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Par
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Titre

Développement racinaire du peuplier en réponse aux signaux fongiques lors de la mise en place de l'ectomycorhize

Poplar root development in response to fungal signals during onset of ectomycorrhiza development

Thèse soutenue le 14 Décembre 2009

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Our head is round, so our thinking can change directions.

Francis Picabia

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Table of contents

Introduction **8**
Développement de la symbiose ectomycorrhizienne (published in Biofutur) **9**
I. Background of root systems **15**
I.1 Evolution and function of roots 15
I.2 Root Anatomy 15
I.2.1 Root types 15
I.2.2 Root apex organization 16
II. Regulation of lateral root development **17**
II.1 The phytohormone auxin 17
II.1.1 Auxin gradients drive root system development 17
II.2 Auxin homeostasis, transport and signalling 18
II.2.1 Auxin biosynthesis 18
II.2.2 Auxin conjugation, recycling and catabolism 19
II.2.3 Polar auxin transport (PAT) 20
II.2.4 Auxin signalling 23
II.3 Mechanisms regulating lateral root development 24
II.3.1 Priming of founder cells in the basal meristem (pre-initiation phase) 25
II.3.2 Lateral root initiation 26
II.3.3 Lateral root primordium progression and new meristem development 30
II.3.4 Lateral root emergence 30
II.4 Phytohormone crosstalk during LR development 31
II.4.1 Brassinosteroids 31
II.4.2 Ethylene 32
II.4.3 Jasmonic acid 33
II.4.4 Cytokinins 34
II.5 Lateral root development in response to soil (micro-)organisms 35
III. Ectomycorrhizal symbiosis **36**
III.1 Background of ectomycorrhizal symbiosis 36
III.2 The development of ectomycorrhizal symbiosis 37
III.3 Signalling molecules in ectomycorrhizal symbiosis 40
III.3.1 Rutin and zeatin in plant exudates 40
III.3.2 Fungal and plant auxins 40
III.3.3 Defence- and stress-related signalling 42
III.3.4 Volatile phytohormones 43
III.3.5 Ion fluxes 44
III.3.6 Polysaccharides, Symbiosis induced proteins and hydrophobins 45

Objectives & Strategy **47**

Results **50**
Chapter I **51**
Ectomycorrhiza development between *Populus tremula x Populus alba* and *Laccaria bicolor* in an *in vitro* culture system (article in preparation) 51
Chapter II **64**
The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signalling (article accepted in Plant Physiology) 64
Chapter III **95**
Defence-related signalling in the early interaction between the ectomycorrhizal fungus *L. bicolor* and roots of poplar and Arabidopsis: new perspectives on fungus-induced lateral root stimulation (in preparation) 95

Conclusion & Perspectives **128**
Conclusion **129**
Perspectives **132**

Annex **136**

Literature cited **140**

Preface

A phenomenon commonly observed during symbiotic interactions of plant roots with ectomycorrhizal (ECM) soil fungi is the stimulation of lateral root (LR) development in the host plant. My thesis focussed on decrypting the molecular events that get activated in the plant upon contact with the fungus and that trigger LR development.

The **introduction** is opened by an invited article (published in *Biofutur*) on signal exchanges between plant and fungus during ECM development. It is followed by a literature-based introduction ("State of the art") on the cellular and molecular mechanisms underlying LR formation, mostly based on the well-studied herbaceous model plant *A. thaliana*. In a second part of the introduction, I recapitulate signalling pathways identified as mediators during during ECM formation. This part will be the basis of a chapter (in preparation) to a new book entitled "Signalling and Communication in Plant Symbiosis" (Springer-Verlag, edited by S. Perotto and F. Baluska).

Based on knowledge gathered in the introduction, the **objectives and strategy** of my PhD thesis are given and a working model is proposed. The **results** are presented in form of three publications (either in preparation (Chapter I and III) or accepted (*Plant Physiology*, Chapter II)). In the final **conclusion** I interconnect the data from Chapter I to III to confirm and affine the working model initially proposed. **Perspectives** on experiments that are needed to complete the articles in preparation as well as a follow-up project are presented.

Introduction

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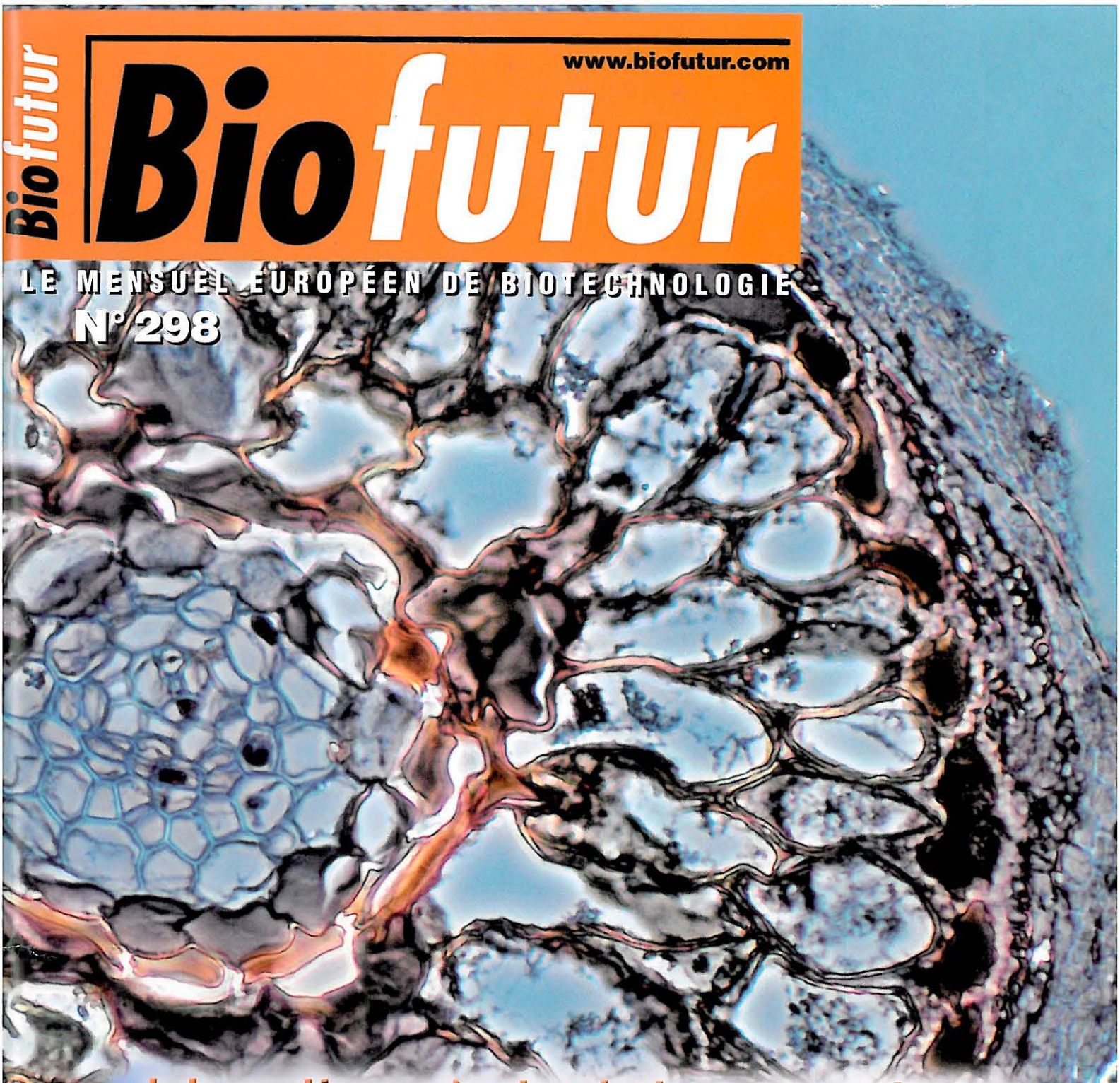

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Développement de la symbiose ectomycorhizienne

La majorité des racines des arbres de nos forêts est colonisée par des champignons, conduisant à la formation d'une symbiose appelée ectomycorhize. Cette interaction est accompagnée d'une forte modification du développement racinaire de la plante hôte. Des expériences physiologiques et moléculaires suggèrent le rôle important des phytohormones telles que l'auxine et l'éthylène dans la modification de la formation des racines du peuplier induit par le champignon ectomycorhizien, Laccaria bicolor.

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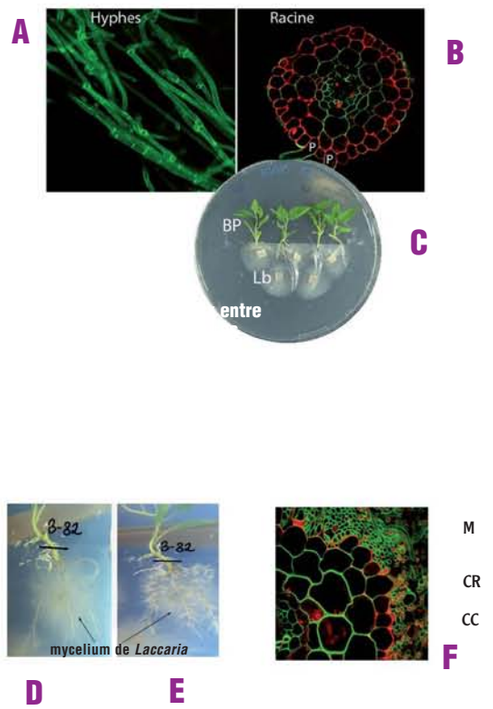
Plus de 80 % des arbres de nos forêts tempérées vivent en association avec des champignons du sol. Cette association à bénéfice réciproque, appelée symbiose ectomycorhizienne, favorise la croissance et la bonne santé des arbres hôtes et présente donc un rôle important dans le fonctionnement de l'écosystème forestier **(1)**. Depuis de nombreuses années, les chercheurs visent à décrypter les mécanismes complexes impliqués dans l'établissement de cette symbiose.

La mise en place d'un réseau plante-champignon

Saviez-vous que les champignons que nous voyons sur les sols de nos forêts ne sont que la partie visible d'un important réseau de filaments fongiques, les hyphes **(figure 1A)**, qui se développent dans le sol ? Ces champignons ont un mode de vie passionnant. Contrairement aux plantes, autotrophes en carbone (qui synthétisent les sucres par la photosynthèse), les champignons sont hétérotrophes en carbone, c'est-à-dire qu'ils ne peuvent pas synthétiser les sucres pourtant essentiels à leur croissance.
Ainsi, les sucres sont apportés aux champignons par les racines des arbres avec lesquels ils sont associés. En revanche, les champignons peuvent absorber plus efficacement que les plantes certaines formes de minéraux tels que le phosphore et l'azote, qui sont ensuite mis à la disposition de l'arbre associé.
La mise en place des mécanismes d'échange de nutriments entre les deux partenaires est la dernière étape du développement de la symbiose, qui est un processus complexe d'étapes multiples **(figure 1)** pouvant durer plusieurs mois. Les études ont montré que l'échange de nutriments se fait uniquement dans une structure particulière nommée réseau de Hartig **(figures 1H et 2)**. Des coupes transversales de racines de peuplier en cours de colonisation, après une double coloration fluorescente spécifique du champignon **(figure 1A, en vert)** et de la plante **(figure 1B, en rouge)**, mettent en évidence le manteau fongique qui entoure la racine **(figures 1F et H)**. Dans des étapes plus tardives de l'association plante/champignon, des hyphes fongiques du manteau pénètrent entre les cellules les plus externes de la racine, les cellules rhizodermiques et les cellules corticales, qui forment le réseau de Hartig **(2)**.
La racine et le champignon forment ainsi un nouvel organe commun, la mycorhize (des mots grecs *mukês* pour champignon et *rhiza* pour racine). La présence d'un manteau et du réseau de Hartig est caractéristique des mycorhizes dites ectotrophes, les ectomycorhizes. À l'extérieur du manteau, un mycélium dit « extra-matriciel » se développe dans le sol et absorbe des nutriments qui sont ensuite transportés vers le manteau **(figure 2)**.

(1) Smith SE, Read DJ (1997) *Mycorrhizal symbiosis,* Academic press, San Diego, USA
(2) Malamy JE, Benfey PN (1997) *Development* 124, 33-44



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Étapes clés conduisant à l'établissement de l'ectomycorhization

A. Hyphes de *Laccaria bicolor* après coloration (microscopie à fluorescence)

B. Coupe transversale d'une racine de peuplier après coloration (microscopie à fluorescence). Les poils absorbants (P) sont présents à la surface de la racine.

C. Système de mycorhization *in vitro*. Les boutures de peuplier (BP) sont déposées sur le milieu de culture gélosé et développent des racines après 3 semaines. Le champignon ectomycorhizien, *Laccaria bicolor* (Lb), cultivé sur une membrane de cellophane, est appliqué sur les racines.

D. Au départ de l'expérience, peu de racines latérales sont visibles.

E. Après 10 jours de contact avec *Laccaria bicolor*, de nombreuses racines latérales se sont développées.

F. Coupe de racine de peuplier lors de la mycorhization. Le manteau fongique (M) entoure la racine.

G. Aspect morphologique des ectomycorhizes *Laccaria*/peuplier fonctionnelles, après 6 semaines de contact. Des racines dichotomiques sont visibles alors que les poils absorbants disparaissent.

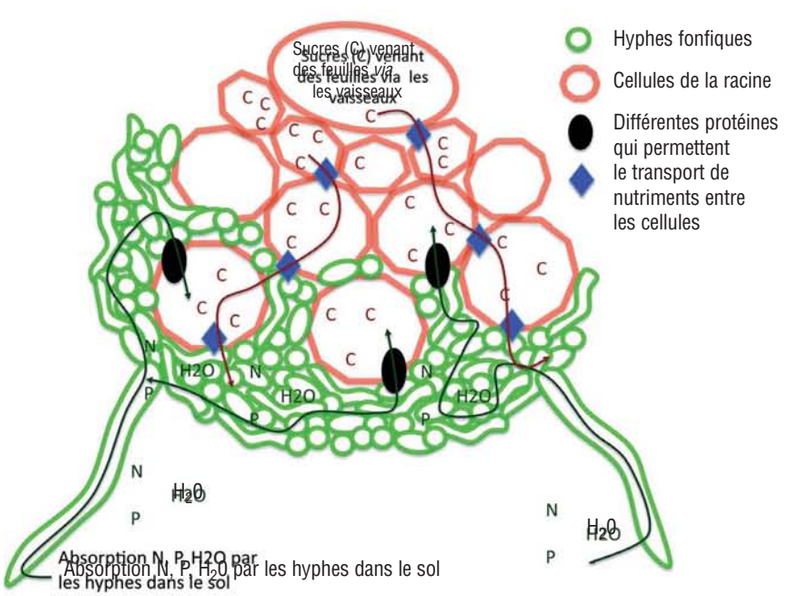
H. Coupe transversale de racine de peuplier ectomycorhizée par *Laccaria bicolor*. Le manteau fongique (M) entoure la racine, des hyphes s'insèrent entre les cellules rhizodermiques (CR) et les cellules corticales (CC) formant le réseau de Hartig (RH).

Une modification précoce du développement racinaire

Comme illustré dans la **figure 1**, la mise en place de la symbiose ectomycorhizienne s'accompagne d'un changement profond du développement racinaire qui se manifeste par une augmentation de la formation des racines latérales.

Le système racinaire d'une plante est généralement formé d'une racine primaire sur laquelle se développent de manière hiérarchique les racines secondaires puis tertiaires, appelées racines latérales **(figure 3)**. À partir d'observations microscopiques précises, les chercheurs ont distingué plusieurs stades dans la formation d'une racine latérale. D'abord, une initiation par une réactivation du cycle cellulaire des cellules du péricycle de la racine, ces cellules retrouvant leur état de cellules souches donnent naissance à une ébauche racinaire (*primordium*). Les divisions cellulaires sont ensuites suivies d'une différenciation des cellules et de l'acquisition de leur fonction spécifique **(2)**. L'allongement des cellules provoque, dans la dernière phase, l'émergence de la racine latérale.

Bien que l'on sache que la mise en place de la symbiose ectomycorhizienne conduit à la construction d'un système racinaire fortement ramifié de la plante hôte **(1)**, les processus impliqués dans le développement racinaire de l'hôte



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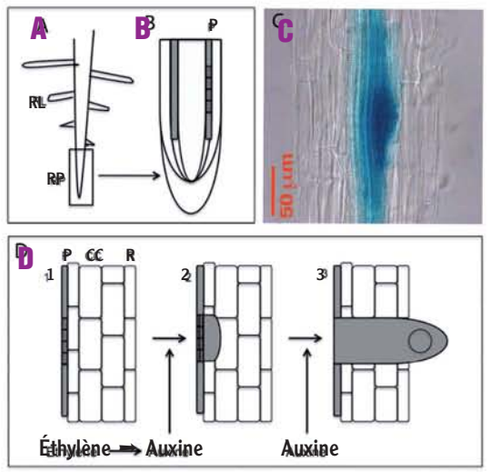
Transfert des nutriments dans une ectomycorhize fonctionnelle



Système racinaire

A. Organisation typique d'un système racinaire d'eudicotylédone. La racine primaire (RP) croit et initie des racines latérales (RL).

B. Coupe longitudinale de l'apex racinaire. Les cellules du péricycle (P, indiquées en gris) sont les cellules souches des racines latérales.

C. *Primordium* de racine latérale en vue longitudinale. La coloration bleue traduit une accumulation d'auxine dans ces cellules.

D. Étapes de développement des racines latérales (en coupe longitudinale). 1) Des cellules du péricycle (P) initient des divisions cellulaires qui seront 2) à l'origine de la formation du *primordium* de la racine latérale. Sa croissance conduit à son émergence à travers les cellules corticales (CC) puis du rhizoderme (R). 3) La racine latérale émerge et le nouveau méristème est activé.

en réponse au champignon sont peu connus. Afin de pouvoir les décrypter avec précision, nous avons développé un système de mycorhisation *in vitro* (sur boîte de Pétri), en utilisant le peuplier et le champignon basidiomycète *Laccaria bicolor* comme modèles **(figure 1C)**. Les génomes de ces deux espèces étant connus **(3,4)**, ces modèles fournissent aux chercheurs une vaste ressource pour des études moléculaires.

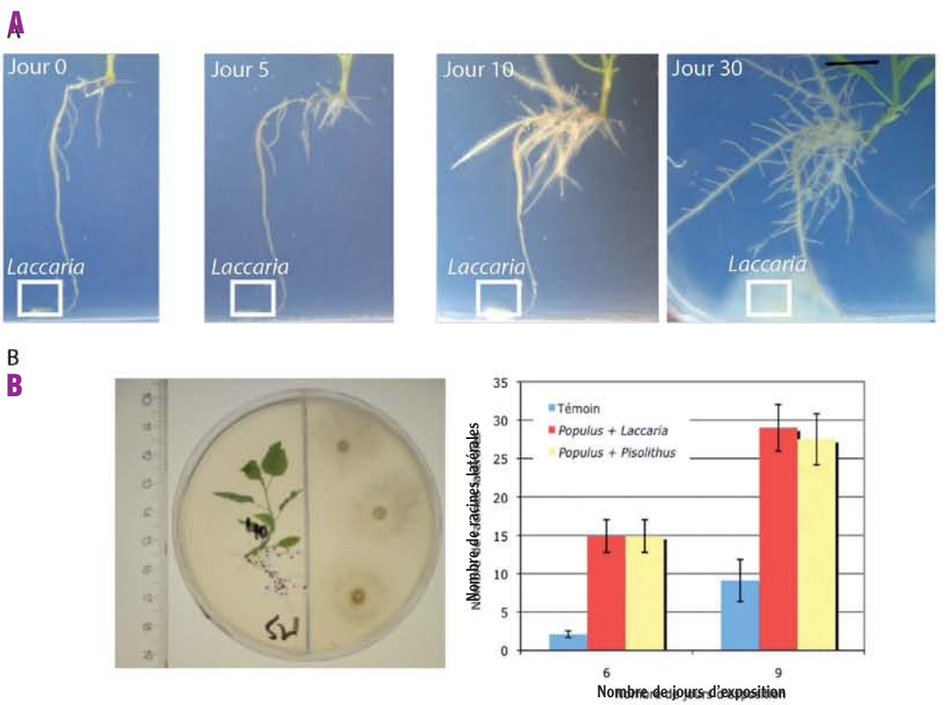
Dans notre système de culture *in vitro*, les études de cinétique de formation des racines latérales ont permis de mettre en évidence une augmentation importante du nombre de racines latérales du peuplier, après dix jours de contact avec *Laccaria bicolor* **(figures 1D et E)**. Ainsi, le champignon induit des changements précoces dans le développement racinaire (comparés à la mycorhize mature obtenue après 6 à 8 semaines de contact). De manière intéressante, nous avons aussi observé que la croissance de ces racines est stoppée assez rapidement après leur émergence, formant alors ce que les spécialistes appellent généralement les « racines courtes ». Curieusement, ces racines présentent parfois une croissance dichotomique (du grec « coupé en deux ») **(figure 1G)**, c'est-à-dire possédant deux pointes

(3) Tuskan GA et al. (2006) Science 313, 1596-604
(4) Martin F *et al.* (2008) *Nature* 452, 88-93

Effets des signaux fongiques sur la formation des racines latérales

A. Le mycélium fongique est ajouté (cadres blancs) à T_0. L'effet après 5, 10 et 30 jours est visible sur l'ensemble du système racinaire, illustrant la mobilité du signal.

B. Le peuplier et le champignon ectomycorhizien (*Pisolithus microcarpus* ou *Laccaria bicolor*) sont cultivés dans une même boîte de culture mais dans deux milieux séparés. Après 6 et 9 jours de co-culture, le nombre de racines latérales est augmenté, suggérant que des molécules volatiles seraient impliquées dans la communication plante/champignon.



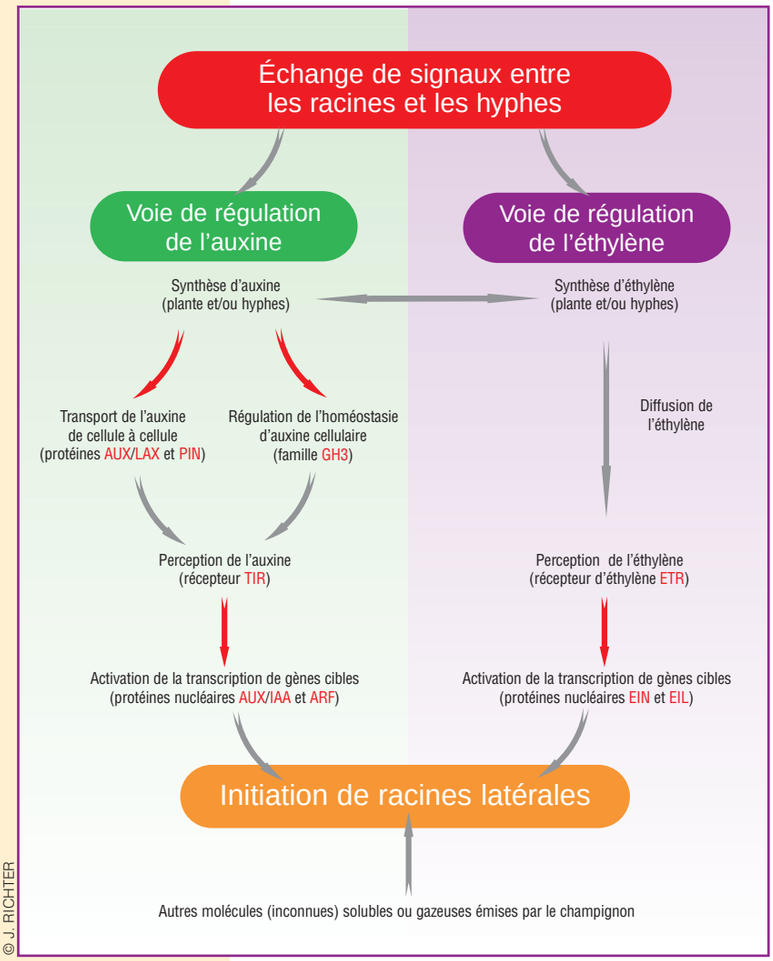


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Figure 5 Principales voies de régulation de l'éthylène et de l'auxine connues chez *Arabidopsis thaliana*

Les protéines clés et les voies susceptibles d'être régulées au cours du contact peuplier/*Laccaria* sont indiquées en rouge.

racinaires. Nous avons aussi observé une disparition des poils absorbants **(figure 1G)**, cellules rhizodermiques **(figure 1B)** spécialisées dans l'absorption de l'eau.

L'implication de petites molécules servant de signal entre les deux partenaires a été suggérée car le champignon induit précocement une modification importante du développement racinaire de la plante hôte, c'est-à-dire avant la mise en place du manteau fongique et du réseau de Hartig.

La symbiose ectomycorhizienne implique des étapes de communication moléculaire

D'une manière générale, l'établissement d'une symbiose implique, dès la première étape de l'interaction, des échanges de signaux entre les deux partenaires. Le champignon émet en direction de la future plante hôte des molécules qu'elle perçoit et qui déclenchent chez elle des réponses morphogénétiques **(figure 1)**.

L'identification de ces molécules signal et de leur perception par la plante est un des objectifs de notre projet de recherche. Trois approches différentes sont utilisées afin de décrypter la communication plante/champignon :

- une étude physiologique du développement de la plante (biotest) est utilisée afin de connaître les caractéristiques des molécules signal (soluble, gaz, transportable dans la plante, stable dans différentes conditions, etc.) ;
- une approche physicochimique (chromatographie et spectroscopie de masse) permet d'analyser et d'identifier les molécules excrétées par le champignon et de comparer leurs profils en présence ou absence de la plante ;
- enfin, une analyse transcriptomique (analyse de l'abondance des ARN de différents gènes) des racines a été réalisée afin de comprendre les événements moléculaires induits dans la plante hôte.

Les biotests ont relevé quelques caractéristiques de molécules sécrétées par *Laccaria bicolor*. Ainsi, un apport ponctuel du champignon induit une stimulation de la formation de racines latérales dans l'ensemble du système racinaire **(figure 4A)**. Ces observations conduisent à émettre plusieurs hypothèses concernant les caractéristiques de la ou des molécules signal :

- la molécule fongique est mobile : elle pénètre dans la racine et est transportée dans le système racinaire ;
- ou/et la molécule est volatile et se propage librement *in vitro*.

L'expérience présentée par la **figure 4B** renforce l'hypothèse d'une implication de molécules volatiles dans la communication plante/champignon : en effet, la culture *in vitro* d'un champignon symbiotique, *Laccaria bicolor* ou *Pisolithus microcarpus*, dans un compartiment séparé de celui du peuplier conduit néanmoins à une augmentation du nombre de racines latérales **(figure 3E)**.

