way that auxin plays an important role in ECM development but also that other factors are involved in the process and regulate the expression of these genes. Nevertheless we identified four new auxin regulated genes in *P. sylvestris* (*AI1*, *Clv1 like*, *MtN21-like-a* and *MtN21-like-b*), that are most likely to be involved in the early stages of ECM symbiosis with *L. bicolor*. Their exact function remains uncertain but the kinetics of the expression of *Clavata1-like* gene during ECM symbiosis suggests a role for this gene during lateral root initiation.

Future perspectives

The investigations published in Paper **I** and **II** led to very interesting paths for future research. They concern all the crucial early steps in ECM initiation: the pre-contact stage when signalling compounds are exchanged between the partners, leading to the next stage when recognition by the plant of the symbiotic fungus occurs, followed by the reprogramming of the plant root cells to produce a new lateral root, the physiological adaptation of the root isolated from the environment

after the mantle has formed and finally the morphological changes of root cells concomitant with the formation of the Hartig net. In parallel to all these steps the fungus and the plant have to overcome general defensive responses due to physical contact and the intercellular invasion of the tree root by fungal mycelium.

The genes highlighted by the micro-array experiments should now ideally be studied in knock-out or RNAi systems to observe the phenotypical alterations and understand better their function. Unfortunately *P. sylvestris* and conifers in general, have extremely large genomes (200 times the size of *A. thaliana*) and very long reproduction times (up to 30 years for the first seed production in natural conditions, without mentioning the obvious practical issues). And as a consequence the transformation, the generation and screening of mutants is, despite some successful attempts, at best extremely slow and technically challenging. The availability of the first tree genome sequence (*Populus trichocarpa*) provides hope that a similar project will be possible for Pine species. However, knowledge gained from this genome, could be used for Pine to decipher the functioning of ECM symbiosis. The ability of Poplar to form both ECM and AM symbiotic associations can also be used to determine whether genes involved in AM symbiosis have been recruited during the evolution of ECM symbiosis. This would be an important feature in the understanding of plant and microbe interactions through a co-evolution perspective. It could be proven by e.g. finding a common component involved in the signal transduction responsible to the cascade of events described earlier, as it is the case now in legumes between AM and nodulation.

In a shorter perspective, of course developing micro-arrays containing more genes and refining the sampling times will allow the identification of more candidate genes. But with the actual technology, it is also possible to understand, localize and put in a time frame with more precision the expression of our genes of interest catalogued in paper **I** and **II**. GUS reporter technology would be the best choice, but, for the reasons explained earlier, this is a cumbersome technology in a pine system. On the other hand, *in situ* hybridization may provide valuable information on the identity of the cells expressing by e.g. the defence related genes during Hartig net formation. The *Clavata1-like* gene could also be studied in a similar way to understand its role in the lateral root initiation triggered by *L. bicolor*. The localization of the gene expression of the nodulin 21 homologs studied in Paper **III** (*5NG4*, *MtN21-like a/b*) could be compared and may show the reprogramming of specific and different cells during the ECM process than those from non-mycorrhizal lateral roots. Furthermore several signal transduction genes have been identified in Paper **II** and can potentially link to other key components of ECM symbiosis. The cloning of their full sequence gene and the construction of a yeast-two-hybrid library or the use of an affinity chromatography system may allow revealing more components of the ECM pathway. It would also be feasible with a similar strategy to identify which plant proteins could interact with fungal proteins. This may help us to understand how the ECM fungus can overcome the plant's defence reaction and if a similar process described in pathogenic interactions (by e.g. the gene for gene theory) is involved in symbiotic interactions.

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