Par ailleurs, il a été mis en évidence que les champignons, comme les plantes, sont capables de synthétiser puis de sécréter certaines phytohormones **(5,6)**. Ces petites molécules régulent à très faibles concentrations les différentes étapes du développement des plantes (développement des feuilles et des racines, floraison, maturation des fruits, etc.). Nos analyses physicochimiques, ainsi que celles d'autres chercheurs, ont mis en évidence la production d'auxine par *Laccaria bicolor*. D'autres équipes ont également démontré que les champignons ectomycorhiziens produisent une autre phytohormone, l'éthylène, se présentant sous forme gazeuse **(5)**. Ces deux hormones jouent aussi un rôle important dans les différents stades de formation des racines latérales **(figure 3) (7,8)**.

Récemment, un certain nombre de voies de régulation et de protéines intervenant en réponse à l'auxine et l'éthylène ont été identifiées et ont conduit à proposer un modèle de régulation des voies de ces hormones **(figure 5)**. Il a été mis en évidence que les protéines responsables du transport de l'auxine (nommées AUX/LAX et PIN) et celles régulant l'homéostasie de cette hormone (les protéines de la famille GH3) sont impliquées dans des étapes en amont de l'induction du cycle cellulaire pendant la « naissance » des *primordia* racinaires. Des analyses moléculaires et génétiques ont mis en évidence que des protéines nucléaires comme les AUX/IAA, ARF (pour la voie auxine) et EIN, EIL (pour l'éthylène), font la connexion entre la présence d'auxine ou d'éthylène et la régulation du cycle cellulaire.

Afin de décrypter les voies de régulation (*via* auxine, éthylène et autres hormones) impliquées dans l'initiation des racines latérales en réponse à *Laccaria bicolor* et d'identifier des marqueurs moléculaires, des études de transcriptomique ont été réalisées à partir des racines du peuplier après trois jours de contact avec *Laccaria bicolor* (correspondant à la formation des nouvelles racines latérales). Les résultats confirment l'implication des voies de signalisation hormonale décrite auparavant lors de la communication *Laccaria*/peuplier. ●

(5) Rupp LA *et al.* (1989) *Can J Bot* 67, 483-5
(6) Karabaghli-Degron C et al. (1998) *New Phytol* 140, 723-33
(7) De Smet I *et al.* (2007) *Development* 134, 681-90
(8) Ruzicka K *et al.* (2007) *Plant Cell* 19, 2197-12

State of the art

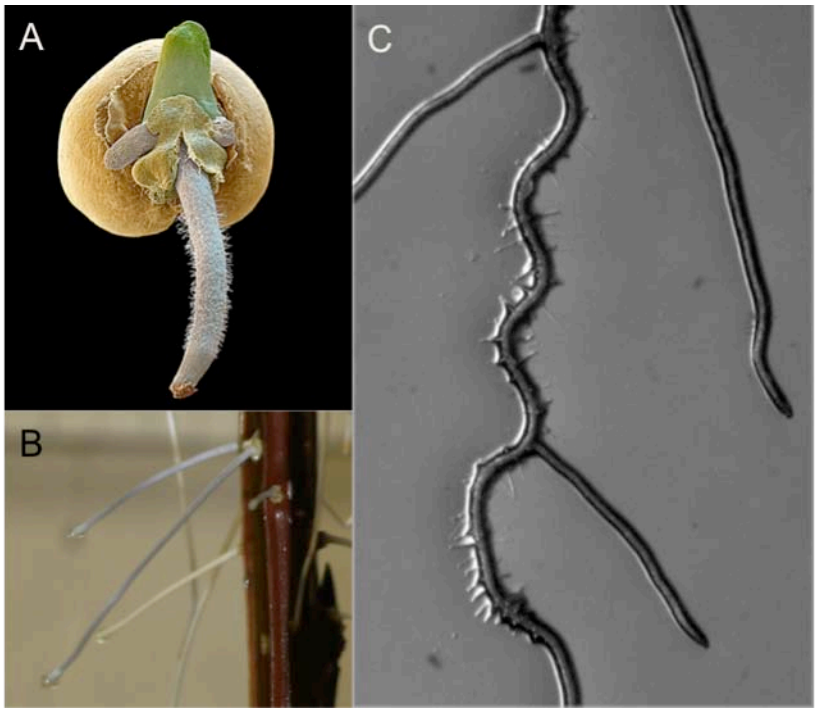

Figure 1: Root types

A embryonic root (radicle) emerging from a wheat (*Tricicum* sp.) seed. Coloured, scanning electron micrograph, Magnification: x17 (from www.eyeofscience.de). **B** Adventitious roots emerging from a poplar cutting (branch of field grown poplar) after culturing in a hydroponic system (courtesy of Adeline Rigal). **C** Lateral roots emerging from an *Arabidopsis thaliana* primary (embryonic) root.

I. Background of root systems

I.1 Evolution and function of roots

When the first primary, multicellular plants started to colonize the land about 500 million years ago (mya), they were still root-less. The need for nutrition led them to absorb minerals, water, CO_2 and O_2 and solar energy through their entire, undifferentiated body surface. Only when different plant organs (e.g. leaves) started to form and took over specific functions, vascular tissues, containing phloem and xylem, started to differentiate (Kolek and Kozinka, 1992). Analysis of fossil remnants of the oldest vascular plant (*Ryniaceae*) from the fern-family, which may have developed small leaves, showed that these species had only simple root-like filamentous structures, corresponding to rhizoids of bryophytes or root hairs of today's vascular plants, which they used to fix themselves on the rocky land surface (Kolek and Kozinka, 1992; Drenou et al., 2006). True roots only appeared 400mya together with the development of shoots and leaf axes in Tracheophytes.

In order to make nutrient absorption from the soil more efficient, the majority of roots are associated with symbiotic soil microorganisms such as fungi or bacteria. Recent work has confirmed that even the earliest land plants without real roots were already colonized by hyphal fungi similar to modern arbuscular mycorrhizas (Smith and Read, 2008). This impressively points out the importance of these beneficial associations.

Roots, whether colonized by microorganisms or not, are an important part of plants and ensure three basic functions: stable anchorage of the plant in the soil, nutrient and water absorption as well as resource storage. But roots, like leaves, also produce specific secondary metabolites that get redistributed throughout the entire plant. Among these metabolites, phytohormones have a significant role in almost all physiological processes. For instance it has been shown that cytokinins are synthesized in the root tip and transported to shoot tissues (Aloni et al., 2005). Root tips also synthesize auxin (Ljung et al., 2005; Petersson et al., 2009).

I.2 Root Anatomy

I.2.1 Root types

We have to distinguish three different types of roots: embryonic roots that are already predetermined in seeds, and two post-embryonic root types, namely adventitious and lateral roots. Adventitious roots can develop on all three plant-organs, stems, leaves and on roots after secondary growth (not founded in the pericycle). A project focussing on molecular key actors during adventitious root development is ongoing in the tree/microbe interaction group at INRA Nancy (PhD Thesis of Adeline Rigal) but will not be further detailed here. The present work will focus on lateral root development. In contrast to adventitious roots, lateral roots only form on other roots, independent of

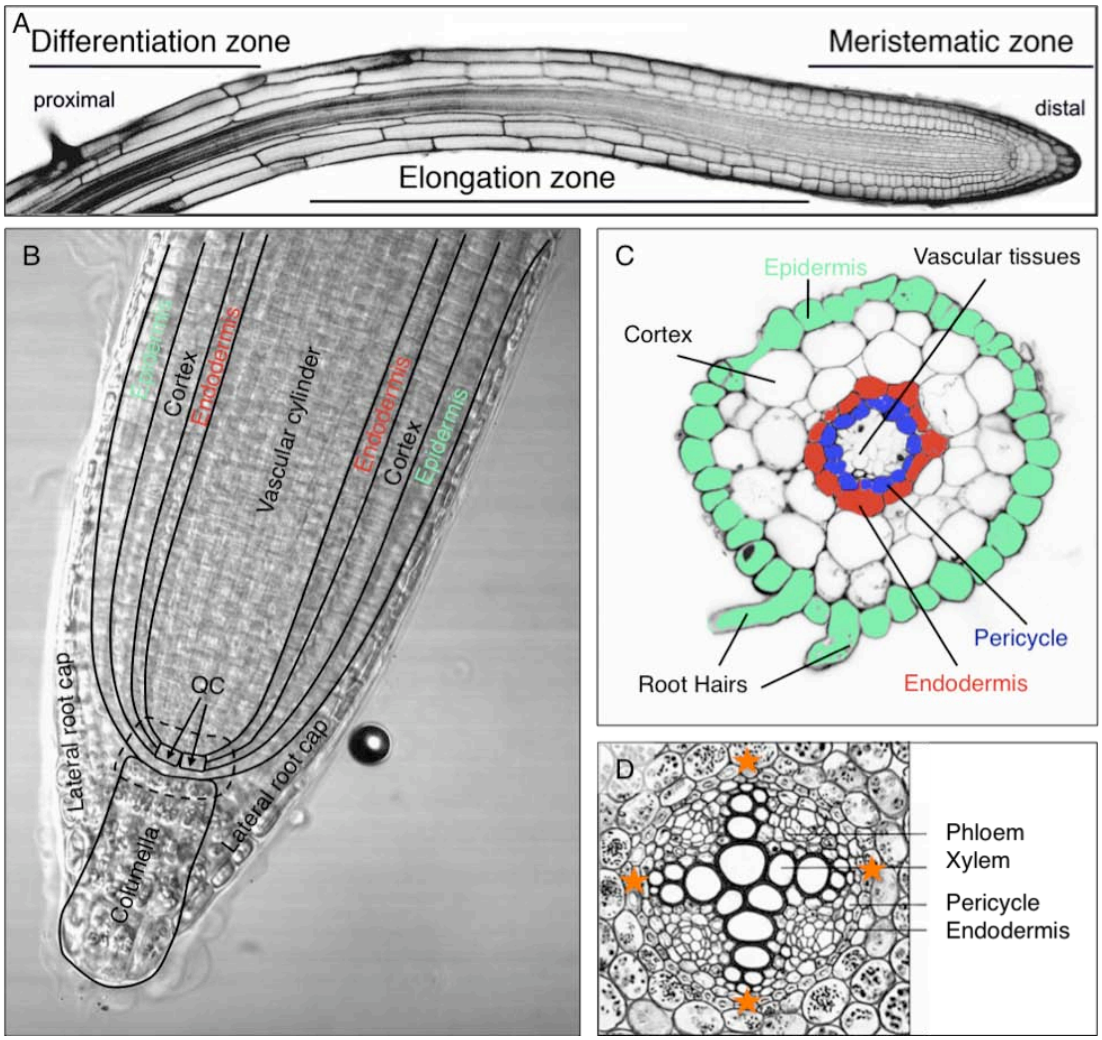

Figure 2: Root tissue and cellular organization.

A Root tissue zonation showing meristematic, elongation and differentiation zone from distal to proximal root parts. Image modified from Laskowski et al. (2008) **B** Longitudinal view into the organization of the Arabidopsis root apex in the meristematic zone Cell files of Epidermis, cortex, endodermis and vascular tissues as well as columella cells arise from initial cells for each specific cell type within the meristem (dotted line). Initials surround the Quiescent Center (QC), which maintains their cell-division activity but has a low division frequency itself**. C** Cross section through the differentiation zone of a *P. tremula x P. alba* root. From outside to inside epidermis, cortex, endodermis, pericycle and vascular tissues are visible. Pericycle cells surround vascular tissues. Some epidermis cells differentiate into root hairs. **D** Microscopic view of a cross section through the center of a root of *Ranunculus sp*. (a dicot like Arabidopsis and poplar). Pericycle, phloem and xylem form together the stele. Xylem cells are arranged in a star-shaped form building four xylem poles (orange stars). D was modified from http://kentsimmons.uwinnipeg.ca/16cm05/16labman05/lb4pg3.htm

whether those are embryonic, adventitious or other lateral roots but only as long as the parental root has not undergone secondary thickening (Kolek and Kozinka, 1992). Lateral roots are important for the plant to enhance its soil surface for nutrient absorption but also in term of anchorage. Moreover, lateral roots present important contact sites for plant interactions with microorganisms in the rhizosphere as discussed later.

I.2.2 Root apex organization

Although impressive progress has been made in crops and trees, the herbaceous model plant *Arabidopsis thaliana* remains the best-characterized experimental system for studying root development and will be the main focus here. Angiosperm roots have a rather stereotypic cellular anatomy. The root apex can longitudinally be divided into three different zones (Fig. 2A) and is transversally formed by nested cylindrical layers representing the tissue types of the root — epidermis, cortex and endodermis — which in turn enclose a central vascular cylinder, containing pericycle, phloem and xylem (Fig. 2B and C). In the meristematic zone (MZ) at the root apex these cell files converge into the meristem, also termed the stem cell niche, where they are initiated. The MZ in Arabidopsis plants contains four Quiescent Center (QC) cells, which are surrounded by initials (dotted line in Fig. 2B) located at the distal end of each cell file. QC cells have a low division frequency. Their role is to maintain the initials in an undifferentiated state and QC cells may eventually replace initials (van den Berg et al., 1997). Initials divide frequently and give rise to new cells in their own cell file and for some (lateral root cap and epidermis) also in a neighbouring cell file. Pericycle and protophloem cells start already to differentiate in the MZ, whereas cell of other cell-files differentiate more proximal to the root apex. The proximal zone of the meristem partially covers the elongation zone (EZ). This overlapping zone is often called the transition zone and has been proposed as the zone of high absorption of and sensitivity to exogenous factors (Baluska et al., 1996, 2001). Due to the high absorption rate, this zone is also extremely sensitive to exogenous signals (e.g. auxin) and has furthermore been proposed to be the starting point for plant/soil microbe interaction. Cells in the EZ, which have stopped dividing, elongate according to the axis of their respective cell file. This is responsible for the growth of the entire root. Phloem or protoxylem start to differentiate in this zone. In the next more proximal zone, called the differentiation zone (DZ), the elongated cells differentiate into epidermis, cortex, endodermis or vascular cells (Fig. 2C). In this part of the root, epidermis cells can undergo further differentiation into root hair cells, which facilitate water absorption into the root. Within the central part of the root, enclosed by the pericycle, the cells differentiate to form the vascular bundles of phloem and xylem (Fig. 2D), named protophloem and protoxylem, at the distal side of the DZ. While *Ranunculus* (Fig. 2D) has four xylem poles with the pericycle, Arabidopsis has a diarch root tissue structure and thus only presents

two xylem poles. LRs in Arabidopsis arise from pericycle cells adjacent to one of the two (proto)xylem poles in the DZ. These cells carry cytological features such as dense cytoplasm, large nuclei and small vacuoles thereby resembling meristematic cells and are different from the quiescent phloem pole pericycle cells (Parizot et al., 2008). As LRs cannot form in a mutant that only has xylem pole pericycle cells but lacks the phloem pole cells, Parizot and co-workers (2008) have suggested that the difference between xylem and phloem pole pericycle cells is crucial for LR development. One possibility for this phenomenon could be that the flow of certain factors that trigger LR development is different between xylem- and phloem-pole pericycle and that fine-tuning of the accumulation of these factors prior to LR initiation requires the coordination of this flow by xylem- and phloem-adjacent cells. A candidate for such a factor, which is crucial for LR development, is the phytohormone auxin.

II. Regulation of lateral root development

II.1 The phytohormone auxin

Phytohormones (plant-growth substances) are metabolites that exist in extremely low quantities and influence in a dose dependent manner the development of all plant organs. The discovery of the effect of the phytohormone auxin more than a century ago marked the beginning of research in plant growth regulation. When Charles and Francis Darwin (Darwin, 1880) observed the curvature of oat coleoptiles toward the light, they suggested the involvement of a growth-regulating substance long before the word 'hormone' was used (first in humans) (Starling, 1905). It took until the 1920s to demonstrate the presence of such a mobile substance, which promoted photo- and gravitropism, respectively (Went, 1926; Cholodny, 1927). The responsible signalling molecule was termed auxin (from the Greek *auxein*, to grow) and identified as indole-3-acetic acid (IAA) (Went and Thimann, 1937). Among the first known biological processes influenced by auxin in addition to tropisms were the growth inhibition of the primary root as well as the stimulation of lateral root initiation and adventitious rooting (Hitchcock and Zimmermann, 1942). Today auxin is understood as a key-regulator of lateral root development and numerous molecular mechanisms have been identified that help us understand the molecular events triggered by auxin during lateral root development.

II.1.1 Auxin gradients drive root system development

A feature that appears universally associated with auxin action is its heterogeneous distribution throughout the plant with the presence of zones (single cells or groups of cells) of high auxin concentrations, called auxin maxima. These zones have been revealed by auxin measurements (Petersson et al., 2009) but also indirectly by the use of an artificial auxin responsive promotor, *DR5*,

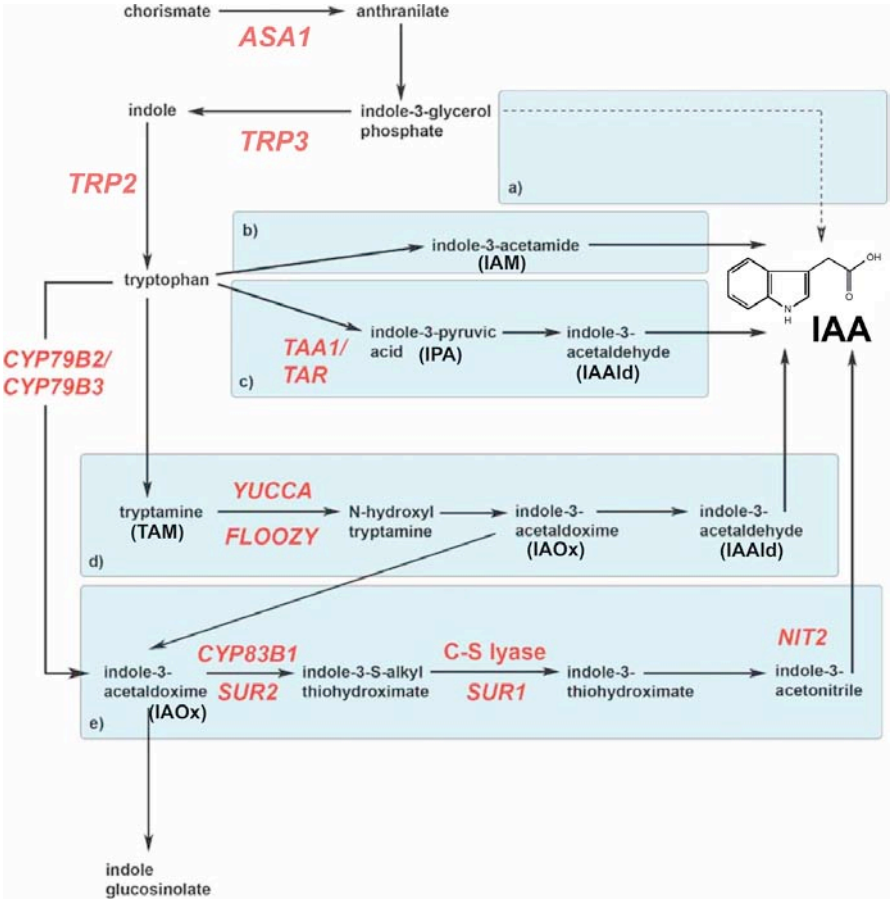

Figure 3: Diversification of auxin biosynthesis pathways

(a) the postulated tryptophan (Trp)-independent pathway and the four main branches of Trp-dependent synthesis via, **(b)** indole-3-acetamide, **(c)** IPA, **(d)** tryptamine or **(e)** indole-3-acetaldoxime. The positions of enzymes encoded by genes that result in a phenotype when mutated or over-expressed are shown. Modified from Chandler 2009.

made up of several auxin-response elements (Ulmasov et al., 1995; Ulmasov et al., 1997) fused to marker genes such *β-Glucuronidase* (*GUS*) (Ulmasov et al., 1997) or *Green Fluorescent Protein* (*GFP*) (Ottenschlager et al., 2003). Their use has uncovered that, within the root system, auxin specifically accumulates at root apices where it regulates meristem identity (Sabatini et al., 1999) and also coincides with the onset of LR development (Dubrovsky et al., 2008). Auxin accumulation may arise from locally increased biosynthesis (also in roots, the usual auxin-sink tissues), altered metabolism (auxin conjugation and catabolism) or through directed polar auxin transport (PAT). Where auxin accumulates, it triggers downstream signalling by binding to its receptors and activating transcription of auxin responsive genes that further tune LR development.

II.2 Auxin homeostasis, transport and signalling

II.2.1 Auxin biosynthesis

The natural occuring and most intensively studied auxin, which is active in all different realized auxin biotests, is indole-3-acetic acid (IAA) (Woodward and Bartel, 2005). Different biosynthetic pathways that lead from distinct precursors to IAA have been identified (Fig. 3 and Woodward and Bartel (2005)). One tryptophan (Trp)-independent (Fig. 3a) and four Trp-dependent- (Fig. 3b to e) pathways can be distinguished. The Trp dependent pathways – named respective to their intermediates – are the indole-3-acetamid (IAM), the indole-3-pyruvic acid (IPA), the tryptamine (TAM) and the indole-3-acetaldoxime (IAOx) pathways. IPA and IAM-auxin-biosynthesis pathways have also been found in microorganisms (Koga, 1995; Patten and Glick, 1996). Woodward and Bartel (2005) had suggested that basic auxin biosynthesis in plants may mostly happen in a Trp-independent pathway and that Trp-dependent IAA biosynthesis may be activated when increased endogenous IAA levels are required, e.g. in a stress situation. In this case, the various possibilities of IAA biosynthesis may be involved in coordinating the response of the plant to distinct environmental stimuli. On the other hand, in each of the Trp-dependent pathways genes were discovered that, when over-expressed or mutated, caused a root phenotype in a distinct developmental process (Fig. 3, reviewed in Chandler (2009)). This would suggest that the diversification of the auxin biosynthesis pathway permits localized auxin synthesis in specific organs and during distinct developmental processes. For example, the quadruple mutant *yuc1 yuc4 yuc10 yuc11* from the *YUCCA* family involved in the tryptamine-pathway did not form roots (Cheng et al., 2007). Also, absence of TAR proteins (in the *taa1(=wei8), tar2* double mutant) in the IPA-pathway led to differentiation of the stem cell niche within the root meristem (Stepanova et al., 2008) and mutations of *SUR1* and *SUR2* in the IAOx pathway impacted adventitious and lateral root development (Boerjan et al., 1995; Delarue et al., 1998; Barlier et al., 2000; Mikkelsen et al., 2004). It has recently been shown that different auxin biosynthetic enzymes are expressed in a tissue-specific manner within the root (Petersson et al., 2009), which would explain the various root phenotypes caused when specific

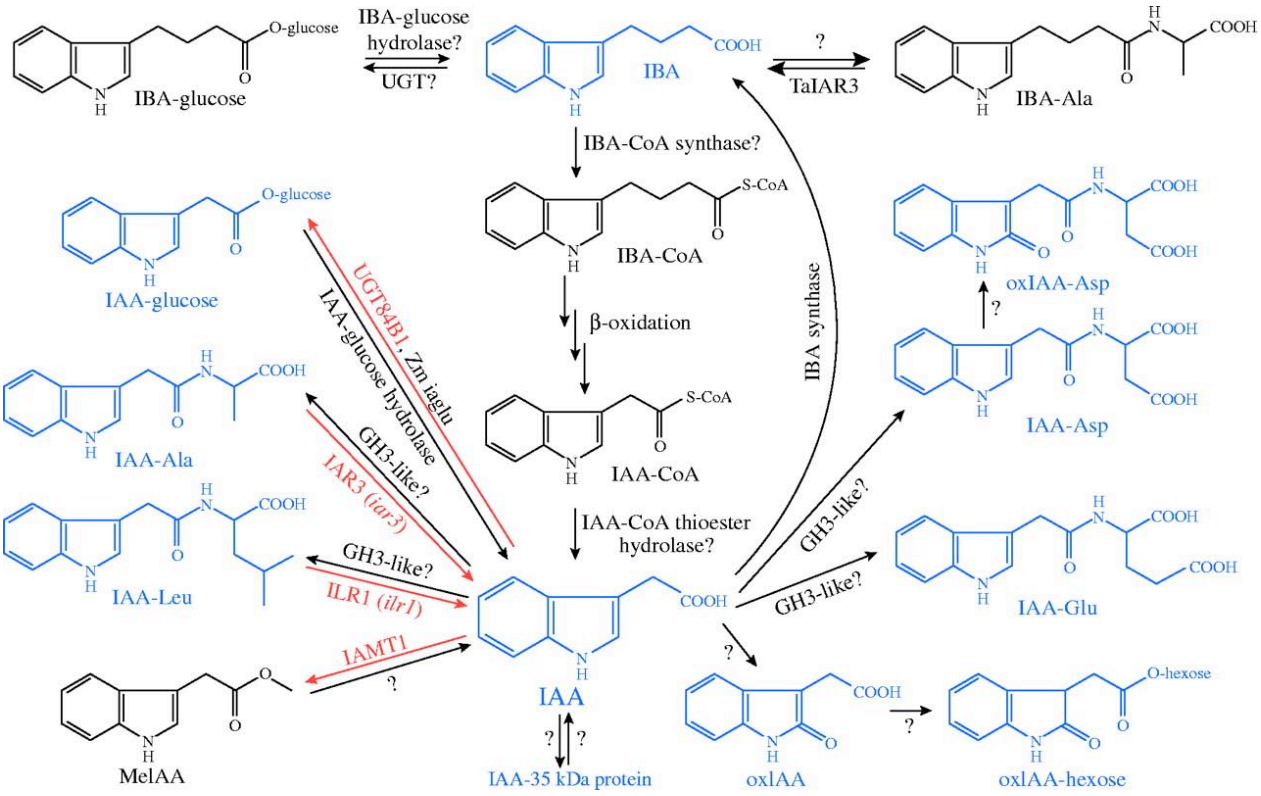

Figure 4: Potential pathways of IAA metabolism.

Compounds detected in Arabidopsis are in blue, enzymes for which the Arabidopsis genes are cloned are in red, and Arabidopsis mutants are in lower-case italics. Suggested conversions for which plant genes are not identified are indicated with question marks. According to Woodward and Bartel (2005). GH3 amido-synthetases conjugate IAA to Ala or Leu for reversible storage and liberation of free IAA through hydrolysas or to Glu and Asp for degradation. IAA can also be converted into IBA from which free IAA can be released through peroxisomal beta oxidation. Oxidation of the indole ring leads to formation of oxIAA that forms sugar-esters and is degraded. Conjugation of IAA to sugars in the absence of ring oxidation is reversible. The carboxyl group of the IAA side chain may be methylated, a reaction suggested to be reversible.

members are mutated. This adds an interesting point to auxin balances in the plant: whereas in classical models auxin is produced in the leaves as a source tissue and only transported into the roots (sink tissue), now there is evidence that auxin biosynthesis in the root also has to be taken into account when looking at auxin gradients.

II.2.2 Auxin conjugation, recycling and catabolism

In addition to auxin biosynthesis, storage of IAA in the form of conjugates or after conversion to indole-3-butyric-acid (IBA) or targeting excess IAA for degradation are important mechanisms to regulate a cell's auxin balance (Woodward and Bartel, 2005)(Fig. 4). Auxin can be liberated from storage specific conjugates upon demand by beta-oxidation or hydrolysis. In Arabidopsis seedlings almost 90% of IAA is present as amide-linked conjugates (IAA connected to amino acids), 10% as esters (IAA linked to sugars) and only 1% is free IAA (Normanly et al., 1993; Tam et al., 2000). Even if these profiles vary between different plant species (Cohen and Bandurski, 1982), this points out the importance of IAA conjugates. Proposed functions of these conjugates include storage, compartmentalization, excess IAA detoxification and protection against peroxidative degradation (Cohen and Bandurski, 1982). Conjugation pathways that are reversible are characteristic for IAA storage whereas irreversible pathways are involved in IAA degradation. IAA amino-acid conjugates found in plants can be classified into these two groups based on bioassay activity and susceptibility to hydrolysis *in planta* or by plant enzymes. IAA-Ala and IAA-Leu are substrates of IAA-amido hydrolases such as ILR1 and IAR3 (Fig. 4) (Bartel and Fink, 1995; Davies et al., 1999). As free IAA can be released, IAA in the form of these conjugates efficiently inhibits Arabidopsis root elongation (Bartel and Fink, 1995; Davies et al., 1999; LeClere et al., 2002; Rampey et al., 2004). IAA-Leu is likely to be the root specific IAA-amide storage (Kowalczyk and Sandberg, 2001). In contrast IAA-Glu and IAA-Asp are neither hydrolysed (Ostin et al., 1998) nor do they activate root inhibition (LeClere et al., 2002). These results are consistent with a catabolic role for IAA-Asp and IAA-Glu. Interestingly, IAA-amido-synthetases of the GH3 family are among the earliest transcripts that respond to an exogenous auxin accumulation (Hagen and Guilfoyle, 1985), suggesting that auxin directly regulates its own balance inside the plant. The GH3 gene family in Arabidopsis consists of 18 members (Staswick et al., 2005), which are likely to have specific affinities for the respective amino-acids they conjugate IAA to, different responsiveness to auxin increases and tissue specific expression. In accordance to their proposed role as a dynamic IAA-source, IAA-Leu and IAA-Ala do not form upon exogenous auxin application (Ostin et al., 1998; Barratt et al., 1999), because degradation and not storage is required. Thus for example in tissues with high auxin levels, IAA-Glu and IAA-Asp can be found and indicate active auxin catabolism to maintain the auxin equilibrium (Kowalczyk and Sandberg, 2001).

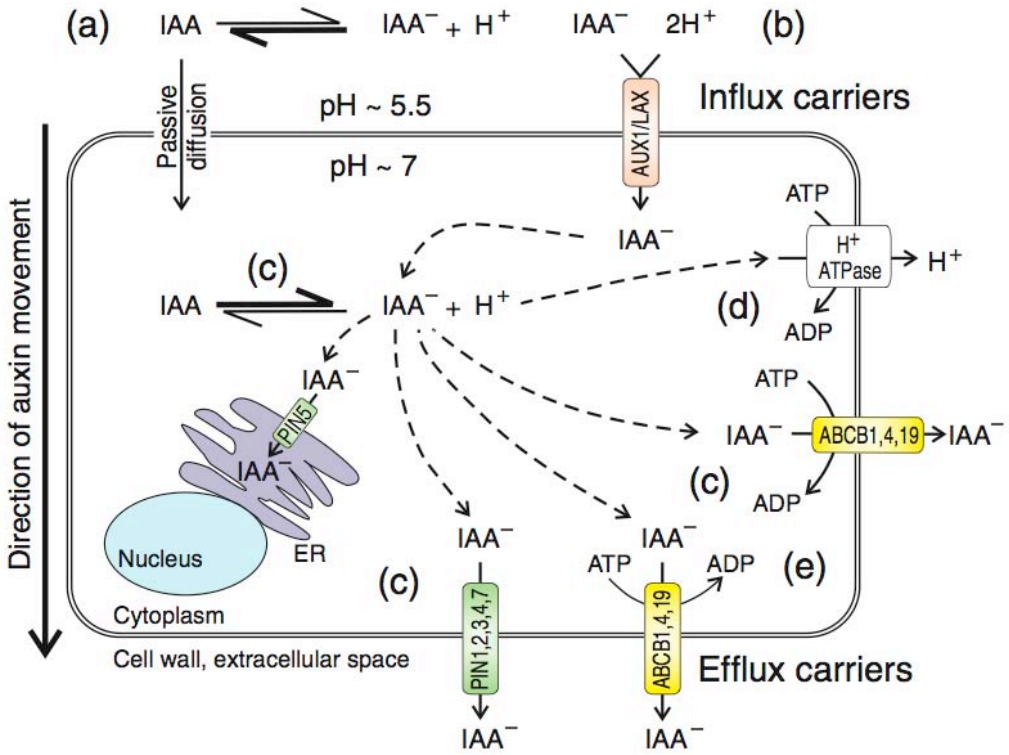

Figure 5: Cellular auxin transport across the plasma membrane.

Auxin can enter the cell through passive diffusion when protonated (**a**) or through AUX/LAX IAA-Proton co-transporters (**b**). Once inside the cell, IAA is de-protonated and can quit the cell only by PIN (**c**) or PGP (**e**) auxin transporters. PIN proteins of the PIN5-like subfamily are suggested to compartmentalize auxin by their ER localization (Mravec et al., 2009). The driving force (proton gradient) for plasma membranous PIN auxin efflux carriers is established by a H^+-ATPase (**d**), whereas ABCB transporters have ATPase activity themselves. Schema from Petrasek and Friml (2009).

II.2.3 Polar auxin transport (PAT)

Transport of auxin into or away from a tissue also modifies auxin concentrations. Two main pathways describe auxin transport, a fast, non-directional transport of conjugated auxin through the phloem (speed about 5-20cm/h) and a ten times slower, directional transport of free auxin, called polar auxin transport (PAT) (reviewed in Friml and Palme (2002)). The major polar auxin (Fig. 6A) fluxes run towards the root apex through the stele (acropetal root PAT). In the root apex the auxin flow is returned through the outer cell layers (epidermis) into the elongation zone (basipetal root PAT), where auxin enters again the acropetal auxin flow through the stele. This creates an auxin reflux loop at each root apex (Blilou et al., 2005).

PAT was discovered in the 1970s. In experiments where IAA was applied to pea buds and IAA distribution was detected in transverse shoot sections, Morris and Thomas (1978) had revealed phloem-independent auxin movement. Other studies had given rise in parallel to the chemiosmotical theory: IAA is a weak acid and is present in its protonated form in the apoplast, which has a low pH (5.5). It therefore can enter the cell passively by diffusion through the plasma membrane. But once it arrives in the cell, where a higher pH (7) is present it gets deprotonated into a charged membrane-impermeable form and is thus trapped in the cell. It had thus been proposed that the export of auxin requires active transport through auxin efflux carriers (Rubery and Sheldrake, 1974; Raven, 1975). It was further postulated that an asymmetric polar membrane localization of the efflux carriers would determine the direction of the intercellular auxin transport. This hypothesis gained strong molecular support when the first auxin carrier proteins were identified in the end of the 1990's (Bennett et al., 1996; Chen et al., 1998; Galweiler et al., 1998; Luschnig et al., 1998; Muller et al., 1998). Today, in Arabidopsis, three protein families with auxin-transport activity are known: (1) the auxin influx carrier family AUX/LAX (Marchant et al., 2002; Yang et al., 2006; Bainbridge et al., 2008; Swarup et al., 2008), which mediates enhanced auxin entry into the cell, (2) the auxin efflux carrier family PIN (Blilou et al., 2005; Petrasek et al., 2006; Wisniewska et al., 2006), which determines the directionality of auxin transport by its subcellular localization, and (3) the ATP-dependent ABCB/PGP transporter proteins (Noh et al., 2001; Noh et al., 2003; Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Mravec et al., 2008) (Fig. 5).

II.2.3.a AUX1/LAX auxin influx carriers

The Arabidopsis AUX1/LIKE AUX1 (AUX1/LAX) family of proton-driven amino-acid permeases that catalyze auxin influx into cells consist hitherto of four members. The first member, AUXIN RESISTANT 1 (AUX1), was identified in a mutant screening as having an agravitropic phenotype and being resistant to the synthetic exogenous auxin 2,4 D (Bennett et al., 1996). Recently, a biochemical characterization of AUX1 and LAX3 has shown that these two influx carriers indeed mediate IAA uptake when heterologously expressed in *Xenopus* oocytes (Yang et al., 2006; Swarup

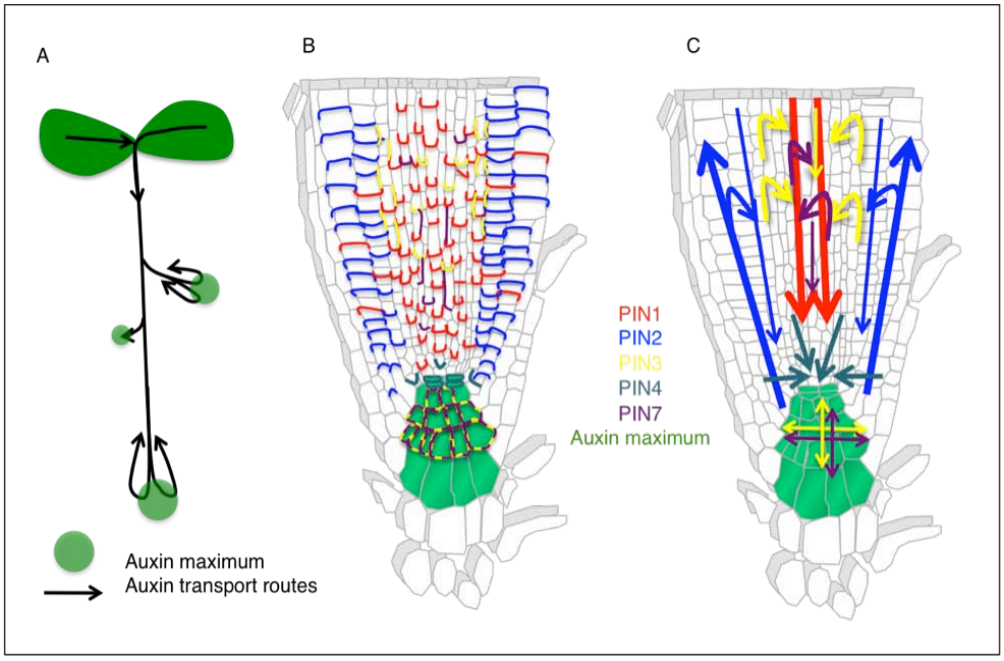

Figure 6: Polar auxin transport through PIN auxin efflux carriers.

A Scheme of general auxin transport routes within an Arabidopsis seedling. Auxin comes from the leaves and is transported into the roots. At roots tips an auxin maximum is formed (in green) and auxin enters the reflux loop. **B** Scheme of polar localization of PIN1-like subfamily PIN proteins and **C** the fluxes that are created by the polar localization of PIN proteins (according to data from Blilou et al. (2005), root model from Yvon Jaillais).

et al., 2008). Concerning localization of AUX1/LAX in root tissues, AUX1 is found in columella and lateral root cap cells as well as in protophloem cells. In the latter, it has been suggested to mediate auxin unload from the long distance transport through the phloem for further polar transport to the root tip (Swarup et al., 2001). AUX1/LAX carriers are regulated on a transcriptional level and through endocytosis on a posttranscriptional level (Kleine-Vehn et al., 2006). The cycling of AUX1 between plasma membrane and intracellular vesicular compartments can be regulated by the ER localized AXR4 (Petrasek and Friml, 2009) (Fig. 7).

II.2.3.b PIN auxin efflux carriers

In Arabidopsis, the PIN family consists of eight members that can be divided into two subfamilies (Mravec et al., 2009), the PIN1-like and the PIN5-like subfamily. Interestingly in poplar we can find each Arabidopsis *PIN* duplicated, so a total of *16* PINs are present. In Arabidopsis, tomato and poplar, PIN1-like family members are involved in auxin transport from cell to cell and are expressed at the plasma membrane. PIN1-like proteins are expressed in Arabidopsis roots in a partially overlapping pattern (Blilou et al., 2005) (Fig. 6B) and their expression sites are important to direct a root's auxin flow. In Arabidopsis, it has recently been shown that PIN5-like family members are localized at the ER membrane and may contribute to a cell's auxin homeostasis through compartmentalization (Mravec et al., 2009) (Fig. 5).

Blilou and co-workers (2005) localized all of the PIN1-like proteins in Arabidopsis by immuno-histochemical techniques or as GFP-fusions proteins. PIN1 was found to be expressed at basal ends of stele cells as well as weakly in an apical orientation in the epidermis and in a basal localization in cortex cells (Fig. 6B). PIN2 localized apically in epidermal and basally in cortical cells. As for PIN3, the protein was detected without pronounced polarity in columella cells as well as at basal sides of vascular cells and at lateral sides of pericycle cells in the EZ. PIN4 was revealed to be around the QC, as well as basally in provascular cells. PIN7 expression overlapped with PIN3 in columella cells and was additionally observed in lateral and basal orientation in provascular cells in the MZ and EZ. The specific localization of PINs drives the direction of the auxin flux through the plant (Fig. 6C). PIN1 and PIN2 are key-mediators for the acropetal auxin transport through the stele towards the root tip (PIN1) or the basipetal auxin transport through the epidermis from the root tip towards the elongation zone (PIN2).

The partially overlapping localization of all other PIN proteins already denotes a possible redundancy in their function. Indeed *pin* single mutants in Arabidopsis only display (if at all) a weak phenotype (Galweiler et al., 1998; Muller et al., 1998; Friml et al., 2002b; Friml et al., 2002a; Blilou et al., 2005), suggesting that other members of the PIN family may replace a defective or missing PIN. This assumption has been reinforced by visualization of ectopic protein expression of the remaining PIN proteins in *pin* mutants (Blilou et al., 2005). Analyzing double and higher order *pin*

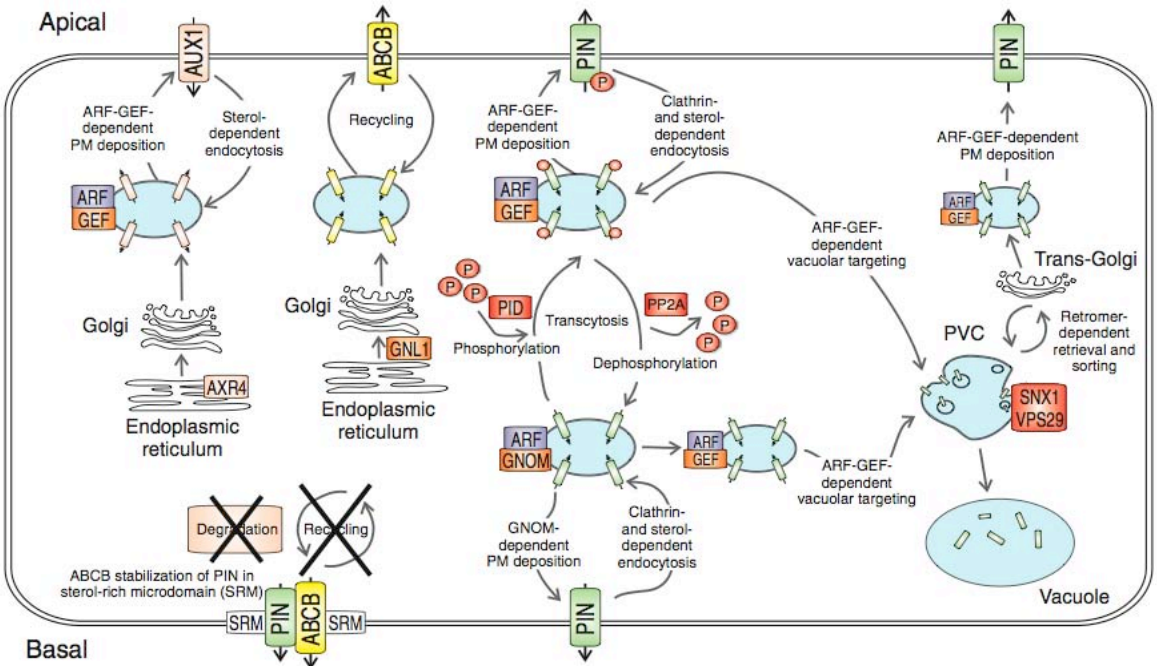

Figure 7: Intracellular trafficking of auxin transporters

A typical feature of plasma membranous proteins such as auxin transporters is their transport through vesicle trafficking. AUX1, PGP (ABCB) and PIN transporters all cycle between the plasma membrane and endosomal compartments (blue). Cycling can be mediated by clathrin (PIN1 and 2) (Dhonukshe et al., 2007) and/or by sterols (AUX1, PIN) (Willemsen et al., 2003; Kleine-Vehn et al., 2006; Men et al., 2008). Interaction of PGP19 with PIN1 in sterol-rich micro-domains stabilizes PIN membranous localization by prohibiting internalization. Different ARF/GEF proteins with endosomal localization target the transporters specifically to apical or basal membranes. AUX1 proper localization is further facilitated by ER located AXR4 as well as PGP by ER localized GNOM-Like (GNL1) (Titapiwatanakun et al., 2009). ARF-GEF-dependent endosomal sorting is also involved in the trafficking of PIN2 to the lytic vacuolar pathway through the prevacuolar compartment (PVC), from which PIN proteins might be retrieved again into the trans-Golgi network through the assistance of the retromer complex subunits SORTING NEXIN 1 (SNX1) and VACUOLAR PROTEIN SORTING 29 (VPS29) (Jaillais et al., 2006). PIN phosphorylation through PID or dephosphorylation through PP2A can modify the polar localization of these carriers through transcytosis (Michniewicz et al., 2007). Scheme from Petrasek and Friml (2009).

mutants, these authors could reveal which PINs mask, by their ectopic expression, the phenotype of close family members. Strikingly, *pin1pin2* and all triple and quadruple mutants containing *pin2* showed more than an additive reduction in root and meristem size. Therefore a pivotal role for PIN2 in cell division control was proposed. Another study has shown that PIN expression is cross-regulated (Vieten et al., 2005). Auxin has a feedback through the basic auxin-signalling pathway (see below) on PIN expression and the absence of one member of the PIN family alters auxin fluxes in a way that activates expression of a remaining member. This data shows how the auxin gradient can be stabilized by a self-regulating system and emphasizes the first process of PIN regulation, which is transcriptional control.

A second way of controlling auxin fluxes through PIN is their polar, asymmetric localization. This is subjected to control on a post-translational mechanism: endocytosis. It has been identified that asymmetric, polar localization of PIN proteins depends on the membrane-associated guanine-nucleotide exchange factors of ADP-ribosylation factor G protein (ARF GEF) GNOM, which regulates endocytic cycling of PINs to distinct sides of the cell (Steinmann et al., 1999; Geldner et al., 2001) (Fig. 6). Internalization of PIN1 and PIN2 is furthermore mediated either by clathrin (Dhonukshe et al., 2007) or by the sterol composition of the plasma membrane (Willemsen et al., 2003; Men et al., 2008) as well as by the actin cytoskeleton (Dhonukshe et al., 2008). A third PIN internalization regulator is the phosphorylation state of the carrier. PIN proteins can be phosphorylated through PINOID kinases or dephosphorylated by PP2A phosphatases (Benjamins et al., 2001; Michniewicz et al., 2007), which are important for proper endocytic polar PIN targeting (Friml et al., 2004). PINOID kinases get transcriptionally activated by auxin and accelerate auxin efflux from cells by inhibiting internalization, thereby accumulating PINs at the plasma membrane (Paciorek et al., 2005).

In addition to transcriptional and endocytic, polar targeting of PINs, auxin efflux can also be regulated by the PIN activity itself. In this context it has been shown that interaction of PINs with phosphoglycoproteins (PGPs, see below) alters substrate specificity and inhibitor sensitivity (Petrasek et al., 2006; Blakeslee et al., 2007). Interaction of PGPs with PINs can also again modify its internalization activity (Fig. 7).

II.2.3.c Auxin transport through ABCB/PGP proteins

A third group of proteins that can transport auxin are homologs of human membrane integral ATP-binding cassette (ABC) multidrug transporting (MDR) phosphoglycoproteins (PGP) (Noh et al., 2001; Blakeslee et al., 2005). Three of the 21 members in Arabidopsis (PGP1, PGP4 and PGP19) have been shown to mediate auxin efflux from Arabidopsis and tobacco protoplast cells as well as from heterologous systems such as yeast and HeLa cells (Geisler et al., 2005; Terasaka et al., 2005; Petrasek et al., 2006). Petrasek et al. (2006) therefore suggested that PGPs and PINs molecularly

characterize different auxin transport systems. Intriguingly, their data, as well as a follow-up study (Blakeslee et al., 2007), showed that auxin transport by PINs or PGPs in the absence of the other was less substrate specific in heterologous systems (Petrasek et al., 2006; Blakeslee et al., 2007) as well as *in planta* (Blakeslee et al., 2007). Furthermore, Blakeslee and co-workers's (2007) uncovered that PIN1 interacted with PGP1 and PGP19 using co-immunoprecipitation and yeast two hybrid experiments. PIN1/PGP19 co-localization *in planta* was dependent on the analyzed tissues. PGP1 was expressed in all root cells, except for the columella (Mravec et al., 2008), whereas PGP19 expression was restricted to the endodermis and the pericycle. This might allow for separation of the acropetal and basipetal auxin fluxes through PIN1 in the stele and the epidermis, respectively (Blakeslee et al., 2007; Wu et al., 2007; Mravec et al., 2008). PGP4 expression coincided with PIN2 and AUX1 in the epidermis (Terasaka et al., 2005; Wu et al., 2007) and indicated a role in basipetal auxin transport. Together, these results suggested that PINs and PGPs characterize coordinated, independent auxin transport mechanisms, but also function interactively in a tissue-specific manner. As shown for PINs and AUX1/LAX, PGPs are also regulated on a transcriptional level by auxin (Geisler et al., 2005; Terasaka et al., 2005) as well as by endocytic cycling (Titapiwatanakun et al., 2009) (Fig 7). PGP anchorage in sterol-rich membrane pits inhibits their internalization.

Taken together, there are three different mechanisms that regulate auxin availability in a distinct tissue: biosynthesis, conjugation and transport. The high diversity of auxin biosynthesis pathways, the different molecules auxin can be conjugated to and the variety of auxin transporters that can be modulated on a transcriptional and various post-translational levels makes the regulation of auxin availability in a certain tissue or in a cell a complex process.

II.2.4 Auxin signalling

When auxin accumulates in a cell, it triggers auxin signalling. In *Arabidopsis thaliana* two types of proteins are known that have affinity to auxin and have been discussed as possible auxin-receptors: the F-box family, Leucine Rich Repeat (LRR) proteins TIR1, AFB1, AFB2 and AFB3 (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski and Leyser, 2005; Tan et al., 2007), and auxin-binding-protein 1 (ABP1) (reviewed in (Napier et al., 2002)). TIR1 is present in the nucleus and thus regulates responses to intracellular auxin. ABP perceives extracellular auxin signals at the plasma membrane. There, respective localization makes one believe that ABP may trigger fast external auxin responses and TIR1 and AFB proteins slower responses (Badescu and Napier, 2006). While little is known about how ABP1 triggers auxin responses downstream of perception (Tromas et al., 2009), signalling through TIR1 has been well studied (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005; Tan et al., 2007). In a recent study of crystallized TIR1, Tan and co-workers (2007) discovered that IAA binds into a binding pocket of TIR1 and, as a consequence, stabilizes the

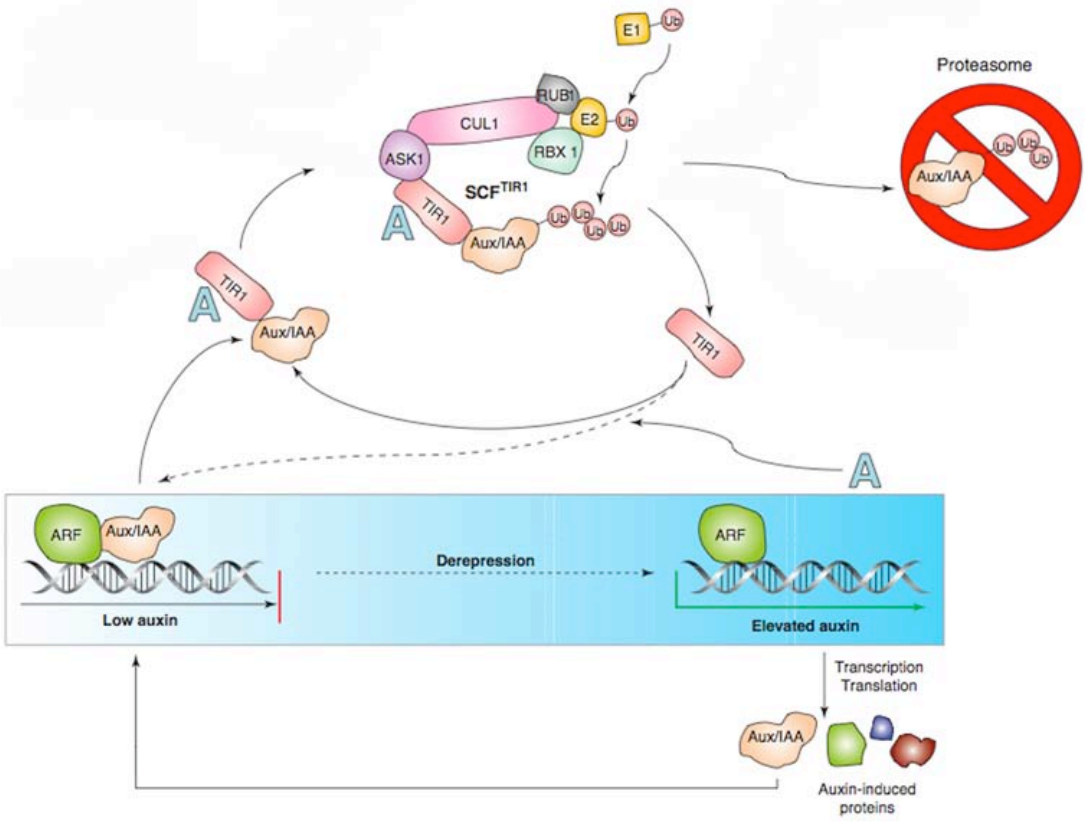

Figure 8: TIR1-dependent auxin signalling

IAA (A) binds to auxin receptor TIR1 and triggers recruitment of transcription factors AUX/IAA into the complex. TIR1/Auxin/AUX/IAA form the SCF^{Tir} complex, where AUX/IAA get ubiquitinated and hence degraded by proteasomes. Aux/IAA degradation de-represses auxin response factor (ARF), which can trigger then transcription of auxin responsive genes. Simplified from Badescu and Napier (2006)

interaction of TIR1 with the transcription factors AUX/IAA. AUX/IAA transcription factors are negative regulators of auxin response genes. They consist of four domains, of which two are necessary for interaction with AUXIN RESPONSE FACTORS (ARF) transcription factors, and the two others form the instability domain, allowing for their rapid turn over (Guilfoyle et al., 1998). In the absence of an auxin signal, AUX/IAAs interact with ARFs and repress their activity. Based on molecular data, a model of auxin signalling has been proposed by Badescu and Napier (2006) (Fig. 8). When IAA binds to TIR1 and thereby stabilizes the TIR1-AUX/IAA interaction, a super-complex of proteins, named SCF^{TIR1} gets recruited around the auxin bound TIR1-AUX/IAA proteins (Badescu and Napier, 2006). The activity of E3 ubiquitin ligases within this complex leads to AUX/IAA ubiquitination that is recognized as a signal by proteasomes, which then degrade AUX/IAA proteins. This de-represses Auxin Response Element (ARE) bound ARFs on auxin responsive genes, which can then activate transcription of their targets. In Arabidopsis, the AUX/IAA and ARF families have 29 and 23 members, respectively. They are large gene families and the role and interaction of different members are not yet extensively studied. Interestingly, even if the second type of auxin receptor, ABP1, still has an uncharacterized downstream signalling pathway, specific AUX/IAAs have recently been reported to lack activation when ABP1 is conditionally repressed and might create a convergence point of ABP1 and TIR1 dependent auxin signalling (Tromas et al., 2009). Most studies on AUX/IAA transcription factors use stabilized versions of these proteins that carry a mutation in the instability domain to prevent their degradation (Fukaki et al., 2002), thereby rendering downstream signalling impossible. A prominent AUX/IAA-ARF couple during LR development is IAA14 and the two redundant ARFs, ARF7 and ARF19 (Fukaki et al., 2002; Fukaki et al., 2005; Vanneste et al., 2005; Okushima et al., 2007). TIR1, IAA14, 7 and 3 as well as ARF7/ARF19 are hereafter referred to as members of the "basic auxin signalling pathway".

In conclusion, in addition to the numerous mechanism regulating auxin-homeostasis, the various proteins in the auxin signalling cascade and the diversification of *ARF* and *Aux/IAA* gene family makes also auxin signalling a complex process and permit its regulation on various levels, such as gene expression, protein interactions and protein degradation.

II.3 Mechanisms regulating lateral root development

According to recently available results that show genetic separation of different phases of LR development, I will present the molecular events controlling LR formation in each of the following four (partially overlapping) processes:

1. Priming of founder cells (pre-initiation phase),
2. Lateral root initiation (LRI),

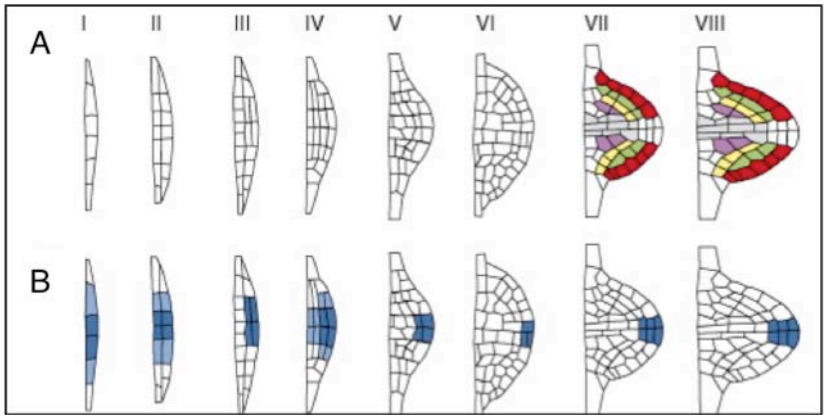

Figure 9: Stepwise model of LR formation

A LRs originate deep within the primary root from the pericycle. Cell divisions during the eight stages of LR formation giving rise to the new tissue of the LR. Epidermis (red), cortex (green), endodermis (yellow), pericycle (violet). **B** illustrates auxin accumulation (in blue) during LRI and subsequent restriction of the signal to the new forming meristem, such as observed with *pDR5:GUS* reporter lines. Images A to B originate from Péret et al. (2009).

3. Lateral root primordia (LRP) development including meristem formation and
4. LR emergence.

From LR initiation until emergence, eight different stages of lateral root primordia (LRP) (Fig. 9A) can be distinguished according to the number of cell layers of the forming LRP (Malamy and Benfey, 1997). When founder cells undergo several rounds of anticlinal cell divisions (Malamy and Benfey, 1997; Casimiro et al., 2001; Dubrovsky et al., 2001), a single layered LRP composed of up to ten small cells of equal length forms (stage I, Fig. 9B). The next divisions are in a periclinal orientation and lead to formation of the inner and the outer cell layer that is first visible in stage II LRPs. Further subsequent cell division in both anticlinal and periclinal directions (stage III to VII) give rise to the dome-shaped LRP before its emergence at stage VIII.

II.3.1 Priming of founder cells in the basal meristem (pre-initiation phase)

The first event in the formation of a new plant organ involves specification of founder cells, which upon further activation start to divide and form a primordium (Beveridge et al., 2007). Dubrovsky et al. (2008) found, using the synthetic auxin responsive promotor DR5 (Ulmasov et al., 1997) fused to green fluorescent protein (GFP), that DR5:GFP expression was present in specific xylem-pole adjacent pericycle cells and that all these, but not GFP negative cells, gave rise to LRP. In the *alf4-1* mutant, which does not form any LRP, GFP expression under the *pDR5* promotor was detectable but no LRI occurred, implying that the *pDR5* driven GFP signal was specific to founder cell identity and not to LRI (Dubrovsky et al., 2008). De Smet et al. (2007) detected founder cell specification in still more distal tissues in the basal meristem (Fig. 10). These authors showed that founder cell specification and auxin accumulation in the basal meristem were independent of auxin signalling through IAA14, which is known to be involved in LRI (see below). This is in accordance with results from Dubrovsky et al. (2008) and strengthens the genetic independence of founder cell specification and LRI. Dubrovsky and colleagues (2008) furthermore demonstrated by artificial expression of auxin biosynthetic genes in root tissues under an inducible, non-specific promotor that an auxin accumulation in one pericycle cell was sufficient to confer founder cell identity to this cell. As they had revealed in a former study that LRs form from a minimum of three or six founder cells depending on whether the initiation is of a longitudinal uni-cellular or bi-celluar type (Dubrovsky et al., 2001), it may be suggested that an unique founder cell can communicate with its neighbouring cells to recruit them into the cell pool for LRI (Fig. 11). They concluded from these results that *pDR5:GFP* expression, which refers to auxin response and to auxin accumulation, at least in Arabidopsis (Petersson et al., 2009), is a marker for LR founder cells. They suggested that auxin may act as a morphogenic trigger, which through unequal distribution of its activity induces a new developmental fate in a cell. The question that arises is what is the origin of this auxin accumulation?

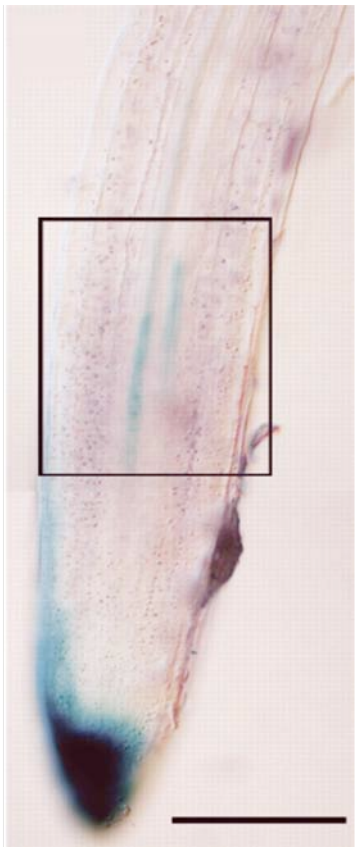

Figure 10 : Founder cell specification in the basal meristem

Arabidopsis plants (40 h after germination on MS medium) harbouring the *pDR5:GUS* marker show a signal in protoxylem cell in the basal meristem (boxed area), adjacent to the pericycle cells that are specified as founder cells for LRI. From De Smet et al. (2007)

In the study by De Smet and co-workers (2007) it was reported that the appearance of *pDR5* directed GUS stained cells in the basal meristem (Fig. 10) was drastically reduced in the *aux1* mutant line, which also has a reduced LR number. Recently the basal meristem has been shown to recycle auxin channelling back from the root tip through the root cap (Blilou et al., 2005) and again back towards the stele (Blilou et al., 2005; Ditengou et al., 2008). De Smet and co-workers (2007), and also Ditengou et al. (2008), have suggested that this redistributed auxin might be involved in the priming of LR founder cells in the basal meristem (Fig. 11A, B). The question remains whether AUX1 positively influences founder cell specification by globally participating in acropetal and basipetal auxin distribution at the root apex (Swarup et al., 2001; Marchant et al., 2002; Swarup et al., 2005), or whether it specifically imports auxin into cells to make them become founder cells. Furthermore *aux1* is still able to form LRs, meaning that it is either not crucial for founder cell specification and can be bypassed or has redundancy with other members of the AUX1/LAX family.

Together, auxin accumulation in pericycle cells in the basal meristem characterizes founder cell specification prior to lateral root initiation. More investigations will be necessary to decrypt the origin of the auxin accumulation in founder cells and to unravel any involvement of polar auxin transport.

II.3.2 Lateral root initiation

II.3.2.a Cell division competency

The event that marks lateral root initiation (LRI) is the first anticlinal cell division of the founder cell(s). On a cellular basis, LRI can either occur through division of a signal cell (longitudinal uni-cellular LRI) or by asymmetric division of two adjacent pericycle cells (longitudinal bi-cellular LRI), with the latter found more frequently (Dubrovsky et al., 2001). Two to three pericycle cells of parallel protoxylem adjacent cell-files are the minimum requirement for LRI. The first process that indicates an upcoming LRI is an induction of the auxin response in a protoxylem adjacent pericycle cell visible for example by *pDR5:GFP* activation (Ditengou et al., 2008). The next visible cellular process of longitudinal bi-cellular LRI is the movement of the nuclei of two adjacent pericycle cells towards each other (for example (De Smet et al., 2007) and Fig. 11) before the first anticlinal cell division occurs.

When grown vertically on solid agar medium, *A. thaliana* shows a wavy root growth and LRs initiate predominantly at the outside of each curve (De Smet et al., 2007). LRI in Arabidopsis seems to be a regular event occurring every 15 hours apically from older LRPs in the DZ (Dubrovsky et al., 2006; Lucas et al., 2008a) (no LRs are initiated more proximally between already existing lateral organs (Dubrovsky et al., 2006)). Curving a root through gravi-stimulus or mechanical bending caused LRI at the convex (outer) side of the curved area within a more reduced time window (5 to 13 hours)

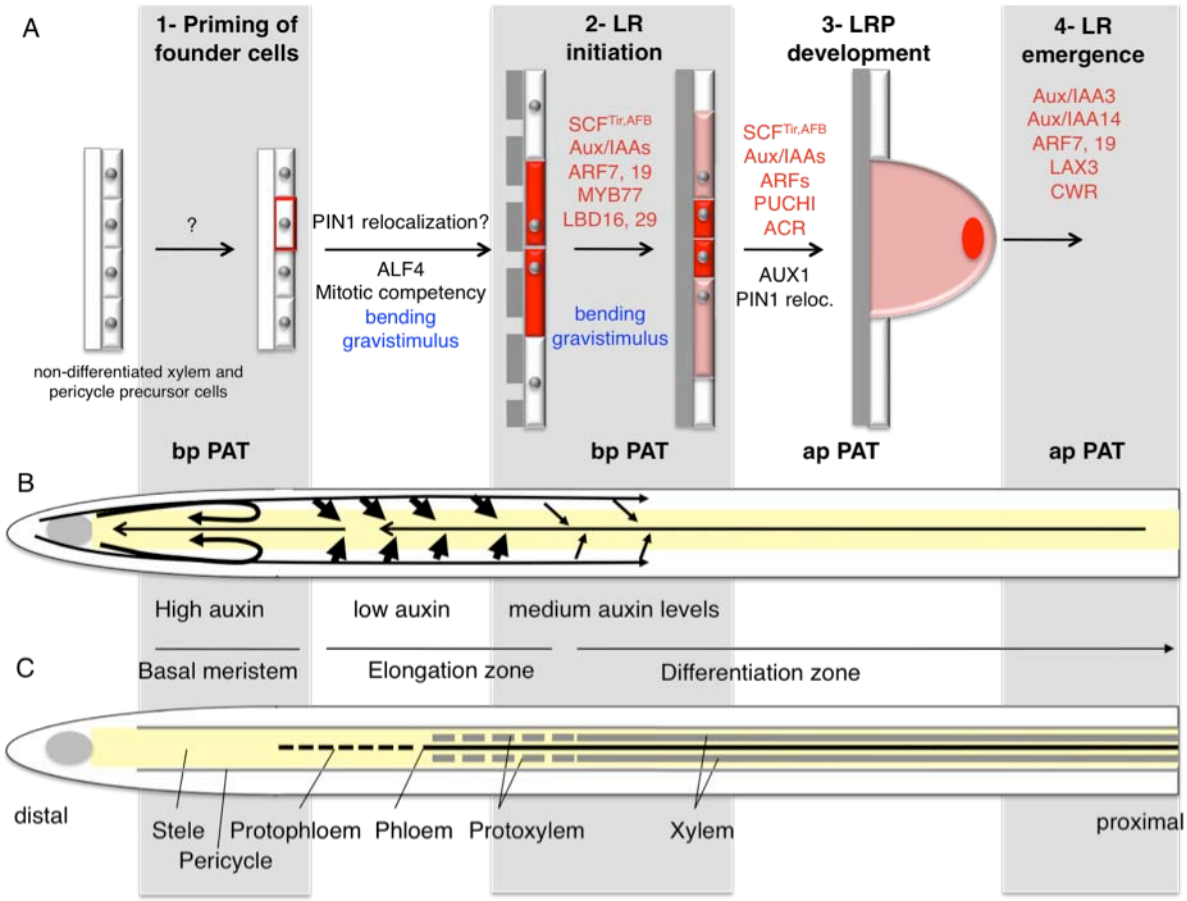

Figure 11: Molecular and cellular events during LR development

A LR development stages, **B** auxin fluxes at the root apex and **C** their tissue specific localization within the root. Priming of pericycle cells occurs in the basal meristem (Dubrovsky et al., 2008, De Smet et al., 2007) where auxin levels are high due to an intense auxin reflux (basipetal PAT) (Blilou et al., 2005). In the EZ auxin levels are lower due to a high reflux based on lateral PIN localization (Laskowski et al., 2005). Probably founder cells take slowly up auxin and expand in this zone, but no LRI occurs. At the proximal EZ pericycle cells have expanded enough to be competent to undergo cell division. Furthermore protoxylem cells differentiate at this part of the root (dashed grey line in A and C). Lateral auxin transport decreases and thereby increases auxin availability (Laskowski et al., 2005). The vicinity of pericycle cells to the here differentiated protoxylem cells together with the higher auxin levels in this zone and the mitotic competency (ALF4) of pericycle cells enables LRI. A PIN1–dependent auxin accumulation in pericycle founder cells (Geldner et al., 2004, Ditengou et al., 2008) makes the cell achieve the auxin-threshold for LRI (Lucas et al., 2008b). Auxin triggers LRI through an auxin signalling dependent way (auxin responsive genes in red). But the auxin signal can also be preceded by bending or a gravistimulus (in blue) (Ditengou et al., 2008, Lucas et al., 2008a). PIN1 relocalization to import auxin in the forming LRP and a positive loop through AUX1 can be suggested to promote the growth and subsequent cell-divisions through an auxin signalling dependent mechanism. This process depends on acropetal PAT. LR emergence, as well dependent on acropetal PAT is due to the auxin-dependent activity of cell-wall remodelling enzymes and auxin accumulation around the growing primordium through LAX3 (Swarup et al., 2008). Acropetal polar auxin transport (ap PAT), Basipetal polar auxin transport (bp PAT).

(Ditengou et al., 2008; Laskowski et al., 2008; Lucas et al., 2008a). It is not clear whether gravi- or mechanical stimuli only accelerate the intrinsic LR program or whether the molecular mechanism may be distinct, but these treatments present a convenient way to study LRI at a predictable site.

II.3.2.b Origin of protoxylem-pole pericycle auxin accumulation

An *in silico* approach used by Lucas et al. (2008a) proposed that auxin needs to accumulate up to a certain threshold in founder cells before the first cell division of LRI, which gives rise to stage I primordia, can occur and consume the local auxin pool. Analysis of the timing of founder cell appearance in the basal MZ and LRI in the DZ in one to two days old *A. thaliana* seedlings have suggested a time period of about 20 hours between both events (De Smet et al., 2007). These data indicated that LRI occurred at a regular rhythm – in the absence of any external stimuli – and might be due to the time required for the cell to achieve the critical auxin concentration for LRI, as well as to reach the right position in the root (the proximal EZ, Fig. 11). But what is the origin of the auxin increase in founder cells that tune LRI?

The first suggestions were that auxin for LRI comes from the root apex through basipetal transport (Bhalerao et al., 2002; Marchant et al., 2002). Their experiments proved that shoot-derived auxin was not crucial for LRI (Casimiro et al., 2001; Ditengou et al., 2008). Nevertheless, as the basipetal auxin-reflux loop (Blilou et al., 2005) depends on the auxin pool available at the root apex, an increase in the acropetal transport of auxin from the shoot feeds the auxin pool at the root apex and in the reflux loop and thus (indirectly) positively influences LRI (Lucas et al., 2008b). Laskowski and co-workers (2008) have proposed that in the elongation zone the lateral transport of auxin from the epidermis and cortex toward the stele is strong and reduces the overall auxin concentration in this zone. However, in the more proximal elongation zone and distal differentiation zone the lateral auxin transport decreased and the auxin content therefore increased (Fig. 11B). This may determine the position of LRI in roots grown without any further stimuli. In addition, Laskowski and co-workers (2008) have used a computer modelling approach to analyze LRI stimulation upon root bending and revealed that AUX1 triggers a positive feedback loop for auxin accumulation in pericycle cells at the outer side of the bent root. Results from Marchant et al. (2002) on untreated roots and Ditengou et al. (2008) on bent roots indicated that AUX1 expression started only at late stage 1 primordia, after the first anticlinal cell division and before the first periclinal cell division. Thus, auxin accumulation just before LRI seems not to dependent on auxin uptake through AUX1. Furthermore *aux1* mutants are still able to form 50% of the LRs compared to wildtype Arabidopsis (Swarup et al., 2008) under unstimulated conditions, indicating that AUX1 has a positive effect on LRI but can be bypassed or replaced. In addition *aux1* mutants are not inhibited in LR formation upon bending (Ditengou et al., 2008; Richter et al., 2009).

Different studies have also proposed a role for PIN dependent PAT during LRI (Casimiro et al., 2001; Geldner et al., 2004; Ditengou et al., 2008). Casimiro et al. (2001) demonstrated that application of elevated concentrations of the auxin transport inhibitor NPA inhibited LRI starting from the first cell division. During NPA treatment IAA increased at the root apex leading to the hypothesis that a lack of basipetal transport upon NPA treatment caused the absence of LRI. Geldner et al. (2004) showed that local NPA treatment at the root/shoot junction phenocopied the *gnom*^{R5} mutant, which is unable to undergo LRI because of a lack of PIN polarisation by endocytic trafficking (Steinmann et al., 1999; Geldner et al., 2003; Kleine-Vehn et al., 2008). They put forward a model where PIN1 in a bipolar localization focalized the auxin flow from three juxtaposed pericycle cells into their central cells, thereby depleting the bordering cells from auxin. This would generate an auxin accumulation in the central cell and would prevent the bordering cells from undergoing LRI, thereby insuring proper (distant) spacing of LRs. Finally, Ditengou et al. (2008) have shown an involvement of PIN1 during gravi-stimulated LRI. While observing *pPIN1:PIN1-GFP* plants after gravitropic curvature they detected PIN1 expression at both the basal and apical side of one specific protoxylem cell within the bent region as well as on the apical side of one to three basipetal protoxylem cells from there. They suggest that this re-localization of PIN1, which occurs before auxin accumulation in the pericycle, focuses auxin into the basipetal neighbouring protoxylem cells. Nevertheless no lateral re-localization of PIN1 in the protoxylem cell towards the pericycle cells was observed. Therefore it remains unknown whether auxin moves into the pericycle cell from the auxin loaded protoxylem cell and how this is accomplished. Additionally, PIN1 over-expression also caused an increase in LRI (Benkova et al., 2003), thus a positive role for this PAT protein may be suggested. The fact that LR development in the *pin1* mutant occurs normally may be due to redundancy of PIN1 to other PINs.

In addition to PAT, Laskowski and co-workers (2008) proposed that during root bending the mechanical extension of cells at the convex side of the curvatures may solely lead to auxin accumulation. However, temporary rapid bending of several seconds that cannot lead to cell size changes, induced LRs at the bend site and led to LRI as well (Ditengou et al., 2008; Richter et al., 2009). Richter et al. (2009) showed the appearance of a Ca^{2+} signal, which might be the mechanism by which the plant rapidly senses the bending. How the Ca^{2+} signal is connected to auxin increase an LRI remains to be investigated.

These data show that 1) LRI underlies an endogenous rhythm but can, to a certain extent, be accelerated by an environmental stimulus such as gravity or mechanical bending and that 2) a common feature that triggers LRI in the stimulated or untreated roots is an auxin accumulation, probably first through PIN mediated PAT and only later, after the first cell division through AUX1.

II.3.2.c Auxin signalling and cell-cycle activation

Lateral root initiation depends on the capacity of LR founder cells to undergo mitosis to give rise to a LRP (Dubrovsky et al., 2000; Dubrovsky et al., 2008). The fact that LR founder cells, primed in the basal meristem, need to pass the EZ and to elongate so as to be able to undergo the first cell division during LRI may again be a reason to explain the 20h delay between priming and LRI (Fig. 11). The nuclear protein ABERRANT LATERAL ROOT FORMATION (ALF4) has been suggested to participate in cell cycle competency through interaction with cell-cycle related genes (DiDonato et al., 2004). Interestingly, ALF4 itself is not auxin inducible, but might be required in interaction with other auxin inducible cell-cycle genes. Even if cell-cycle competency is a need for LRI, over-expression of cyclins led to increased growth rates of LRP but did not induce LRI (Doerner et al., 1996; Cockcroft et al., 2000; Vanneste et al., 2005). Vanneste et al. (2005) suggested that cell-cycle activation and a change in cell-fate are both required for LRI.

What happens downstream of auxin accumulation to initiate cell division? Different studies on Arabidopsis lines carrying mutations in the ARF or AUX/IAA auxin biosynthesis signalling pathways, showed that auxin signalling through these genes was crucial for LRI (Tatematsu et al., 2004; Vanneste et al., 2005; Okushima et al., 2007). A comparative analysis by Vanneste et al. (2005) on gene expression during synchronized LRI in stabilized IAA14 (*slr1* mutant) and wildtype plants demonstrated absence of transcriptional activation of several other *AUX/IAAs* and *ARFs*, auxin transport (*PIN1*, *3*, *7*, *AUX1*, *LAX3*) and homeostasis-related genes (*GH3*) and cell-cycle regulating cyclins. This emphasized that auxin signalling activates different processes during LRI. Over-expression of cyclins in the *slr1* mutant did not restore LRI and led Vanneste et al. (2005) to conclude that cell cycle activation needs to be accompanied by an auxin-signaling dependent cell fate to enable LRI. Nonetheless, more recent studies have shown that LRI can occur under certain conditions (stimuli) independently of auxin signalling through AUX/IAAs and ARF (De Smet et al., 2007; Ditengou et al., 2008; Lucas et al., 2008a). Thus the first cell division of LRI was independent of AXR3 (IAA17) (Lucas et al., 2008a) in the gravi-stimulated root curves or of ARF7/ARF19 upon mechanical bending (Ditengou et al., 2008). De Smet et al. (2007), furthermore, observed the approach of nuclei in two juxtaposed pericycle cells as an indication for starting LRI in Arabidopsis that expressed a stabilized (non-degradable) form of IAA17 in the pericycle. However, even if the first cell division could occur in these plants, further LRP development was arrested in all conditions suggesting that auxin signalling is required for the latter process.

In conclusion, even if IAA signalling can be circumvented during LRI by environmental stimuli, it is required for the further formation of the LRP (Fig. 11).

II.3.3 Lateral root primordium progression and new meristem development

The signalling cascade downstream of auxin leads to activation of several genes involved in the patterning of the new meristem. Directly downstream of ARF7 and ARF19 genes, the *LATERAL ORGAN BOUNDARIES-DOMAIN/ASYMMETRIC LEAVES-LIKE (LBD/ASL)* gene has been identified (Okushima et al., 2007). When over-expressed, *LBD29/ALS16* and *LBD16/ALS18* rescued the *arf7/arf19* phenotype, indicating that they act downstream of the basic auxin signalling pathway (Fig. 11). Their further characterization will help to understand how these transcription factors tune LR patterning. The auxin dependent signalling cascade also activates the receptor-kinase ACR that promotes divisions in the LRP and represses cell division in surrounding pericycle cells to delimit the new developing LRP (Peret et al., 2009).

Geldner et al. (2004) reported that proper PIN1 polarization through GNOM dependent endocytic trafficking was necessary to create auxin fluxes that promoted development of the new LRP. Furthermore, multiple *pin* mutations (*pin1,4,7* or *pin1,3,7*) caused dramatic defects on root patterning, including LRP organization (Benkova et al., 2003). PIN phosphorylation participates in the polar targeting of PINs. PID Kinases are among the genes activated during LRP formation (Vanneste et al., 2005). This shows that PIN-dependent PAT is involved in and required for LR development. The LR primordium is dependent on the auxin flux from the parental root until exceeding the three to five cell layer stage, when a proper meristem forms and the new LRP gets self-sustainable on auxin biosynthesis (Laskowski et al., 1995). It has been shown that for LRP progression, and also for later emergence, parental shoot auxin is necessary as young LRP were arrested when these auxin fluxes were interrupted (Casimiro et al., 2001; Bhalerao et al., 2002).

II.3.4 Lateral root emergence

Intriguingly, even if all founder cells were to give rise to LRP (Dubrovsky et al., 2008) not all LRP come to emergence (Dubrovsky et al., 2006; Lucas et al., 2008b). It has been reported that only 50 to 66% of acropetally initiated LRs directly continue to develop and emerge and 1/3 can be found more basipetally in between of older LRs (Dubrovsky et al., 2006; Lucas et al., 2008b). LRP that do not emerge directly may be arrested or slower developing and LRs subsequently emerge from those LRP as the plants age. As a common auxin pool is available for LRI and LRP development and emergence, both processes are negatively correlated. Therefore the fact that primordia are arrested may reflect how the root holds a balance between LRI and LRP emergence. Also, arrested primordia may present a pool of silent LRs that can emerge upon an environmental stimulus.

Scanning electron microscopy has revealed that cells adjacent to emerging lateral root primordia in Arabidopsis remain intact but separate from one another along their middle lamella (Laskowski et al., 2006). Different genes, such as pectolyases, were identified in this study, which may be responsible for separating endodermal, cortical and epidermal cells to let pass the emerging root. It is

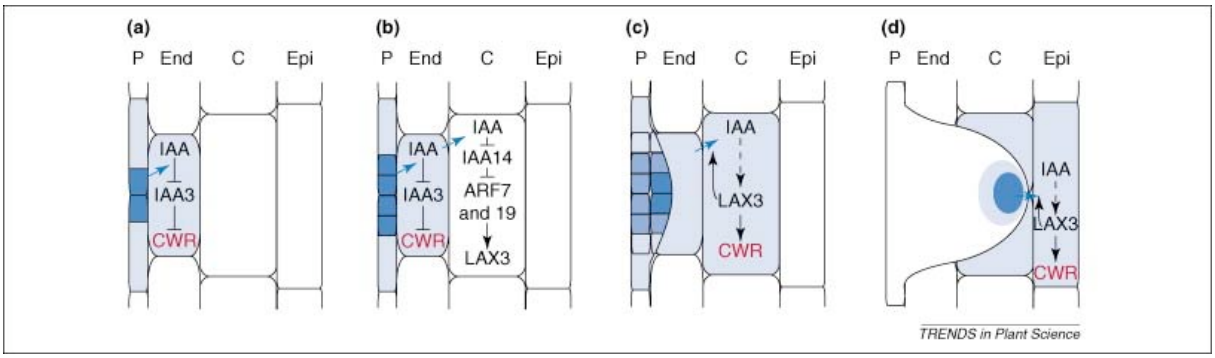

Figure 12: Molecular and cellular events during LR growth and emergence

(**a**) Auxin accumulation in the endodermis triggers Cell-Wall-Remodelling enzymes (CWR) through IAA3 signalling. (**b**) In the cortex, auxin released from the growing primordia activates *LAX3* expression through the basic auxin signalling pathways. LAX3 proteins trigger a positive feedback loop that increases the auxin content of the cell until activation of CWR (**c**), which lose cell walls of cortex (**c**) and epidermis (**d**) cells to facilitate LR emergence. From Péret et al. (2009)

assumed that differences in pectin methylation between the parental cells and the LRP protect the emerging LRP from being degraded.

A recent study has revealed how increased auxin levels in the LRP and enzymatic cell separation during LRP emergence correlate (Swarup et al., 2008) (Fig. 12). A knock out mutation in the auxin influx carrier LAX3 was found to provoke a decrease in LRP emergence. It was suggested that auxin released from the forming LRP leads to degradation of IAA3 that hence activates cell wall remodelling enzymes (CWR) in endodermal cells necessary for LR emergence. In the cortex and epidermis another mechanism is present. As revealed by *pLAX3::GUS* expression, LAX3 is activated in an IAA14 and ARF7/ARF19-dependent manner in a small number of cortical (up from stage II) and later (stage V) epidermal cells adjacent to the developing LRP. LAX3 expression led to auxin accumulation in these cells. LAX3 amplifies in a positive feedback loop the auxin response in the close proximity to the LRP. This response triggers downstream CWR enzyme activation to let the root pass through these tissues during emergence.

In summary, polar auxin transport is a key-regulator of LR development: It mediates the auxin accumulation that triggers LRI, relocalizes the auxin flux in the developing LRP to control proper cell organization and permits a coordinated emergence of the new LR by activating cell-spacing in the parental root.

II.4 Phytohormone crosstalk during LR development

Even if the literature shows that auxin plays a crucial role in LRI, other phytohormones have been suggested to interact with auxin and are therefore likely to interfere with LR formation (Fig. 13). We will examine here those that might be of interest in root/microbe interactions: brassinosteroids, ethylene, jasmonic acid and cytokinin.

II.4.1 Brassinosteroids

Brassinosteroids (BR) and auxins have a partially overlapping response spectrum when exogenously applied to plants. BRs, such as exogenously applied brassinolides (BL), promoted LR initiation in *A. thaliana* through a mechanism that can be blocked by the auxin transport inhibitor NPA, suggesting that BRs interact with PAT (Bao et al., 2004). Furthermore BLs and IAA applied to roots induced a response of the *pDR5:GUS* reporter (Bao et al., 2004; Mouchel et al., 2006). The *pDR5:GUS* response was reduced in the BR insensitive mutant *bri1* or after seedlings were treated with the brassinosteroid biosynthesis inhibitor Brassinazole (BRZ) and it was shown that this was not due to a reduced auxin content but to a probable change in sensitivity (Bao et al., 2004). In accordance with the enhanced auxin response in BL treatments, transcript profiles of Arabidopsis seedlings treated with BL or with IAA, showed overlapping induction of genes from the early auxin responsive gene families (Hagen and Guilfoyle, 1985) *SMALL-AUXIN UP RNA* (*SAUR*), *GH3* and *Aux/IAA* (Goda et

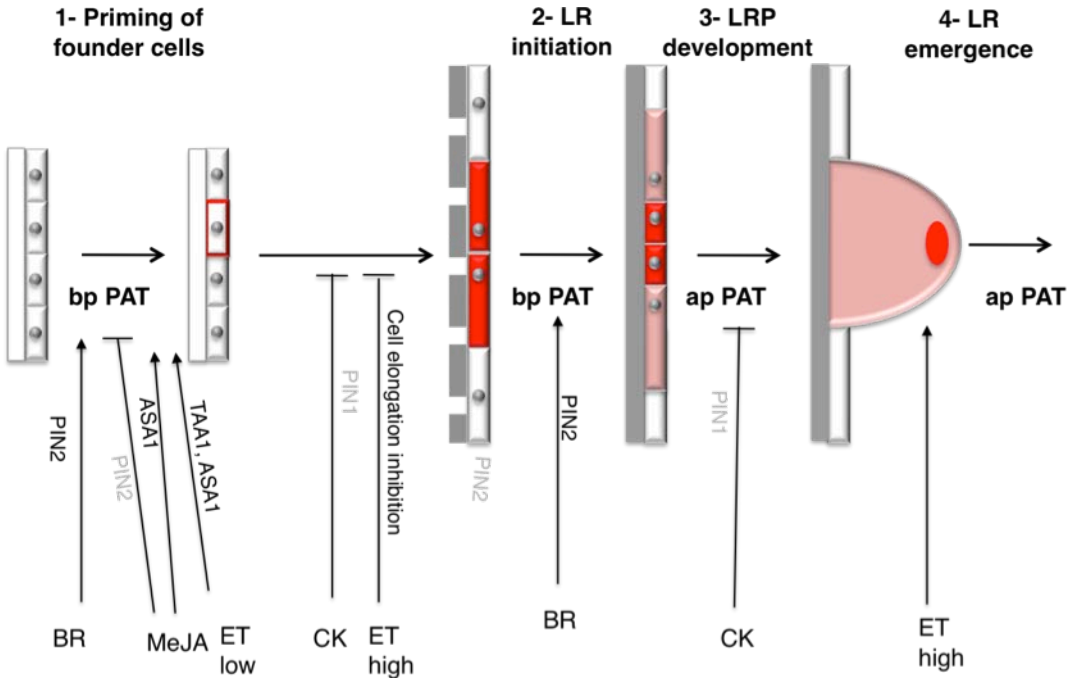

Figure 13: Hormone crosstalk during LR development

Hypothetical key steps at which photohormones ethylene (ET), Brassinosteroids (BR), Cytokinin (CK) and Methyl-jasmonate (MeJA) may interfere with auxin regulated LR development. Brassinosteroids are suggested to interfere with basipetal PAT through PIN2 (Li et al., 2005) and may therefore stimulate priming of founder cells in the basal meristem as well as LRI at more proximal root parts. MeJa changes the net IAA levels at the basal meristem, through increased ASA1-dependent auxin biosynthesis and decreased (grey) PIN2 dependent PAT and stimulates founder cell specification (Sun et al., 2009). Low levels of ET increase auxin levels through TAA1 and ASA1-dependent biosynthesis (Ivanchenko et al, 2008, Stepanova et al., 2005, 2008). As ET alters LR spacing (Ivanchenko et al., 2008), most probably it increases the frequency of founder cell priming. High ET levels inhibit LRI (Ivanchenko et al., 2008, Ruzicka et al., 2007). It is assumed that due to the decreased cell elongation in the EZ LRI cannot take place (Ivanchenko et al., 2008). However, high ET levels stimulate emergence of already existing LRI likely by positively impacting acropetal PAT (Negi et al., 2008, Ivanchenko et al., 2008). CK negatively interferes with LRI but also with LRP development (Laplaze et al., 2007). PIN1 that is repressed by CK has been suggested to interfere with auxin accumulation in founder cells before LRI and during LRP development and may explain the cross-point of CK with auxin regulated LR development.

al., 2004). Bao et al. (2004) had furthermore shown that BL strongly increase the acropetal auxin transport from leaves to roots and Li et al. (2005) have detected a change in PIN gene expression upon BL treatment. According to data mentioned earlier (Lucas et al., 2008b), an increased acropetal transport was shown to enhance LRI, most probably by feeding the auxin reflux loop that has been suggested to be important for LRI. This fits with data from Li et al. (2005), which indeed showed an enhance gravitropic response through BL stimulated accumulation of PIN2 protein in the root EZ. PIN2 is one characteristic mediator of basipetal auxin transport (Blilou et al., 2005) and its localization has also been suggested, together with PIN1, to mediate the founder cell priming auxin reflux loop at the root apex (Ditengou et al., 2008). Thus, BL might enhance priming of founder cells in the proximal basal meristem. The activation of early auxin responsive genes by BL may strengthen the assumption that BRs activate auxin transport through auxin signalling. To validate this, the effect of BL in srl1 (stabilized IAA14), or in arf7/arf19, should be addressed. As BL was shown not to alter the auxin content of the plant, an indirect response of the auxin signalling pathway through increased auxin concentrations may be excluded. This leaves the question of whether or not BL can interact with and directly activate the auxin signalling pathway. Due to the activation of the auxin reflux loop by BRs, they may favour both the priming of founder cells as well as auxin accumulation in founder cells prior to LRI and furthermore stimulate the auxin response necessary for LRI (Fig. 13).

II.4.2 Ethylene

The effect of ethylene (ET) on LR formation has recently been investigated and it was uncovered that ethylene has a dose-dependent antagonistic or agonistic effect on LRI (Ivanchenko et al., 2008; Negi et al., 2008). The stimulatory effect of low exogenous ACC levels led to increased LRI and LRP were often found closely spaced, which was rarely the case in untreated plants (Ivanchenko et al., 2008). Thus ET may increase LR frequency. This study also demonstrated that low exogenous ET application stimulated auxin biosynthesis (through ASA1, ASA2), an already known interaction between these two hormones (Stepanova et al., 2005). Another ethylene-responsive enzyme of the IAA biosynthetic pathways, which leads to increased auxin biosynthesis in roots upon ACC treatment, is TRYPTOPHAN AMINO TRANSFERASE1 (TAA1) (Stepanova et al., 2008). Increased auxin levels in roots promote LRI through the primary auxin response machinery relying on AUX/IAA and ARF proteins (Ivanchenko et al., 2008). This suggests that low ethylene may also impact LR founder cell specification in the basal meristem through increased auxin availability, thus explaining the closer spacing of LRs (Fig. 13).

Negi et al. (2008) observed LRI decrease in all conditions that led to high ET response in the plant such as an exogenous application of ACC or the use of mutant lines with increased endogenous ET levels (*eto1*) or those with constitutive ET response (*ctr*). They furthermore showed that the LR

inhibition was caused by an ethylene-signalling-dependent stimulation of acropetal and basipetal auxin transport through AUX1. In accordance with these findings, ethylene failed to inhibit LRI in ethylene insensitive mutants (*etr1, ein2*) or in *aux1-7*. Ruzicka et al. (2007) had observed transcriptional activation of *AUX1* and *PIN1, 2* and *4* in ACC treated plants. Again *AUX1* and *PIN2* activation by ET was prohibited when ET-signalling was blocked by Ag^+ ions. Strikingly, like high ET doses, BRs also stimulated acropetal and basipetal PAT, but contrary to ET, lead to more LRI events. Ivanchenko and co-workers (2008) explained that the inhibitory effect of higher ET by a cellular rather than by a molecular effect: high ET levels inhibit cell elongation at the root apex and therefore pericycle cells may be too short to undergo cell division for LRI (Fig. 13). This is in agreement with the model presented in Fig. 11 where we propose that founder cells need to cross the EZ and to elongate before an auxin-triggered LRI can take place. In addition to inhibiting LRI high ET levels promote LR emergence. Lucas et al. (2008b) had reported that LR emergence and LRI are negatively correlated, thus blocking one enhances the other. Furthermore, stimulation of shoot-root PAT is known to positively correlate with LR emergence (Bhalerao et al., 2002). Thus blocking LRI might advantage LR emergence, as more auxin could be available for this process.

II.4.3 Jasmonic acid

Jasmonic acid (JA) is known to inhibit root growth (Ueda and Kato, 1980) and this response has commonly been used to screen mutant populations for JA insensitivity (Wasternack, 2007). Only very recently has a connection between JAs and LR development been established (Sun et al., 2009). In a way that reflects the effect of ET, JA Methyl-jasmonate (MeJA) also promotes LRI when applied at low concentrations (where it does not inhibit root growth). However, in contrast to ET, elevated concentration of JA does not affect LRI. Sun et al. (2009) identified the *asa1* mutant, which lacked LRI stimulation in response to low levels of MeJA. Looking at the cell-cycle marker *CYCB1:GUS* in *asa1*, it became clear that MeJA failed to promote LRI due to an inhibition of initial anticlinal pericycle cells division. ASA1 (ANTHRANLITAE SYNTASE 1) is an enzyme of the Trp biosynthesis pathway, presenting a rate-limiting step in synthesis of IAA precursor (Woodward and Bartel, 2005). MeJA also failed to stimulate auxin biosynthesis in *asa1*, causing altered MeJA response. Interestingly MeJA activation of *ASA1* expression was independent on ET, another activator of this enzyme (Stepanova et al., 2005), but required a functional MeJA signalling cascade. The increase in *ASA1* transcript correlated with an auxin response increase (visualized through *IAA2:GUS* expression activation) at the basal meristem but not in *asa1* nor in *aux1* mutants. Concerning auxin transport, MeJA enhanced expression of *proPIN1:GUS*, *proPIN2:GUS* and *proAUX1:GUS*. However in the MeJA treated *asa1* mutant expression induction of *PIN1* and *PIN2* was lower than in MeJA treated wildtype and completely absent for *AUX1*. Intriguingly, the MeJA increase in *PIN1* and *PIN2* transcripts (in wildtype) paralleled a decrease in protein amounts as revealed by immunolocalization experiments (Sun et al., 2009). The protein decrease was even more

important in case of *asa1* plants treated with MeJA, and is therefore suggested to occur in an ASA1-independent pathway. The authors proposed a model for MeJA action on LR development in which a net auxin increase in the basal meristem is provoked by (i) the activation of auxin biosynthesis through ASA1 and (ii) a decreased basipetal PAT (Fig. 13). However, when MeJA-stimulated ASA1-dependent auxin biosynthesis is absent – as in *asa1*- the decreased basipetal PAT negatively influences LRI. These examples stress the fact that the regulation of PAT can have opposite effects on LRI when acting in combination with another auxin-dependent pathway, as here auxin biosynthesis.

II.4.4 Cytokinins

The antagonistic effect of cytokinins (CK) and auxin on plant development is commonly used to regenerate respectively shoot or root tissues from callus. Similarly, during LR formation IAA and CK showed antagonistic effects, with exogenous CK inhibiting LR development (Laplaze et al., 2007; Kuderova et al., 2008). In accordance to this, lowering CK signalling, such as in Arabidopsis mutants with low CK levels or inhibited CK response led to an increase in LRs (reviewed in (Fukaki and Tasaka, 2009))while mutations to negative regulators in the CK signalling cascade increase LR numbers.

The regulation of LR development by CKs has recently been investigated (Laplaze et al., 2007). Laplaze and co-workers (2007) showed that CK's effect on LR development occurred in an ethylene biosynthesis and signalling independent manner. CK caused a delay of LRI together with a delayed or blockage LRI. Interestingly, CK overproduction by an isopentyltransferase (IPT) either directed to xylem pole pericycle cells or to the developing LRP (stage I to IV) demonstrated that pericycle cells are sensitive to CK and that they represent the site of its action as opposed to in LRP. IPT expression in the pericycle disturbed LRI but had no impact on LR development when expressed after LRI had already occurred. Further experiments showed that CKs did not interfere with the auxin-sensitive induction of cell division during LRI but negatively impacted the developmental program of LRI. The target of CK seems to be auxin transport, as the presence of CK lowered transcript accumulation of *PIN1, 2, 3* and especially of *PIN7*, thereby prohibiting the formation of a proper auxin maximum and hence disturbing LRI. Ruzicka et al. (2009) also had addressed the question of the interaction of CK with PAT. Expression data and transport analysis in BY2 tobacco cells (when ethylene signalling was inhibited) showed that CK repressed *PIN1* and thereby lowered auxin efflux from these cells. They confirmed this finding *in planta* and showed furthermore that the repression of *PIN1* was indeed dependent on CK signalling. Their data also showed only a slight (ethylene independent) decrease in *PIN2* expression, but contrary to Laplaze et al. (2007) they reported an increase in *PIN7* expression. Taken together, this shows that an improper PIN polar localization alters auxin distribution and is likely to cause the observed delay or decrease of LRI and the defects

Table I: Signalling in soil (micro)organism/plant interactions through Ligand/Receptor (Rcpt) and phytohormone pathways and their impact on plant endogenous auxin pathways.

Organism	Recognition Ligand/Rcpt Signalling	Phyto-hormones released	Impact on plant endogenous auxin pathways	Literature source
Ectomycorrhizal fungi 	?	Auxins Hypaphorine Ethylene Cytokinins	? PAT inhibitors (TIBA/NPA) decreased LR stimulation and colonization	(Graham and Linderman, 1980; Ek et al., 1983; Karabaghli et al., 1998; Karabaghli-Degron et al., 1998; Splivallo et al., 2009)
Endomycorrhizal fungi 	Myc/? SYMRK=> Ca2+ => ENOD	Auxins Gibberellin Cytokinins	Auxin increased in roots no increase in fungal tissues GH3:GUS patchy signal (beyond fungal material in root). PAT inhibition (TIBA) increased colonization.	(Barea and Azcon-Aguilar, 1982; Hooker et al., 1992; Olah et al., 2005; Jentschel et al., 2007; Markmann and Parniske, 2009)
Other fungi (Trichoderma) 	?	Auxins	Increase of DR5:GUS signal in Arabidopsis primary and lateral roots Stimulation of LR emergence by the fungus was absent in *pin2* and *aux1* mutants	(Contreras-Cornejo et al., 2009)
Rhizobia 	Nod/NFR SYMRK=> Ca2+ => ENOD	Auxins Gibberellins Cytokinins Ethylene Abscisic acid	MtPIN2 (AtPIN2 homolog) expression in early LRP and in root nodules. Less root nodules in *Mtpin2, 3, 4* mutants	(Olah et al., 2005; Huo et al., 2006; Boiero et al., 2007; Erum and Bano, 2008; Grunewald et al., 2009; Markmann and Parniske, 2009)
Frankia bacteria 	Nod/? SYMRK=> Ca2+? => ENOD?	Cytokinins Auxins	*CgAUX1* activation during infection process (root hairs, cortical cells) Nodules arrested when AUX1 inhibited (NOA)	(Peret et al., 2007; Markmann and Parniske, 2009)
Nematodes 	?/? ??=> ENOD	Auxins	Repression of acropetal PAT (AUX1, PIN1) Relocalization of PIN3 and PIN4 at feeding site	(Favery et al., 2002; De Meutte et al., 2005; Grunewald et al., 2009)

in cellular organization of LRP. As CKs have been shown to negatively interfere with PIN1, which is suggested to be important before and after the first cell division during LRI (Fig. 11), a possible inhibitory effect has been indicated in Fig. 13 in these two processes.

Taken together, all four different exogenously applied phytohormones that impact LR development significantly affect polar auxin transport. PAT may therefore be suggested as a central element for the incorporation of environmental stimuli into LR development.

II.5 Lateral root development in response to soil (micro-)organisms

A plant root system in its natural environment has to face a heterogeneous milieu from which various stimuli are released. Even if root system development is determined by intrinsic mechanisms, response-pathways that modulate these intrinsic programs make the root being plastic to adapt to these diverse stimuli (Malamy, 2005). We will focus here on the interaction between rhizospheric derived stimuli and plant roots. By 'stimuli' we mean signals released by soil beneficial microbes (e.g. mycorrhizal fungi, rhizobia or *Frankia* bacteria) or parasitic organisms (e.g. nematodes) (Table I) important for the interaction with roots. When a symbiotic interaction is established a new, common plant/(micro-)organism organ forms through which they exchange nutrients.

Even though the outcomes of rhizobia-, *Frankia*-, mycorrhizal- and nematode-plant interactions are very different, many aspects concerning host invasion or signal transduction are shared between these interactions (Table I) (Abe, 2001; Markmann and Parniske, 2009). Ectomycorrhizal (ECM) and endomycorrhizal fungi as well as rhizobia bacteria all stimulate the plant-partner's LR development (Hooker et al., 1992; Karabaghli-Degron et al., 1998; Olah et al., 2005). Furthermore, rhizobia and actinorhizal (*Frankia*) root nodules share common features with LR development, as they are also initiated from the xylem-pole pericycle. The signalling pathways between endomycorrhizal fungi or rhizobia bacteria and their host plant that leads to cell-reprogramming to induce symbiosis establishment are already well described (Oldroyd et al., 2009). Plants release with their root exudates flavonoids and strigolactones that attract the micro-symbiont, which in response release Nod factors (lipochitooligosacharides), in the case of rhizobia bacteria, or Myc factors (unknown chemical structure) in the case of endomycorrhizal fungi. Nod and Myc factors are recognized by Symbiosis Receptor Kinases (SYMRK) in plant cells. The activation of SYMRK triggers downstream responses leading to cell reprogramming necessary for symbiosis establishment. Intriguingly, it has been shown that SYMRK is required for LR induction (Olah et al., 2005). Accordingly, just an application of the bacterial Nod factor, the infection trigger, or of endomycorrhizal diffusible factors led to a SYMRK dependent LR stimulation. Moreover, mutants with increased nodulation and mycorrhizal capacity show increased LR numbers (reviewed in Abe

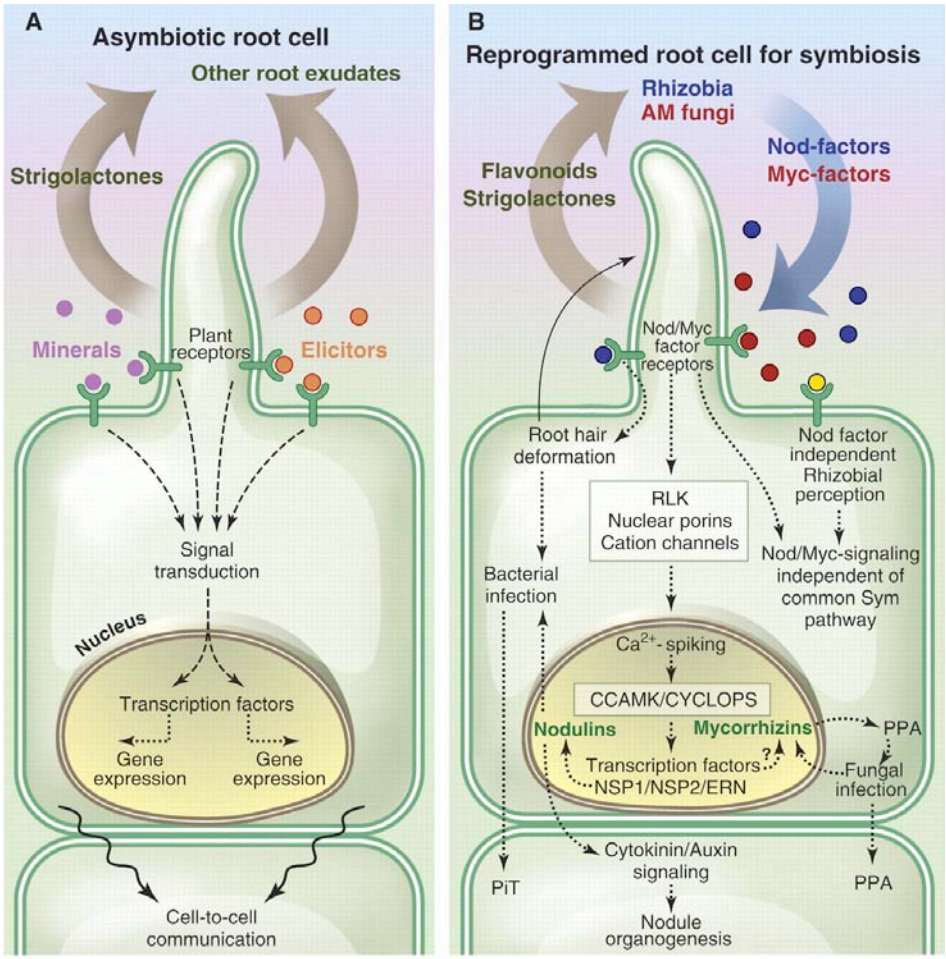

Figure 14: Signal exchange during arbuscular mycorrhiza (AM) and rhizobia symbiosis.

(**A**) An asymbiotic cell constitutively releases root exudates, including strigolactones. The root cell monitors the concentration of minerals and microbial organisms in the soil and transduces the respective signals. Integration of the signals includes cell-to-cell communication. (**B**) A root-hair cell primed for interaction with rhizobia or AM fungi, respectively. Plant roots release flavanoids and strigolactones that prime the rhizobia and AM fungi. Nod and Myc factors act as signals from the symbionts to plant root cells that activate calcium spiking via the Sym pathway (boxed). The potential differential activation of CaMK/Cyclops leads to differential induction of nodulation-specific transcription factors (NSP1, NSP2, and ERN) and unknown mycorrhizal-specific transcription factors. Rhizobial and mycorrhizal infection require the common Sym pathway but also exhibit recognition and signalling independent of this pathway. The path for fungal infection and the infection thread (IT) is predicted by the pre-infection thread (PiT) and the pre-penetration apparatus (PPA), respectively, indicating directed signalling to neighbouring cells. Nodule organogenesis is induced in inner cortical cells after Nod-factor perception by epidermal cells. This requires cytokinin signalling and is associated with changes in auxin levels. From Oldroyd et al., 2009

(2001)). These data show that there is a link between the infection process, triggered by recognition of both partners, and LR stimulation. To date we are lacking the signalling cascade that connects SYMRK activation with LR initiation. However, for all interactions plant/soil microorganisms described, there are data showing that these organisms interfere with their host-root's endogenous auxin levels or transport (Table I). In addition, several soil-organisms are also able to produce auxin and other phytohormones that might further impact plant development.

As it becomes evident from Table I, ECM symbiosis' are the least understood. For all other root-(micro-) organisms interactions, recent results point out the importance of PAT. ECM symbiosis compared to rhizobia, *Frankia* and endomycorrhizal symbiosis are different because the latter examples are root endosymbiotic (the interaction is taking place inside root cortex cells) whereas during the ECM symbiosis the fungus never enters the cell. All the colonization in ECM occurs outside of the root. Therefore pericycle-cells stimulation for LR initiation and further root colonization by the fungus may not be directly mechanistically connected. 'Myc' factors equivalent to the bacterial Nod factors are poorly studied in ECM symbiosis (Melin, 1954; Albrecht et al., 1994) but they may consist of chitineous compounds from the fungal cell wall (Salzer et al., 1997). Thus, both the mechanism leading to LR stimulation and the symbiotic trigger are so far unknown, which shows that progress is needed to understand ECM symbiosis. However, some signalling molecules that are released by ECM fungi have been identified and some root internal pathways have been shown to be activated upon plant/fungus interaction. The following chapter will give an overview on these and suggest how they could interfere with LR development in the plant partner.

III. Ectomycorrhizal symbiosis

III.1 Background of ectomycorrhizal symbiosis

Ectomycorrhiza from the Greek *mukês*: fungus, *rhiza:* root, have for the first time been described by Frank in 1885 (Frank, 1885) and de Bary (de Bary, 1887). Ectomycorrhiza (ECM) typically form between Gymno- or Angiosperms plant partners and Basidio- or Ascomycotina fungal partners (Smith and Read, 2008). According to Meyer (1973) only 3% of seed plants are ECM, but their global importance originates in their disproportionate occupancy of the terrestrial land surface and their association to economically important woody species (e.g. for timber production). ECM can be found in boreal forests of the Northern Hemisphere, temperate forest in the Northern- and Southern Hemisphere, as well as with significant importance also in tropical forests of South-East Asia (Smith and Read, 2008). It is estimated that about 5000 to 6000 fungal species can undergo ECM symbiosis (Molina et al., 1992). The beginning of ECM fungi evolution is dated on a large time period

probably about 180 to 130 mya, and it is most possible that they arose at different occasions when saprotrophic fungi formed symbiotic partnerships (Hibbett et al., 2000; Alexander, 2006; Moyersoen, 2006). Interestingly, this situates ECM emergence to an era close to the origin of conifers (180-140 mya) (Bell et al., 2005). It has been suggested that these fungi had an important contribution to plant evolution (Le Tacon and Selosse, 1997). ECM fungi are therefore younger than endomycorrhizal fungi, which developed 450 to 350 mya (Simon et al., 1993). Distinct ECM fungi can be more or less specific to their host plants. Furthermore the same plant can be colonized by many different ECM (and other) fungi; for instance on a single tree tens of ECM fungi and on a mono-specific plantation several hundreds of ECM fungi can be found associated to the tree roots (Bruns, 1995; Buee et al., 2009).

The functional ectomycorrhiza is characterized by the presence of a **mantle of fungal hyphae** enclosing the root, a labyrinthine inward growth of hyphae between epidermal and (depending on the species) cortical cells, called the **Hartig net** (Blasius et al., 1986), and an outwardly growing network of hyphal elements, the **extramatrical mycelium**, from which may arise sporocarps. The typical structural elements of the mature ECM are important to drive the nutrient flow that makes this symbiosis beneficial for both partners (Martin, 2007). Extramatrical hyphae gather nutrients (mostly N and P) from the soil and transport them to mantle hyphae, where they may be stored. From there nutrients are transported to Hartig net hyphae and delivered to and taken up by root epidermis (and cortex) cells. Vice versa, photoassimilates (sugars) are released by plant root cells and taken up by the fungus, before (partially) being transported back into extramatrical hyphae that use them for further growth. Together the different structures and nutrient flows stimulate growth and health of the plant on different levels: (i) thanks to the wide network of extramatrical hyphae it benefits from nutrient resources of a bigger soil volume than it could exploit by its roots alone (Rousseau et al., 1994), (ii) the fungal enzyme activities, which increase mineral solubilisation in the soil, can enhance nutrient absorption by plant roots as well (Landeweert et al., 2001) and (iii) the enclosure of the root by the fungus protects plant roots against pathogens but also against inorganic agents. In specific cases the ability of fungi to absorb and store heavy metals (Blaudez et al., 2000) can even open an ecological niche for the plant it would not be able to grow in without being associated to the fungus.

III.2 The development of ectomycorrhizal symbiosis

ECM development can be separated into different phases (Fig. 15) (Martin and Tagu, 1995). The first consist of a **pre-contact phase**, as well termed **early phase**, during which plant and fungus only communicate via diffusible molecules. Root exudates released by the plant attract the fungus, for example from a soil propagule, by increasing hyphal branching (Lagrange et al., 2001) (Fig. 15.1). In the case where the fungus originates from spores, root exudates may activate their germination.

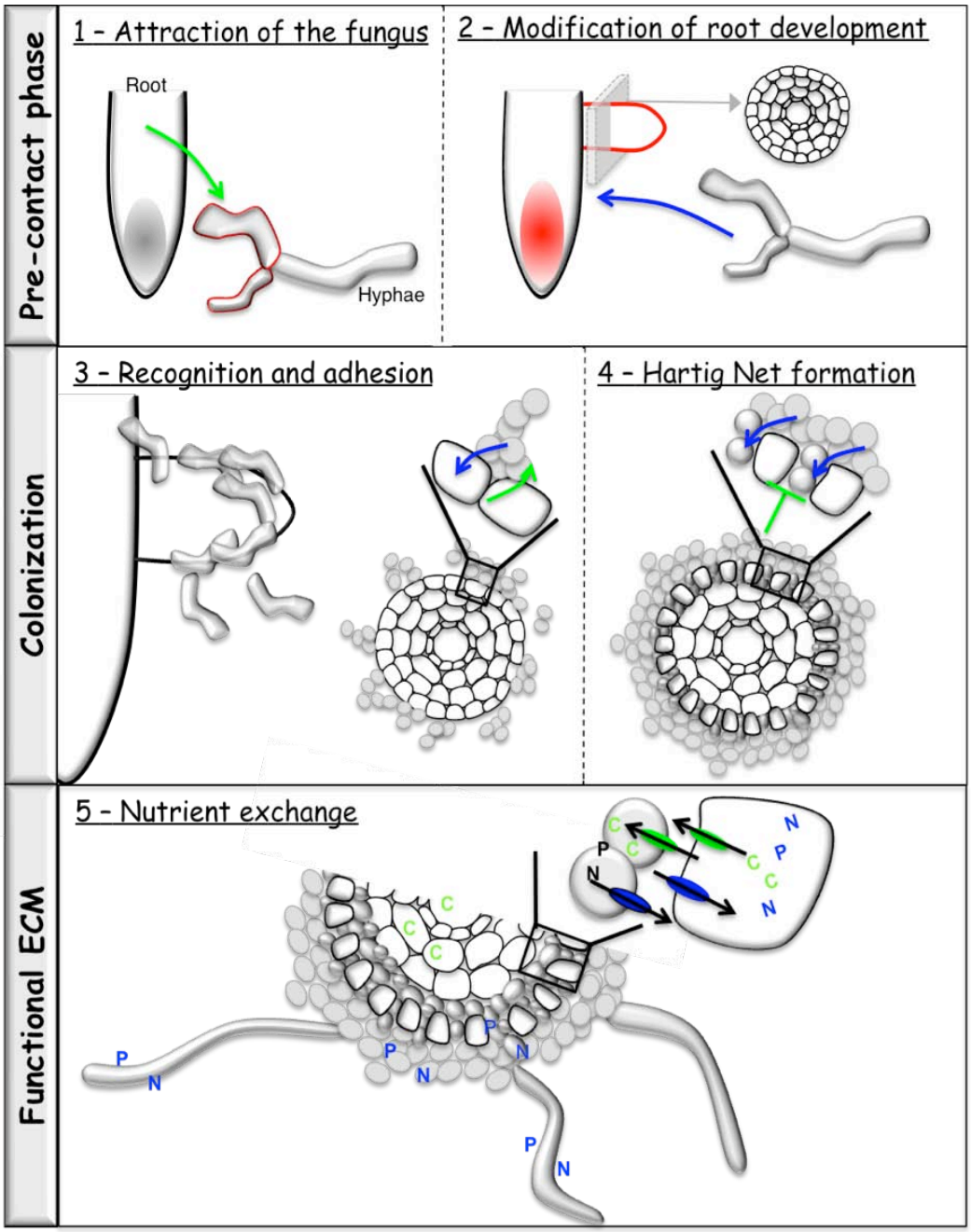

Figure 15: The different phases of ECM development

ECM development can be separated into three main phases: (i) Signal exchange prior to physical contact, (ii) colonization and (iii) nutrient exchange in the functional ECM. Signals are exchanged from fungus to plant (blue arrowheads) and from plant to fungus (green arrowheads). Developmental changes caused in plant or fungus are highlighted in red. The pre-contact phase is characterized by a plant-to-fungus signalling to induce hyphae branching and attract the fungus (1) and to a fungus-to-plant signalling, which stimulates lateral root development of the plant (2). This phase is thought to coincide with changes in root auxin gradients (red sphere at root tip). The colonization phase may be separated into a first recognition and adhesion of hyphae on the root (3) and of penetration of fungal hyphae in between epidermal (and cortex) cells of the plant (4, represented as a transverse root section). The functional ectomycorrhiza is characterized by active nutrient exchange (ammonium (N) and phosphate (P) from the fungus to the plant and sugars (C) from the plant to the fungus). Extramatrical hyphae explore nutrients in the soil and transport them to the mantle and Hartig Net where specific transporters insure the exchange.

Signalling molecules secreted by the fungus stimulate LR development in the plant (Fig. 15.2) (Karabaghli-Degron et al., 1998; Rincon et al., 2001; Rincon et al., 2003; Splivallo et al., 2009). Under laboratory conditions this early phase can artificially be mimicked when a barrier, which is permeable for signalling molecules but impermeable for hyphae and roots is present between both partners. This kind of experiment has confirmed that hyphal branching (Horan and Chilvers, 1990) and LR development (Karabaghli-Degron et al., 1998) are activated without requiring any physical contact between the partners. The second phase, called **colonization-phase or intermediate phase** (Fig. 15.3 and 15.4) consists of a recognition sub-phase that triggers adhesion of fungal hyphae on the root surface (Rincon et al., 2001) prior to mantle and Hartig net formation. The docking process that consists of hyphae attachment to root epidermal cells starts with the formation of an "adhesion pad" through aggregation of hyphae (Jacobs et al., 1989). It has been proposed that the initial interaction of hyphae and roots during colonization occurs close to the root tip and that subsequently hyphae invade the root from root cap cells in- and upwards to the epidermis (Horan et al. 1988). Depending on the species either first a mantle of multiple layers of hyphae that multiply and differentiate (Horan et al., 1988) forms around the root from which the Hartig net develops then inwards, or occasionally the Hartig net can establish first and the mantle forms afterwards (Nylund and Unestam, 1982). With preceding root colonization changes in root development occur, such as root growth arrest, root cap decay, dichotomy of the root apical meristem in conifer species, and cytodifferentiation of root cells (radial elongation, root hair decay) (Horan et al., 1988; Dexheimer and Pargney, 1991; Ditengou et al., 2000). Once colonization of a certain root part is accomplished, a third phase can be set aside, which will be termed **functional mycorrhiza** or **late phase** (Fig. 15.5). This phase is characterized by nutrient exchanges occurring between both partners in the Hartig net. Furthermore an equilibrium is established in terms of colonization: depending on plant and fungal species the Hartig net establishes within a certain depth into the cell layers of the root (from the epidermis cells to cortex cells) and once colonization is accomplished the fungus does not penetrate any further. The equilibrium (biotrophic) phase where efficient nutrient exchange occurs may be maintained for a certain period of time, which depends on the species involved (Smith and Read, 2008), until the mycorrhiza ages and undergoes senescence (Dexheimer et al., 1986). It has been shown that ageing mycorrhiza may loose their fungal mantle, even if the Hartig net is maintained and that their contribution to plant/fungus nutrient exchange diminishes (Al-Abras et al., 1988). The fungus can then go back to a saprophytic lifestyle and invade senescent root cells (necrotrophic phase).

In ECM development, there is a certain overlap in the different events of each phase and a strict limit of each phase cannot be assigned. For instance increased LR formation triggered in the pre-contact phase continues into the colonization phase and nutrient exchange can already start within the colonization phase.

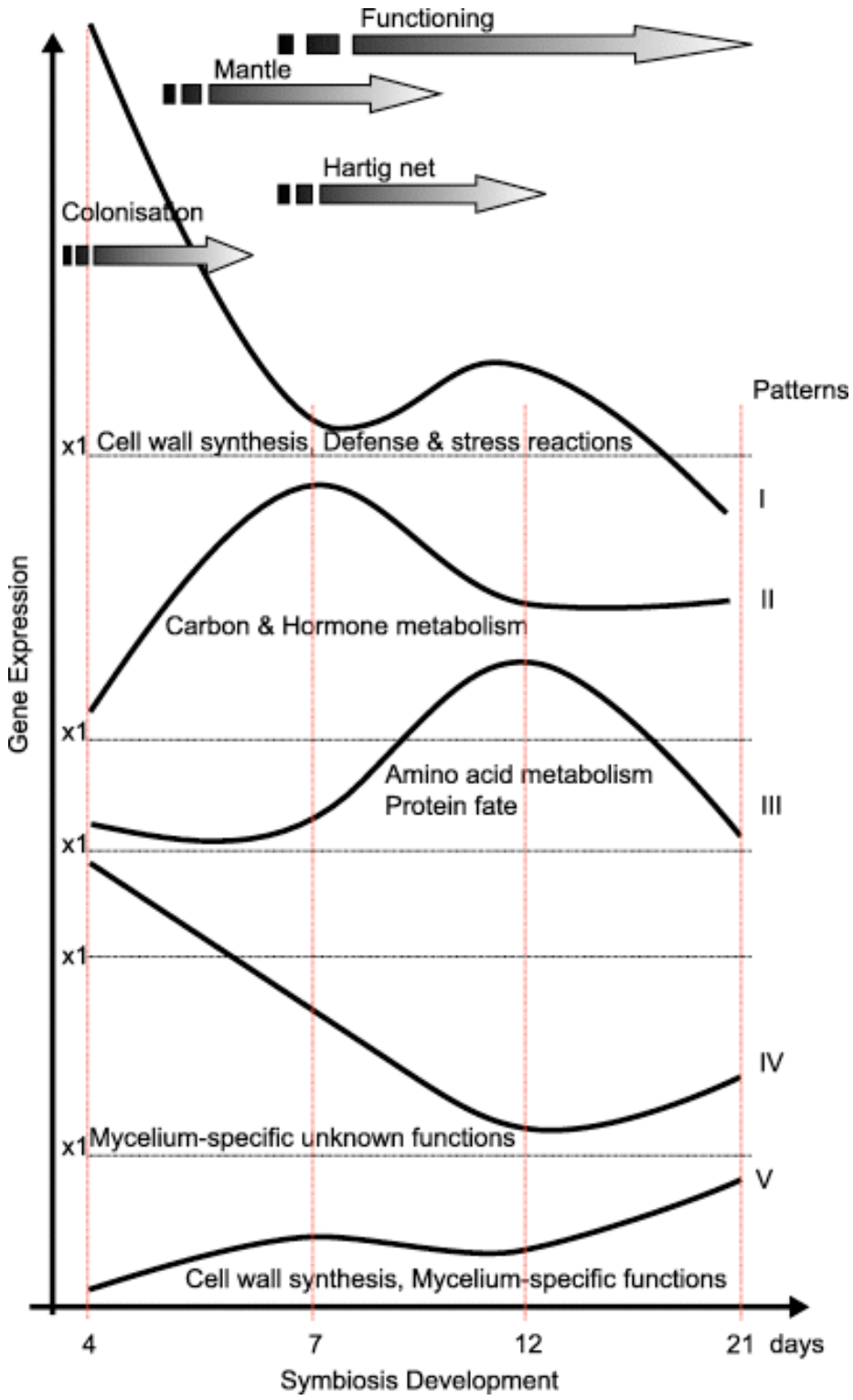

Figure 16: Gene expression profile during ECM symbiosis establishment

Scheme describing the five major gene expression pattern of plant and fungal genes during the development of ECM symbiosis between *Pisolithus tinctorius* and *Eucalyptus globulus*. Mean expression values for each cluster are shown. The pre-colonization phase has not been analyzed. The colonization phase could be attributed from approx. 4 to 12 days (colonization, mantle and Hartig net formation) and the late phase starting at 12 days. From Duplessis et al. (2005)

A transcript profiling realized by Duplessis et al. (2005) on *Pisolithus tinctorius* and *Eucalyptus globulus* over time of root colonization highlights also at a molecular level distinct phases in ECM establishment, with each phase involving a set of characteristic differentially expressed genes (Fig. 16). But also these molecular events show a certain overlap (note that in their study the pre-contact phase is lacking). In terms of molecular events in the intermediate phase a strong defence and stress reaction that decreased further on was observed (Duplessis et al., 2005). It may be suggested that once recognition and adhesion is achieved, this defence response is actively repressed to enable colonization. Defence reactions were also observed by Sebastiana et al. (2009) in chestnut challenged with *P. tinctorius* as early as 6 hours after contact of roots and fungi (adhesion of the fungus started within 2h of contact (Baptista et al., 2007)). Cell wall modification, probably due to adhesion, followed the same pattern as stress responses pattern (Duplessis et al., 2005), thereby stressing that recognition and adhesion permit the stress response to decrease. Colonization and Hartig Net formation coincided with alterations in carbon and hormone metabolism. The carbon metabolism activation at these steps indicates a preparation for nutrient exchange (Duplessis et al., 2005). Different phytohormones may regulate the colonization phase. At the transition from intermediate to late phase, a transient induction of genes involved in amino acid and protein metabolism was observed (Fig. 16). Those may be related to adjusting plant and fungal metabolism to the newly established common life of plant and fungus. Interestingly a slight transient defence and stress response was observed again, which coincided with the end of Hartig net development. This stress response may be a mean to accomplish Hartig net formation, that then will not proceed further and to get into equilibrium between defence and offense of both partners. In the functional ECM (late phase), a steady state induction of carbon and hormone metabolism was observed, characteristic for the ongoing nutrient exchange.

Taken together, ECM development is characterized by specific, partially overlapping developmental phases that can be distinguished on the basis or physiological and developmental aspects as well as on a molecular level (genes involved).

Even though several studies have addressed the specific signals that trigger ECM symbiosis establishment, the molecules and recognition mechanism involved remain largely unknown. Time course data is only available for few studies. In the following we will revisit molecules, which have been identified and make an attempt to assign them to a specific phase in ECM development. However, as data from time course analysis is available only for few molecules, it is possible that certain molecules are involved in more phases than the one they have been allocated to in our model.

III.3 Signalling molecules in ectomycorrhizal symbiosis

III.3.1 Rutin and zeatin in plant exudates

Root exudates have been suggested to contain compounds that are recognized by fungi and that attract them (Horan and Chilvers, 1990). In *Eucalyptus* root exudates the flavonol rutin has been identified that stimulates growth of the fungus *Pisolithus tinctorius* already at a picomolar concentration (Lagrange et al., 2001; Martin et al., 2001). It was furthermore shown that cytokinins are able to induce branching of ECM hyphae (Gogala, 1991; Martin et al., 2001). These factors might be the first communication between a plant and its symbiotic ECM fungus in the early phase (Fig. 17). Their attribution to the early phase can be made because it was shown that also when a barrier is present between roots and fungi, compatible fungi will recognize the root and grow towards it (Horan and Chilvers, 1990), suggesting that molecules present in root exudates are sufficient for this step. The fact that a flavonol is within these "branching factors" is an interesting finding, as also rhizobia bacteria are attracted by flavonoids secreted by the plant (Aguilar et al., 1988). This indicates that similarities may occur between different symbioses concerning attraction of the microbial symbiont by the plant partner. Interestingly the perception of plant exudates does not only trigger development of hyphae but also accumulation of metabolites, such as hypaphorine, which may be involved in fungus to plant signalling and will be discussed below (Beguiristain and Lapeyrie, 1997).

III.3.2 Fungal and plant auxins

Slankis (1950) was one of the first, who suggested an important function of auxin released by ECM fungi during ECM development. Since then different research groups have addressed auxin production by ECM fungi and their effects in axenic cultures. Even if quantification techniques were different and reached from thin-layer chromatography experiments for the earliest analysis (Ho, 1987b, a) over quantification with ELISA techniques and IAA antibodies (Karabaghli-Degron et al., 1998; Rincon et al., 2003) to GC-MS analysis (Splivallo et al., 2009), all revealed a low fungal auxin-production in a nanomolar range (10 to 300 nM for different fungi (Ho, 1987a, b; Rincon et al., 2001; Rincon et al., 2003; Reddy et al., 2006)). In comparison, the overproducing mutants of *Hebeloma cylindrosporum* released 900nM of IAA (Reddy et al., 2006). As it is a diffusible molecule, auxin could be assigned to be part of the early (pre-contact) communication between fungus and plant that induce LR development. Furthermore, auxin may also be involved in cell-wall modification during colonization and Hartig net formation in the intermediate phase. In this context auxin has been reported as part of the "Acid-Growth Theory" to modify cell wall compositions and to permit growth of cells (Rayle and Cleland, 1992). Mensen et al. (1998) proposed that fungal auxin could reduce the peroxidase-catalyzed linkage of the cell-wall constituents, allowing hyphae to penetrate between the cortical cells to form the Hartig net.

Looking into the different results reported in these various studies can clarify whether fungal auxins are involved in both the early and the intermediate phase of ECM development. For instance in Norway spruce LR induction required 100 to 500 μM exogenous IAA and below this no effect was observed (Karabaghli-Degron et al., 1998), while when challenged with *L. bicolor* that secretes about 10nM IAA a visible LR induction occurred. Splivallo et al. (2009) had revealed an induction of the auxin responsein roots during contact with truffle fungi, as visualized by *Arabidopsis thaliana pDR5:GFP* lines. However the fungi only released about 100nM IAA into the medium, which may be considered as too low to induce this effect. Accordingly, the authors were not able to reproduce the effect of the fungus by exogenous auxin application. Results from Tranvan et al. (2000) had shown that the IAA-overproducing *Hebeloma cylindrosporum* strain induced more LRs than the wildtype strain during pine root colonization but also colonization (rapidity and Hartig net depth) itself was enhances. Reddy et al (2006) as well as Charvet-Candela et al. (2002) had reported about the differential expression of auxin-inducible genes *PpGH3-16* and *PpIAA88* in *Pinus pinaster* roots root during colonization by *H. cylindrosporum* or *Rhizopogon roseolus*. Interesting their study demonstrated no difference in transcript amount between roots challenged with wildtype or the IAA-overproducing strain but a faster transcript change when the auxin overproducer was used. This indicates that the transcript changes parallel mostly the faster colonization. Niemi et al.'s (2002) results pointed out that also an incompatible strain of *Paxillus involutus* enhanced LR formation when applied to Scots pine despite the fact that it did not colonize. Lastly Karabaghli-Degron (1998) and Rincon (2001) and colleagues had addressed whether inhibition of polar auxin transport (acting on the plant and apparently not on auxin secretion from the fungus) impacted fungus-induced LR stimulation as well as root colonization. They discovered that the polar auxin transport inhibitor TIBA completely inhibited both processes during the *Eucalyptus/Pisolithus* interaction whereas the inhibitor NPA lowered LR induction and lead to an irregular colonization of roots. We may therefore summarize the current hypotheses concerning the role of auxin during plant ECM fungus-interaction:

(i) the amounts of IAA secreted by the fungus are too low to induce LRs on their own,

(ii) fungal auxin alone is not sufficient to induce colonization, if the fungal strain used is incompatible,

(iii) induction of auxin responses inside the plant does not seem to be dependent on the amount of fungal auxins,

(iv) root colonization requires an operative auxin transport system in the plant and

(v) high amounts of auxin released by the fungus enhance and accelerate root colonization by the hyphae.

In conclusion, the component that is likely to be responsible for **LR induction** in the plant upon contact with the fungus is not fungal but probably **plant endogenous auxin and its polar transport.**

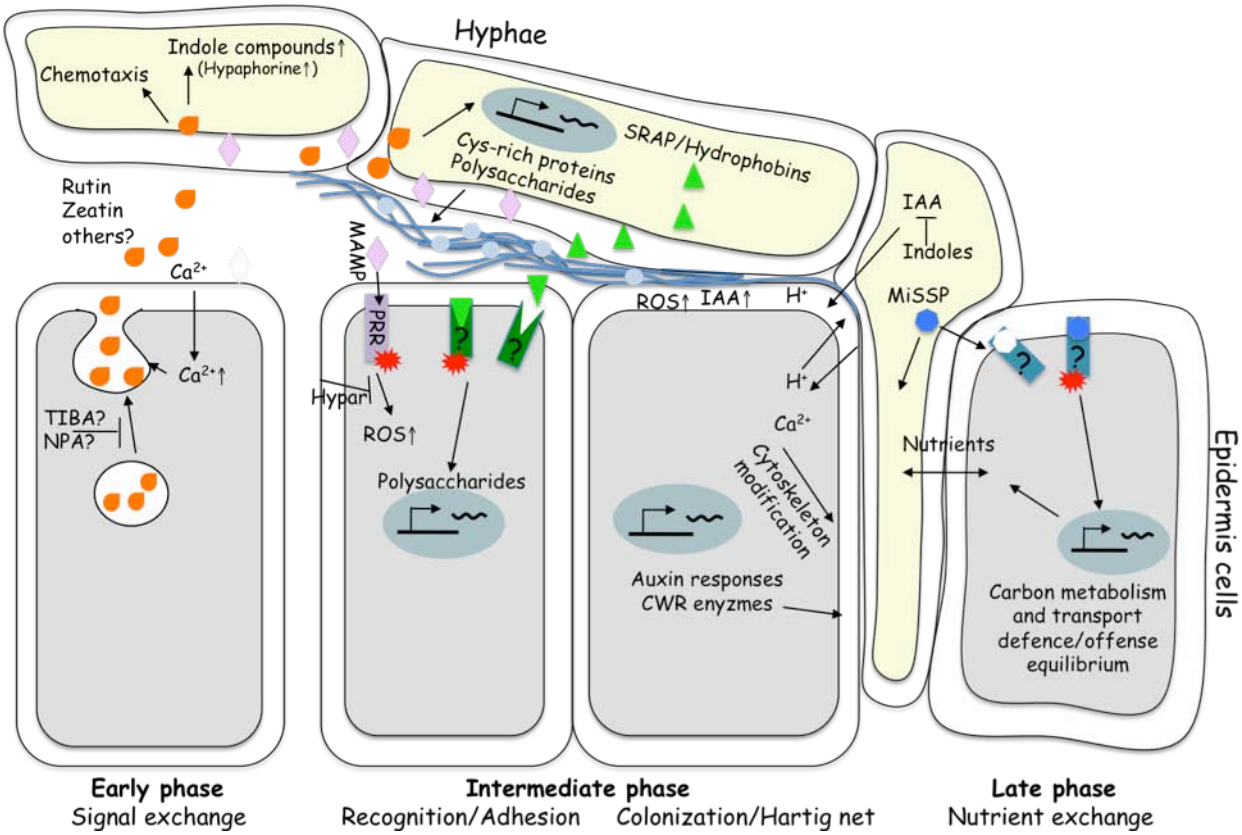

Figure 17: Signalling during ECM development

In the **early phase** root cells constitutively secrete (maybe via exocytosis) compounds such as rutin or zeatin, which trigger chemotaxis in the fungus as well as accumulation of hypaphorine or other unidentified indole-compounds. Indole compounds trigger Ca^{2+} influx, which might increase exocytosis of signalling molecules and amplify the signalling. During the **intermediate phase** microbe associated molecular pattern (MAMP) are perceived by a pattern recognition receptor (PRR) and trigger a defence-response, which may be repressed by indole compounds by activating *Hypar* Glutathione-S-Transferase. This might as well already occur during the early phase (not shown). The fungus produces symbiosis related acidic proteins (SRAP) and hydrophobins, which are recognized by a specific receptor in the plant. The recognition triggers adhesion via polysaccharide production by both partners and cysteine-rich protein release by the fungus. The fungus continues to secrete indole compounds as well as IAA, which accumulate in the apoplast. A proton efflux through plasma membrane H^+-ATPases acidifies in addition to IAA and indoles the apoplast. IAA may as well stimulate cell wall remodelling enzymes (CWR) and induce ROS production. Ca^{2+} accumulation in the cell may trigger cytoskeleton rearrangement. Together apoplast acidification, peroxides and enzymes modify the cell wall, render it flexible and permit penetration of the fungus for Hartig net formation. In the **late phase** mycorrhiza induced small-secreted proteins are released and may as well trigger nutrient metabolism and exchanges as well as a defence/offense equilibrium in both partners that arrest Hartig net formation.

On the other hand, **fungal auxins** may **facilitate colonization downstream of recognition**. Also in case of LRP emergences in the absence of any fungi, distinct auxin maxima have been shown to trigger cell-wall remodelling enzymes and to permit cells to separate (Swarup et al., 2008). A similar mechanism may be involved that permits plant endogenous auxin to trigger cell separation upon LRP emergence and fungal auxin to activate cell separation during Hartig net establishment. Due to the lack of identification of auxin biosynthesis pathways in ECM fungi (contrary to other fungi (Reineke et al., 2008)), hitherto no mutants deficient in auxin production are available. Those could confirm in which ECM developmental processes fungal auxins is the main trigger.

Interestingly also an antagonist of auxin has been shown to be involved in ECM formation. This molecule is the tryptophane-derivative hyphaphorine that is produced in large amounts by *Pisolithus tinctorius* upon ECM formation (Beguiristain and Lapeyrie, 1997). It has been proposed that hyphaphorine secreted by the fungus influences root endogenous auxin and thereby regulates symbiosis establishment (Ditengou et al., 2000; Ditengou and Lapeyrie, 2000; Jambois et al., 2005). Hypaphorine had been shown to interfere with the actin and microtubulin cytoskeleton (Ditengou et al., 2003) and with calcium fluxes (Dauphin et al., 2007), at least in root hairs. However, not all ECM fungi produce hypaphorine. Thus a general mechanism based on this compound cannot be proposed. Nonetheless it cannot be ruled out that fungi, which do not produce hypaphorine may produce other indole-compounds with similar activities.

Taken together, due to its low concentration, fungal auxin is unlikely to be the trigger of LR stimulation in the early phase of root/ECM fungus interaction. Sufficient fungal auxin accumulation in plant tissues is probably only possible when the contact of both partners is close (mantle/Hartig net) and one could hypothesize that fungal auxin is involved in the intermediate phase in Hartig net development (Fig. 17).

III.3.3 Defence- and stress-related signalling

Different transcriptome studies have revealed that a defence reaction arises in the plant upon early contact with the fungus, which is repressed at later stages of colonization. (Duplessis et al., 2005; Le Quere et al., 2005; Sebastiana et al., 2009). This distinguishes this symbiotic interaction form a pathogenic one, during which defence reactions increase continuously. Stress/defence responses involve an accumulation of reactive oxygen species (ROS). ROS production during colonization of roots by ECM fungi is a known phenomenon and is likely to depend on the compatibility of the interacting partners (Gafur et al., 2004). Baptista et al. (2007) showed that three peaks of ROS production can be seen during ECM formation, with the first occurring already within 2 hours of co-culturing of plant and fungus. They described this time-point as the earliest where fungal material was detected on the root surface. We may thus assign ROS production as an event occurring during adhesion (intermediate phase).

Commonly during microbe/plant interactions an innate immunity response triggers the defence reaction in the plant partner. Innate immunity relies on the perception of microbe/pathogen associated molecular pattern (MAMP/PAMP) by plant cells via receptors that activate the defence response (Boller and He, 2009). But microbes and pathogens can release effectors (polysaccharide, proteins, phytotoxins etc.) that repress this immune (defence) response. During ECM symbiosis an innate immunity response has not yet been revealed, but as it is a rather general mechanism, we may suggest it to take place upon root/ECM fungus interaction. The fact that during ECM symbiosis establishment the stress-response is suppressed implies that the fungus secretes an effector.
Nehls et al. (1998) identified induction of *EgHypar* expression, coding for a Glutathion-S-Transferase, early in *Eucalyptus* roots challenged with the ECM fungus *P. tinctorius*, or after exogenous auxin (or 2,4D) or liquid fungal extract application. Signalling molecules released by the fungus are therefore sufficient to stimulate *EgHypar*, which can be assigned to the early phase of plant/fungus interaction. Interestingly, *EgHypar* was also activated by sole application of hypaphorine. Duplessis et al. (2005) confirmed that *EgHypar* was repressed at later stages of interaction. In a further study, Tagu et al. (2003) have pushed further the characterization of EgHypar and confirmed its activity as a Glutathion-S-Transferase. They suggested that EgHypar may be part of the mechanism the fungus uses to repress the defence response of the plant (Fig. 17). Hypaphorine could be understood as an effector that represses MAMP induced defence in the plant in the early phase of root/ECM fungus interaction.

In summary we suggest that the colonization of the root by the ECM fungus requires repression of defence responses that occur during the early plant/fungus interaction. Interestingly, there are molecular mechanisms connecting ROS as well to auxin, thus the stress-response and LR stimulation may originate from a common trigger. For instance, an auxin increase is known to favour ROS production in cells (Jiang and Feldman, 2005). Furthermore brassinosteroids, which are involved in ROS production (Xia et al., 2009), also influence polar auxin transport (Li et al., 2005).

III.3.4 Volatile phytohormones

Different studies have shown that ECM fungi are able to produce the volatile phytohormone ethylene (Graham and Linderman, 1980; Rupp et al., 1989; Splivallo et al., 2009). Others have demonstrated that fungi are able to produce jasmonates (Miersch et al., 1999). Both are diffusible molecules and may act in the signalling events during the pre-colonization phase. Ethylene and methyl-jasmonates are furthermore known for their stimulatory effect on LR development (Ivanchenko et al., 2008; Sun et al., 2009) and may be regarded as possible actors during fungus induced LR stimulation. Splivallo et al. (2009) considered ethylene released by ECM truffle fungi as a stimulator of LR development in Arabidopsis. They observed that in the ethylene insensitive Arabidopsis line *ein2* LR stimulation by

these fungi was decreased and that in feeding experiments ethylene mimicked fungal LR induction only when applied together with exogenous auxin. In addition there are also ECM fungi, such as *Pisolithus tinctorius*, which do not produce ethylene but nevertheless stimulate LR development (Rupp et al., 1989). **This suggests that ethylene may be part of the LR stimulating signals exchanged between fungus and plant during the early phase of interaction, but that it is not the only.** Interestingly the exogenous application of methyljasmonate accelerated the first mycorrhizal contact of spruce roots and *L. laccata* (Regvar et al., 1997), which also suggest a role for methyljasmonate during the early phases of ECM establishment.

Experiments on ethylene levels during colonization have not distinguished between ethylene derived from the plant or from the fungus. The finding of Rupp et al. (1989) that there was an increase in ethylene levels released by roots colonized either with *L. bicolor* (produces ethylene) or with *P. tinctorius* (does not produce ethylene) suggest that also the plant partner produces ethylene upon colonization. The same could be valid for jasmonates. Jasmonate and ethylene are usually associated with the defence against necrotrophic pathogens or herbivore insects (Bari and Jones, 2009), but act as well in biotrophic (mutualistic) associations (Gutjahr and Paszkowski, 2009). In consequence, they may be involved in the early defence response during root/ECM fungus contact.

Together ethylene and jasmonates may be produced by both partners during ECM development and interconnect, like ROS, defence responses and LR development.

III.3.5 Ion fluxes

At the root surface, proton and Ca^{2+} fluxes are observed with each presenting a specific oscillation profile. A proton (H^+) efflux from the root is characteristic for the apex, meristematic and elongation zone and regulates the cytoplasmic pH of the root. Ramos et al. (2009) have reported that these fluxes as well as the periodicity of oscillation were increased specifically in the elongation zone of *Eucalyptus/Pisolithus* ECM in comparison to un-colonized roots. The increase of H^+ efflux through a plasma membranous H^+-ATPase may be important for Hartig Net development, because cell wall loosening is mechanistically achieved by acidification of the apoplast (Rayle and Cleland, 1992). H^+ fluxes are connected to Ca^{2+} fluxes. Intriguingly during colonization with *Pisolithus tinctorius* Ca^{2+} fluxes were decreased, their periodicity was disrupted and in the EZ Ca^{2+} fluxes were inverted from effluxes to influxes. Calcium is an important second messenger and may be involved in signalling upon colonization, comparable as what is known from endomycorrhizal and root nodule symbiosis (Fig. 14, Oldroyd et al. (2009)) The increasing acidification of the apoplast may further facilitate Hartig net formation and cell-spacing.

Together this data shows that different diffusible metabolites and ions are involved in signalling during ECM development. Those have rather non-specific effects on plant development and trigger broad responses during the interaction. Furthermore, fungi also secrete

peptides and small proteins that can have a developmental role (e.g. pheromones in inter-hyphae communication) (Kües and Navarro-Gonzales, 2009). Those small proteins may serve as well as a ligand and bind to specific, yet unknown receptors, at the plant cell surface, which then trigger for example recognition and adhesion during ECM development.

III.3.6 Polysaccharides, Symbiosis induced proteins and hydrophobins

Hyphae aggregation and formation of the adhesion pad at the root surface involves recognition between both partners and synthesis of different cell-surface structures. Those are for instance highly oriented glycoprotein fibrils, polysaccharides and cysteine-rich proteins (Lei et al., 1990; Lei et al., 1991; Rincon et al., 2001). Hyphae also induced "cracks" into the outer cortical cell wall layer ("cuticule") and attached to host cell walls through a polysaccharide interface formed by both partners. When this process was inhibited using the auxin transport inhibitor TIBA, the adhesion as well as mantle and further Hartig Net development failed (Rincon et al., 2001), showing that this first process in adhesion is crucial for further colonization. In fungus collected from forest soils under pine and applied to eucalyptus the polysaccharide interface was absent (*Eucalyptus/Pisolithus* partnership) and no mycorrhiza formed, indicating that a recognition process between compatible partners is required. In the incompatible association a pathogen resembling cell wall thickening was observed in the plant (Lei et al., 1990). It was hypothesized that inhibition of fungal IAA transport inside the root through TIBA inhibits the plant-fungus recognition process and the production or the efflux of host elicitors responsible for the increase of fungal polysaccharide fibrils associates with cysteine-rich proteins. Here fungal IAA was proposed as a recognition process of both partners. Interestingly it has recently been reported that TIBA acts on vesicle trafficking and cytoskeleton (Dhonukshe et al., 2008). There might be the possibility that the effect of TIBA does not go solely through auxin, but also that it inhibits secretion of vesicles loaded with small proteins needed for host/symbiont recognition prior to mantle and Hartig Net establishment.

Certain fungal cell-wall polypeptides are specifically expressed in *Pisolithus* before or at early phases of *Eucalyptus* root colonization and are downregulated later in the mature mycorrhiza (Hilbert and Martin, 1988; Hilbert et al., 1991; Martin and Tagu, 1995; Duplessis et al., 2005). Those were termed 'Symbiosis Related Acidic Polypeptides' (SRAP). Another group of cell-wall related proteins that were over-expressed specifically at the same time points in *P. microcarpus* were hydrophobins. Their specific expression pattern may assign them a role during the recognition and adhesion phase as soon as plant and fungus get into contact.

Microarrays based analysis of *L. bicolor* ectomycorrhiza induced proteins have revealed the strong induction of transcripts coding for so-called 'Mycorrhiza induced Small Secreted Proteins' (MiSSP). One specific *MiSSP* (*MiSSP7*) that was strongly induced in the mature ECM, but neither expressed in the free-living mycelium (Martin et al., 2008), nor at earlier steps of colonization (J. Plett,

personal communication) was investigated more closely. Its protein localized to the Hartig net at mature ECM. It has been suggested that these peptides may be involved in triggering either nutrient exchange or maintaining the equilibrium between offense and defence in the mature ECM (Martin and Nehls, 2009).

In addition to the signalling molecules with broad signalling functions (phytohormones, metabolites and ions), the peptides and proteins mentioned here might have more specific functions in recognition processes. A complex cross-talk between all signalling molecules is likely to regulate the different phases of ECM establishment (Fig. 17). Interestingly, diverse phytohormones are part of the signals likely to govern ECM establishment. These phytohormones might cross-talk with auxin pathways in the root and may thereby alter LR development.

Objectives & Strategy

Box1: The *Populus* genus - pop(u)lar model trees

The *Populus* genus is compost of about 30 species within the family *Salicaceae* commonly named poplar, aspen or cottonwood. Related to leaf and flower characters they have been divided into six sections. Native from the northern hemisphere they occur in areas from the boreal forest to the banks of the Loire valley (France). Their inter-American *(Populus trichocarpa x deltoides*) and Euroamerican (*P. deltoides x nigra*) hybrids constitute large planted areas in Europe, Asia and North America. *Populus tremula* is one of the world's most widely distributed tree species, with its natural range extending throughout Europe to northeastern Asia and into northern Africa. It is a pioneer species that tolerates a wide range of climatic and soil conditions. *Populus* species are characterized by being deciduous, having mainly dioecious flowers and presenting fast and high growth.

Because of their fast rapid development, poplar trees have today various economical applications. In France, poplars are the second most important deciduous species for the production of logs and as a raw material for the veneer industry. They also constitute a crucial factor in the maintenance of the diversity of the ripysilves (forest ecosystems along rivers) and are a large sink for carbon dioxide in the objective of carbon sequestration (Lemus and Lal, 2005). Furthermore they are used in short rotation forestry as energy plants (Dickmann, 2006) and for phytoremediation and phytoextraction of contaminated sites (Robinson et al., 2000).

The importance of poplar trees for industrial and environmental forestry explains why forestry research has shown so much interest in poplars in the last few decades. The existence of protocols for vegetative multiplication and efficient genetic transformation, the construction of several genetic maps, together with rapid growth, have raised the poplar to the rank of a model tree for forest biochemistry, physiology and genetics. The relatively small size of its genome, 520 Mb, is a supplementary asset (Martin et al., 2004; Tuskan et al., 2006) and the public availability of its genome sequence is a powerful tool in molecular biology and genetics.

Box 2: *Laccaria bicolor* – the model ECM fungus

L. bicolor is the first ECM fungus whose whole genome sequence was published (Martin et al., 2008). *L. bicolor* is a basidiomycete that is widely distributed as an ECM symbiont of many autotrophs (within those poplar). An advantage, which has significantly influenced its selection as a model ECM fungus is its tractability as a laboratory organism. Compared to other basidiomycota whose genomes have been analyzed, the one of *L. bicolor* is with 60Mb much larger. A total of about 19,000 genes on 12 chromosomes among which 20% are transposable elements, making the genome extremely flexible to evolve (Martin and Nehls, 2009). Among the 50% of genes with predicted functions many are linked to degradation of soil-polymers, they are for instance chitinases, cellulases, glucanases, proteases, lipases and phytases. This is plausible because *L. bicolor* is of saprophytic origin and therefore required these functions to extract nutrients from the dead soil matter. However, today it is limited to symbiotic lifestyle. Interestingly, compared to saprophytic *C. neoformans* and *P. chryosporium*, the larger size of the genome of *L. bicolor* led to new functions especially in signal transduction pathways that may have important roles for interaction with roots after having achieved a symbiotic lifestyle (Martin, 2007).

Objectives

In the introduction it has become clear that, even if LR stimulation during plant/fungus interaction is a commonly observed phenomenon, the molecular mechanisms that governs this process is yet unknown. However, from intensive studies on LR formation in the herbaceous model plant *Arabidopsis thaliana* we know that polar auxin transport (through auxin efflux carriers (PIN) and influx carriers (AUX/LAX)) and auxin signalling constitute the most downstream regulators of LR initiation in plants. This is why in 2005 in our lab (interaction arbres/micro-organismes), with the Master's thesis of Stéphane Hacquard, studies were initiated on the impact of ectomycorrhiza (ECM) formation between poplar and *Pisolithus tinctorius* on auxin efflux carrier (*PIN*) expression in plant roots. Interesting results revealed that the presence of the fungus stimulated the expression of some members of the *PIN* gene family and strengthened the idea that plant auxin pathways might be involved in fungus-induced LR stimulation. Based on these findings, the phytohormones involved in plant/fungus interaction and the possibility that these phytohormones cross-talk with plant endogenous auxin pathways, we propose a working model (Fig. 18). This model highlights anchor-points where the hormones involved in plant/fungus crosstalk could impact LR influencing auxin pathways.

Thus, the **objective** of my PhD project was to **identify these auxin-related molecular key actors inside plant roots** that **respond to fungal signals** and that are **required for fungus-induced LR stimulation** during the early root/fungus interaction. I furthermore assessed how other **phytohormone- and defence-related pathways** are altered in roots during contact and might **explain the impact of the fungus on auxin-related** genes.

The work was carried out as a German-French PhD project involving the group of Klaus Palme, Freiburg University (Germany) and the group of Francis Martin, Tree/Microbe Interactions, INRA Nancy (France). The first group specializes in LR development in the herbaceous model plant *A. thaliana* and its regulation by auxin distribution through PIN auxin efflux carriers, where the latter is working on tree/microbe interactions, comprising ECM symbiosis.

Strategy

We selected for this study the basidiomycete *Laccaria bicolor* and the hybrid tree *Populus tremula x Populus alba* as ectomycorrhizal partners. The genomes of both species have been sequenced (Box1 and 2) and in addition numerous tools and techniques are available enabling a detailed analysis of the molecular processes activated upon their interaction. We have also used the non-mycorrhizal herbaceous plant *Arabidopsis thaliana* in interaction with *L. bicolor*. Even if it does not undergo symbiosis, LR development is stimulated during contact with the fungus. Because various mutants

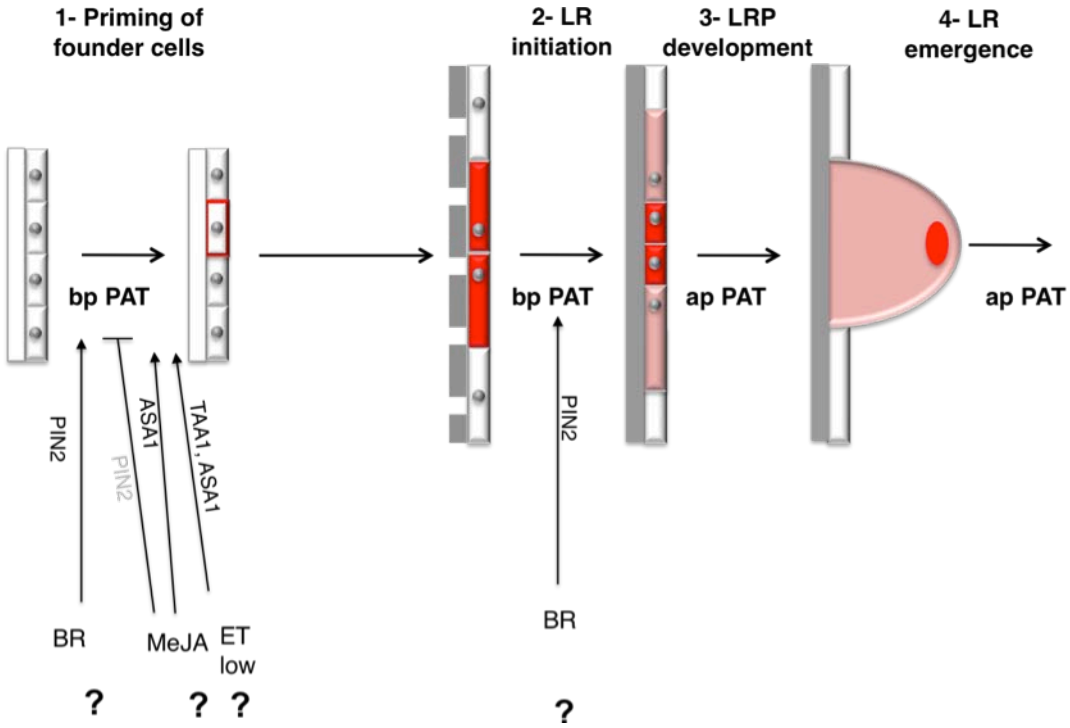

Figure 18: Working model - possible impact of ECM signalling molecules on auxin pathways and LR development

Scheme indicating the four steps of LR development and their regulation by polar auxin transport (PAT). Phytohormones Brassinosteroids (BR), Methyl-jasmonates (MeJA), Ethylene (ET) and Cytokinin (CK) interfere with LR development when applied exogenously or altered in mutants by altering auxin transport or biosynthesis. Jasmonates and ethylene are released by fungi and stimulate auxin biosynthesis and Brassinosteroids may be involved in ROS production and in parallel influence PAT. Question marks indicate possible cross-points for plant/fungus signalling and LR development.

with altered hormone sensitivity, auxin transport and LR development are available, experiments with *A. thaliana* permit screening for pathways involved in fungus-induced LR stimulation.

The strategy chosen to elucidate the molecular events that accompany and are involved in fungus-induced LR stimulation consisted of the following approaches:

- Setting up an *in vitro* plate culture system as a tool for mycorrhiza formation of poplar with *L. bicolor* under controlled conditions and easy observation of root development during colonization
- Comparing LR stimulation by the fungus in a mycorrhizal (poplar) and a non-mycorrhizal plant (Arabidopsis) in the presence and absence of drugs employed to inhibit certain auxin-related pathways
- Studying gene expression in poplar based on whole genome microarrays and Real-time PCR during interaction with *L. bicolor*
- Screening Arabidopsis marker-lines or transgenics with defects in auxin transport or signalling for signal localization or responsiveness during interaction with *L. bicolor*
- Assessing LR formation and ECM formation in wildtype poplar and poplar transgenics defective in key actors identified for fungus induced LR development (collaboration of F. Martin's team with other poplar research groups)
- Investigating plant stress-related responses that might interfere with auxin-signalling based LR stimulation (in collaboration with the group of Prof. Dr. Klaus Palme and the laboratory of Tree Physiology leaded by Prof. Dr. Heinz Rennenberg at the University of Freiburg)

The **results** obtained based on these approaches are presented in form of three publications:

- **Chapter I** (article in preparation) deals with the characterization of poplar/*L. bicolor* ECM development in the newly optimized *in vitro* plate culture system.
- **Chapter II** (article accepted in *Plant Physiology*) points out how auxin-related pathways get modulated in roots upon contact with *L. bicolor* and how this activates LR development.
- **Chapter III** (article in preparation) reports on the activation of defence-responses in poplar roots and considers those as a possible upstream mediator of plant auxin-pathways and a trigger of LR stimulation.

Results

Chapter I

Ectomycorrhiza development between *Populus tremula* × *Populus alba* and *Laccaria bicolor* in an *in vitro* culture system

- Article in preparation -

In the beginning of my PhD project I optimized an *in vitro* Petri dish culture system for *Populus tremula x Populus alba/Laccaria bicolor* ECM formation under controlled conditions as a tool for all further experiments (for details see Annex A1). The culture system was adapted from Chilvers et al. (1986) and Burgess et al. (1996). It permits synthesizing ECM or to study the signal exchange between both partners while colonization is prohibited (Fig. 19). In this first chapter the time-course and developmental processes of ECM formation in this culture system are described. These data permit to integrate the molecular data from Chapter II and III into an ECM developmental context.

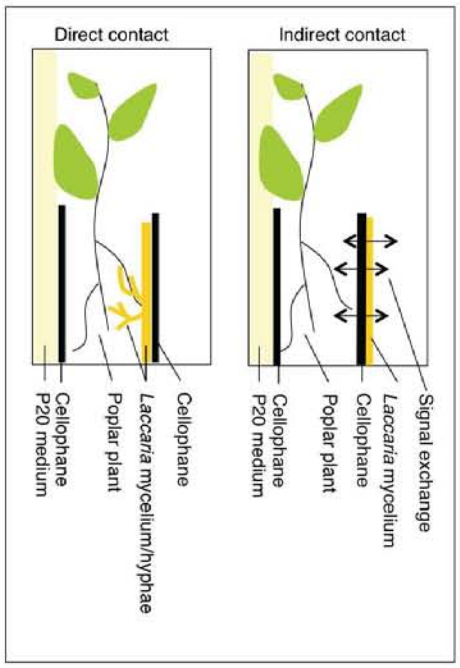

Figure 19: Sandwich culture system for *in vitro* poplar/*L. bicolor* ECM development

Side view on vertically arranged cultures plates containing rooted poplar cuttings and *L. bicolor* mycelium after pre-culturing of each alone on cellophane-membrane overlaid agar plates. In order to obtain direct contacts with mycorrhiza formation cellophane membranes with *L. bicolor* mycelium were laid upside down on the roots. Hyphae then colonized the roots. To achieve indirect contacts the cellophane membrane conveying the mycelium was laid upside-up on the roots. Hyphae could not grow through the membrane and thus colonization was prohibited. However signal molecules could be exchanged through the membrane.

Ectomycorrhiza development between *Populus tremula* × *Populus alba* and *Laccaria bicolor* in an *in vitro* culture system

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Abstract

The symbiotic interaction between the poplar tree and the ectomycorrhizal basidiomycete *Laccaria bicolor* has emerged as a model system for research in ectomycorrhiza (ECM) development and functioning since the sequencing of their genomes. We have developed an *in vitro* culture system for controlled, standardized *Populus tremula x Populus alba*/*L. bicolor* ECM formation based on a plate-sandwich culture using cellophane membranes as a vehicle for the fungal mycelium. We then describe stepwise root development and ECM formation in this *in vitro* co-culture system. Using macroscopic root observations and fluorescent-double stained root sections we show that development of a paraepidermal Hartig net occurred after only one week of interaction at the basis of newly formed lateral roots (LRs). Complete colonization of LRs, from the basis to the tip, took three to four weeks and paralleled the decay of the root cap. The LR elongation rate during forming ECM was decrease compared to controls between weeks two and three of co-culturing, thereby coinciding with hyphae starting to colonize the LR tip. The first two weeks of interaction between plant and fungus were paralleled by an enhanced LR formation, which was absent at later stages. LR stimulation was also observed in conditions that allow signal exchange between both partners but prohibit root colonization, suggesting its origin in signal molecules and not colonization itself. Together these data give a detailed vision on ectomycorrhiza development from a root developmental point of view in the symbiotic-model system poplar/*L. bicolor*.

Introduction

With the emerging outcome of sequences of fungal and tree genomes, researchers can face today an upcoming richness of tools for genetics and genomics that will help to understand ECM formation in more detail. The use of the poplar/*L. bicolor* interaction as a model partnership for ECM symbiosis becomes particularly evident since both genomes, *Populus trichocarpa* and *Laccaria bicolor*, have been sequenced (Tuskan et al., 2006; Martin et al., 2008; Martin and Selosse, 2008). Furthermore the annotation is well advanced and tools such as whole genome microarrays (NimbleGen), genetic maps (Kelleher et al., 2007; Labbe et al., 2008) and transformation protocols are now available (Han et al., 2000; Kemppainen et al., 2005). Using poplar/*L. bicolor* ECM it is therefore for the first time possible to study and to interconnect molecular processes of ECM establishment in order to reconstruct on a molecular level each step of symbiosis development, from the first recognition of plant and fungus up to nutrient exchange in the functional ectomycorrhiza. However, to date poplar/*L. bicolor* ECM formation has only been realized in greenhouse experiments in pot-cultures and only very little is known about their interaction in plate cultures *in vitro* (Langer et al., 2008). Because of the numerous advantages of axenic plate-culture systems (control of contact site, stable

culture-conditions, non-invasive observations, cost- and material efficiency) we set up *P. tremula x P. alba/L. bicolor* ECM formation in an *in vitro* plate culture system based on techniques of (Chilvers et al., 1986; Burgess et al., 1996) with cellophane as a vehicle for *L. bicolor* mycelium. This culture-system presents an essential tool to generate poplar/L. bicolor ECM for all different types of molecular and biochemical analysis. However, in order to be able to interpret molecular results based on a developmental context, there was the need to assess the time course and particularities of ECM formation between those partners.

Here we report about root colonization and root developmental changes associated with ECM establishment in this culture system. Macroscopic root development observations together with confocal laser scanning microscopy of fluorescent doubled stained transverse and longitudinal root sections were used to study root colonization. In addition, lateral root stimulation was quantified, the position of emergence of LRs assessed and LR elongation during colonization measured. Thus, our data give insights into changes in root development in the context of root colonization during the establishment of the poplar/*L. bicolor* ECM under standardized *in vitro* conditions.

Material and Methods

Plant and fungal material

Experiments were performed with the hybrid *Populus tremula x Populus alba* (clone INRA 717-1-B4). Plants were micropropagated *in vitro* and grown on half MS medium (Murashige and Skoog, 1962) in glass culture tubes under a 16 h photoperiod at 24°C in a growth chamber.

The dikaryotic vegetative mycelium of the ECM fungus *Laccaria bicolor* strain S238N (Maire P.D. Orton) was maintained at 25°C on modified Pachlewski medium P5 (Paschlewski and Pachlewskia, 1974; Deveau et al., 2007).

Co-culture system

For mycorrhiza formation on *in vitro* poplar plants (*P. tremula x P. alba*) by *L. bicolor,* we modified an existing sandwich co-culture system initially developed for the *Eucalyptus*/*Pisolithus* interaction (Chilvers et al., 1986; Burgess et al., 1996). The protocol is described in Felten et al. (in press). Briefly, poplar explants were synchronized for rooting for one week on half MS containing 2mg/L IBA and then cultured another three weeks in the absence of hormones on cellophane covered half MS. Rooted plants were arranged together with 10 days old *L. bicolor* mycelium pre-grown on cellophane-covered, sugar reduced Pachlewski medium (Deveau et al., 2007). The contact was realized on sugar reduced Pachlewski medium in square 12x12 plates supplemented with 1g/L MES

sodium salt and 1.2% (w/v) agar-agar, pH 5.8. When membranes harbouring the fungus were laid upside down on roots of the fungus, colonization was enabled and ECM formed (termed direct contact). In the case where the membrane was laid upside-up on the roots, colonization was prohibited and only signal exchange was allowed (termed indirect contact). The bottom part of the plate was covered with a black plastic bag to reduce illumination and plates were maintained vertically at 24°C and under a 16h photoperiod in a growth-chamber.

Microscopic observation of stained root sections

For longitudinal analysis of the root apex anatomy 1cm long, colonized root apices were used. For transversal sections the following 1cm was harvested on the same material. Roots were fixed in 4% (w/v) *para*-formaldehyde in phosphate buffered saline (PBS) (pH 7) overnight at 4°C. Roots were washed in PBS and embedded in 6% (w/v) agarose. Thirty µm transversal sections were prepared using a vibratome (LEICA, Germany). For longitudinal sections, fixed roots were dehydrated stepwise with Ethanol/H_2O solutions (30% (v/v), 50% (v/v), 70% (v/v) and twice 100%, each step lasting 30 min). Roots were kept in 100% ethanol and the next day subsequently transferred into Ethanol/Ottix X Plus solutions (Ethanol:Ottix X Plus 2:1, 1:1, 1:2 for each 30 min). Ottix X Plus was discarded so that material was only covered slightly in liquid and Diawax pellets were added and samplex indubated at 60°C. Diawax was changed four times during the following day before embedding the samples. Eight µm longitudinal sections were prepared using a rotating microtome (MICRON, France) and mounted on SuperFrost slides. Sections were dried over night at 40°C before deparafinizing the following day through successive washes twice 20min in Ottix F Plus, once 5 min in each EtOH:Ottix X Plus 1:2, 2:1 min and twice in 100% EtOH. Samples were then rehydrated in 10% steps, each lasting 2min from 100% EtOH to 0% EtOH/100% H_2O. Fluorescent double staining with propidium iodide for plant cell walls as well as plant and fungal nuclei and UVitex 2B for fungal cell walls of transversal and longitudinal sections, as well as microscopic observation was realized as previously described (Felten et al.).

Macroscopic root development analysis

For root elongation measurements pictures of Petri dishes were taken using a Canon camera and root measurements were realized on these pictures with ImageJ. For LR quantification, 10 to 12 individual poplar plants (2 to 3 per plate) in the respective conditions were observed once a week over five weeks using a Discovery V.8 stereo microscope (ZEISS, Germany). Samples were taken for sectioning at 1, 3 and 5 weeks.

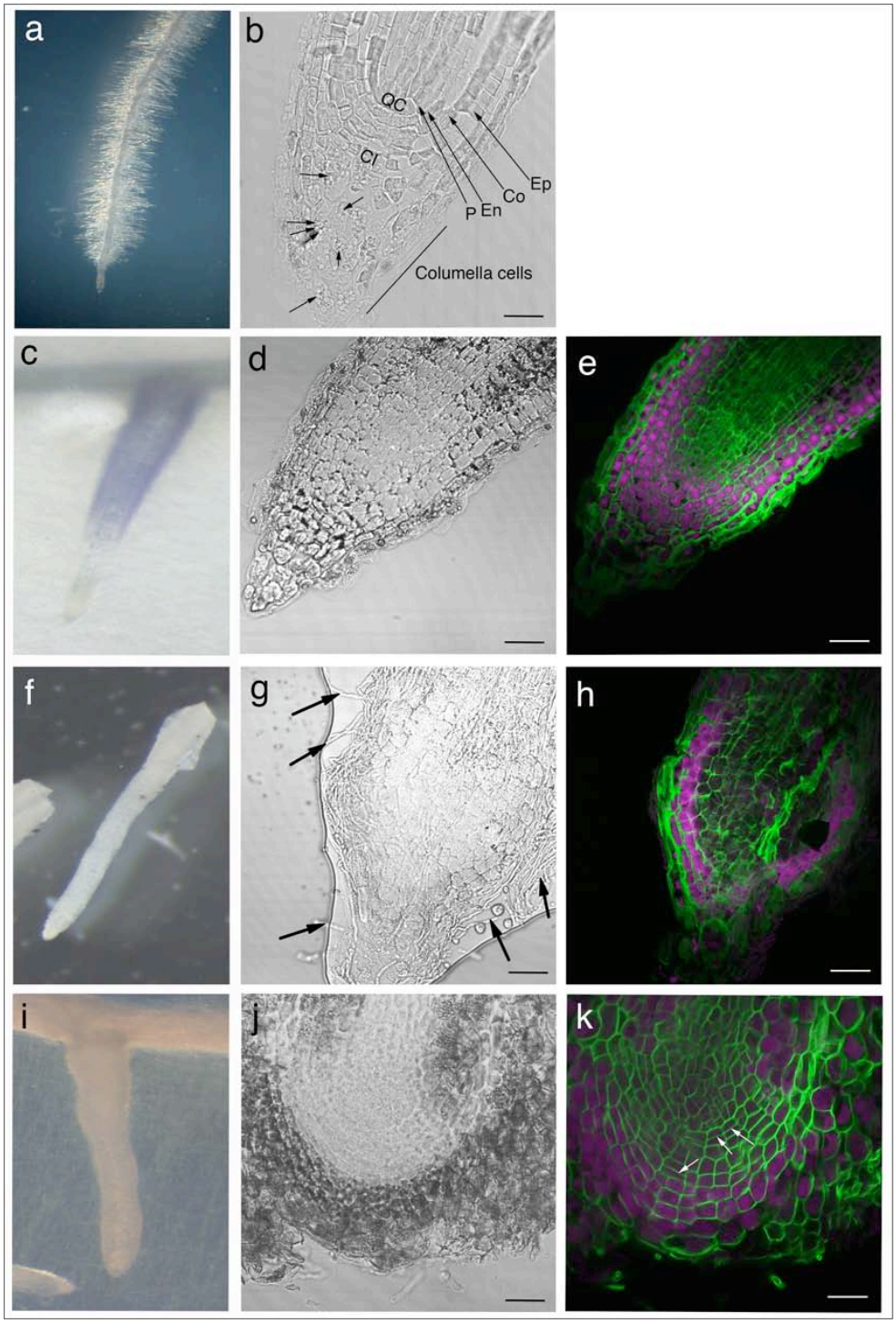

Fig. 1 Poplar LR colonization by *L. bicolor*. **a, c, f, i** observation using a stereo microscope. **b, d, g, j** differential interference contrast microscopic observation on 10µm longitudinal sections. **e, h, k** confocal laser scanning microscope observation after propidium-iodide (magenta) – Uvitex (green) double staining of longitudinal sections. **a, b** before contact ; Quiescent Center (QC), Columella Initials (CI), Epidermis (Ep), Cortex (Co), Endodermis (En), Pericycle (P), arrowheads indicate some of the numerous starch grains in mature columella cells. **c-e** 1 week of contact, **f-h** 3 weeks of contact (f after harvest and fixation), arrowheads indicate hyphae, **i-k** 5 weeks of contact. Root colonization started at the LR basis, hyphae reached the root tip at within weeks (arrowheads in g) and surrounded the tip at 5 weeks. The root cap started to disappear at three weeks (g, h) and no root cap was observed at 5 weeks (j,k) Arrowheads in h indicate division planes of cells. Scale bars = 20 µm

Results

Time course of in vitro Poplar/L. bicolor ECM formation

ECM formation was observed over a five weeks time period (Fig. 1) on lateral roots (LRs) that emerged during the interaction. Already within the first week of co-culturing the mycelium colonized the poplar roots thereby adopting a violet colour at contact sites with the root (Fig. 1c). Roots hairs that were easily visible on control roots (Fig. 1a) were no longer observed at 1 week of colonization. Interestingly colonization and violet mycelium colour occurred first at the basis of LRs, close to the junction with the parental root, thereby omitting the LR tip (Fig. 1c). At 3 weeks of colonization fungal hyphae had attained the root apex (arrowheads in Fig. 1g) and entirely surrounded it by five weeks (Fig. 1j). The violet colour of the mycelium was specifically observed within the first 10d of colonization and faded at later time points, thereby giving rise to a light brown aspect of colonized roots (Fig. 1i).

During root colonization typical cellular changes in root apex development are observed (Massicotte et al., 1987). In order to study cellular aspects during root colonization of poplar we have developed a fluorescent dual-staining reported in Felten et al. (in press). It is based on the use of propidium iodide and UVitex that colour plant and fungal cell walls as well as nuclei. Applying this staining on longitudinal lateral root sections followed by microscopic observation together with transmission light microscopic observation allowed investigating root apex morphology in detail (Fig. 1). In control conditions (Fig. 1b) the root apex presented the typical anatomy of eudicotyledonous plants: the parallel to the root laying provascular tissues in the central part of the root, which end in the Quiescent Center (QC), were surrounded by four concentric cell layers at the tip, referring to the meristematic cells for epidermis, cortex, endodermis and pericycle cell files at each side of the provascular tissues. The root cap protruded from the tip. It was formed by columella initials (root cap meristem cells) below the meristematic cells for the mentioned tissues. These cells give rise to columella cells that have a particular rectangular shape and that are arranged in a way, which gives the root tip its tapered form. Differentiated columella cells contain starch grains that were visible as small round points (arrowheads in fig 1b). At 1 week of colonization, where no hyphae were observed at the root tip, the root organization was not changed compared to controls and so the pointed form of the root tip was maintained (Fig. 1d, e). At three weeks of colonization the most exterior columella cells were no longer present, which shortened the acuminated tip (Fig 1g, h). At five weeks the difference to un-colonized roots was striking (Fig. 1b, j, k). The root tip had a complete round shape and no differentiated columella cells were visible anymore. The shape of the outer cells of the apex was regular and often squared resembling meristematic cells. Several cell division planes were visible (arrowheads in Fig. 1k). As more than the four concentric layers (that refer to the above mentioned cell files of the root) were visible, it is possible that the meristematic

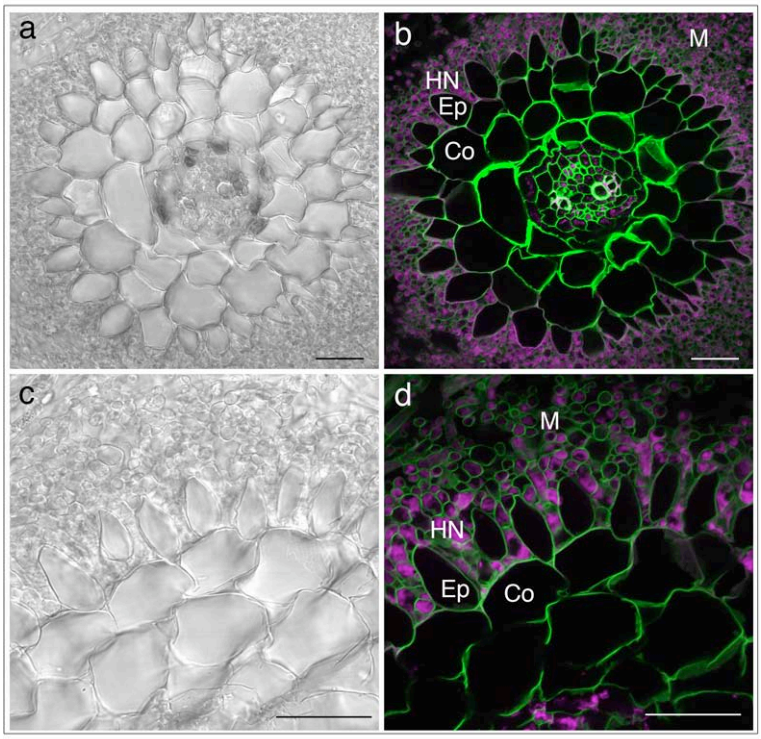

Fig. 2 Seven days old Poplar/*L. bicolor* ectomycorrhiza in the sandwich culture system. Transversal sections (30 µm) through the basis of LRs, where strong violet coloration of mycelium was observed. **a, c** differential interference contrast microscopic observation. **b, d** confocal laser scanning microscope observation after propidium-iodide (magenta) – Uvitex (green) double staining. c and d is a zoom into a and b. A Hartig Net limited to the epidermis cell layer was already visible at 7d of colonization. Mantle (M), Epidermis cells (Ep), Cortex cells (Co), Hartig Net (HN). Scale bars = 20 µm

columella initials were still present. We conclude from this that the differentiated columella cells had completely disappeared when the fungal mycelium had surrounded the root tip and that the most exterior cells of the root tip were cells of the root meristem, most probably also including columella initials.

We next prepared transverse sections of the basis of LRs, where colonization started in order to analyze the presence of mantle and Hartig Net, the characteristic structures of ECM. At 1 week of colonization transverse, double-stained sections showed that a mantle of fungal hyphae enveloped the root. As hyphae were represented as small circles, we conclude that they were orientated longitudinally to the growing root and that their observed shape results from a cross-section through them. From the mantle inwards hyphae penetrated between epidermis cells, thereby establishing a para-epidermal Hartig Net (Fig. 2). Where cells were not yet spaced by the penetration of mycelium, intercellular notches started to form at their surface (Fig. 2b right side of section) indicating beginning penetration of hyphae. Epidermal cells had changed their shape from a usually rectangular (see Figure 1 in Felten et al. (in press)) to a more oval contour (Fig. 2). Also at further time points (3 to 5 weeks, see also Figure 1 in Felten et al. (in press)) the Hartig Net was restricted to the epidermal cell layer.

Lateral root development and positioning

We next investigated two aspects of root development, being LR stimulation and LR elongation. The quantification of LR emergence once per week during colonization showed a strong LR stimulation during the first two weeks with about 19 new LRs emerged per week in direct contacts and only 15 LRs in the first week and 6 in the second week in control plants (Fig. 3a). When comparing LR stimulation in an indirect contact, where a cellophane membrane was present between roots and mycelium that prohibits root colonization but allows signal molecule exchange, we found an equivalent profile than during root colonization in direct contacts. For direct and indirect contacts LR stimulation declined at three weeks to 8 LRs per week and was no longer different from the LR emergence rate in control plants (6 LRs/week). At five weeks of contact plants in direct and indirect contact with the fungus had developed an average of 55 and 62 LRs respectively, whereas control plants only presented 35 LRs (Fig. 3b). We further analyzed the spatial distribution of the eight youngest LRs on each adventitious root (AR) after the initial LR stimulation (14d). In control plants, no LR was detected in the first cm from the apex, suggesting that LR root emergence starts beyond this point. Strikingly compared to control plants, during indirect (data non shown) and direct contact (Fig. 3c) LRs emerged more closely to the root apex, from 0.5 cm. We did not detect a significant altered elongation growth of the parental adventitious root at this time point (Fig. 3d), suggesting that the altered LR distribution observed is not an indirect effect of parental root growth.

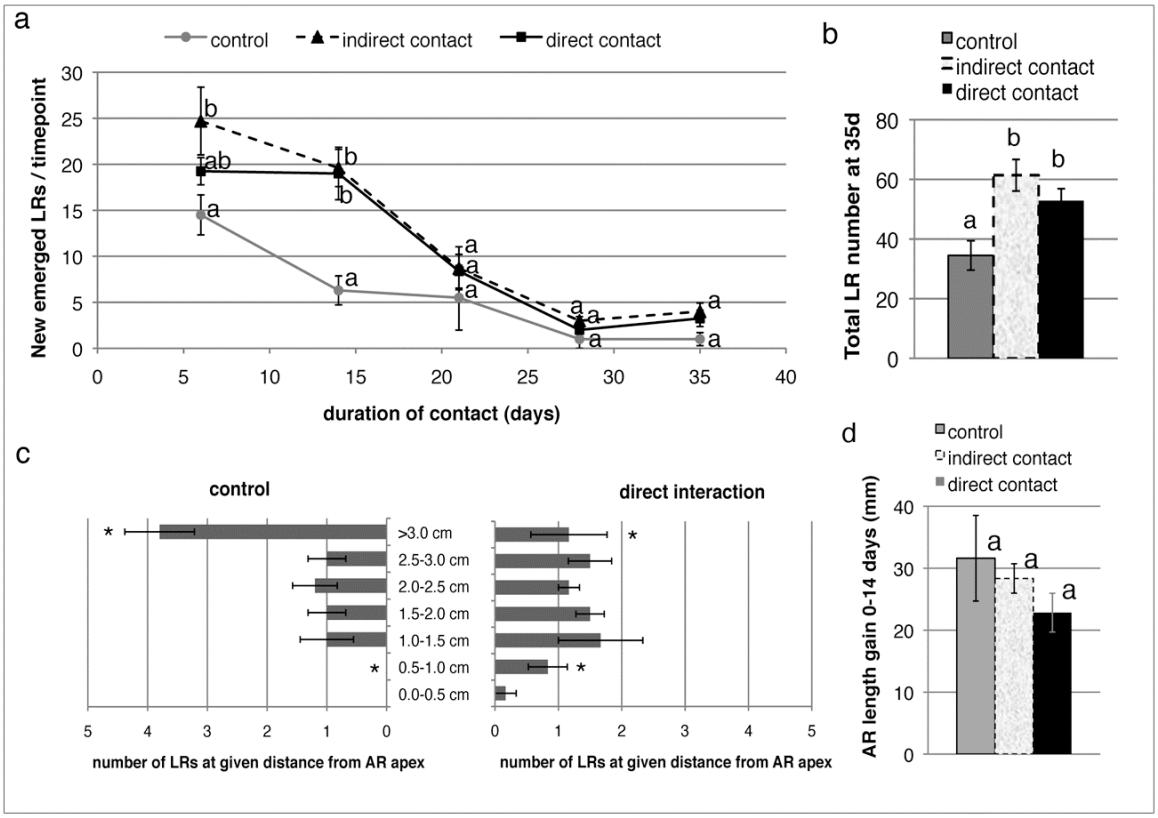

Fig. 3 Lateral root development in poplar during interaction with *L. bicolor*. **a**, **b** Number of new LRs that developed per week during direct and indirect interaction of poplar with *L. bicolor*. A strong LR increase was observed during the first two weeks of direct and indirect interaction compared to control plants. At later time points (> 3 weeks) LR stimulation declined and the rate of LR development in control plants and plants in contact was similar. At 35d LR stimulation during contact led to twice the number of LRs compared to control plants (**b**). **c** Spatial distribution of the eight closest LRs from the root apex upwards on 4 to 7 ARs per condition. The root apex was dessignated as 0cm. At 14d of interaction a shift in LR distribution was observed, with LRs developing closer to the root apex than in control plants. The distribution in direct and indirect interaction was similar (only direct contact is represented). d Elongation of the adventitious roots (AR) on which LR distribution was measured in (c) between co-culture day 0 to day 14. No statistical difference in root elongation was observed (number of ARs measured : 3 (controls), 12 (direct contact), 18 (indirect contact). Different letters (in a,b) or asterisks (in c) indicate statistical difference (Student T Test, $p<0.05$) between respective conditions.

Growth arrest of lateral roots during colonization

We assessed on five root systems per conditions LR elongation of four chosen LRs, which had emerged between the starting day and day seven of co-culturing over the following three weeks, until complete colonization of lateral roots in direct contact was achieved (Fig. 1i). Our data demonstrated that over time there was an intrinsic decrease in LR elongation in control plants (Fig. 4). Between weeks one and two they elongated by about 8 mm, between weeks two and three by 5mm and between weeks three and four by 1mm, thereby being significantly different at every taken time point. LR elongation in indirect contacts was not significantly affected by the presence of the fungus compared to controls. Anyhow in the direct contact there was a significant decrease in LR elongation between weeks two and three compared to controls (2mm compared to 5mm per week). This time point referred to root tip colonization by the fungus as well as the first changes in root cap development (Fig. 1g).

Discussion

Development of *in vitro* ECM

In the present study we have investigated ECM formation between *P. tremula x P. alba* and *L. bicolor* under controlled conditions in an *in vitro* sandwich culture system. Our results showed that mycorrhiza formation under these conditions occurred rapidly, leading to the presence of a Hartig Network already within one week. Compared to *P. trichocarpa/L. bicolor* ECM formation in pot culture systems in the greenhouse that require co-culturing for two to three month (Martin et al., 2008), *P. tremula x P. alba/L. bicolor* ECM formation in the *in vitro* culture system presents a major advantage by being less time-consuming. The faster colonization is likely to be due of the increased interaction surface and the instantaneous contact of root and fungus from the starting day of co-culturing on. In pot cultures the mycelium has to grow towards the roots before colonization can take place. *Poplar*/*L. bicolor* ECM obtained in pot-cultures in the greenhouse showed a different anatomy (Martin et al., 2008) than the *in vitro* mycorrhizae described here, with the major difference consisting in the Hartig Net that was found only para-epidermal under *in vitro* conditions and that proceeded until the first or second cortex cell layer in green-house mycorrhiza. This may be due to the fact that the greenhouse mycorrhizae analyzed were older (three month of colonization). Co-culturing in the in vitro system is limited to about 6 weeks, when shoot and root of poplar plants start to touch top and bottom of the plate and further undisturbed growth is no longer possible. Thus, we were not able to analyze whether mycorrhizae of the same age in greenhouse and *in vitro* cultures would show the same cellular anatomy. Nevertheless, the morphology of ECM obtained in our study is comparable to other *in vitro* ECM obtained on leafy tree species such as eucalypt, birch or poplar

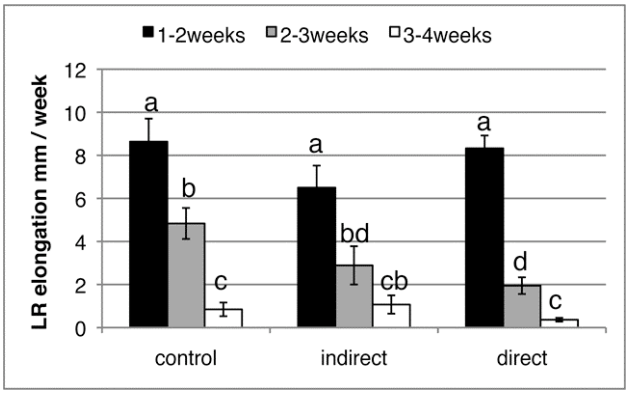

Fig. 4 Lateral root elongation during poplar/*L. bicolor* interaction. Root elongation of LRs that emerged during the first week of poplar/*L. bicolor* interaction was followed over time for 20 LRs in each condition. In all conditions LR elongation decreased significantly over time (different letters indicate statistical difference (Student T-Test, $p < 0.05$)). Compared to control plants LR elongation in direct contacts was significantly lower after 3 weeks of colonization.

with different fungi (Chilvers et al., 1986; Horan et al., 1988; Brun et al., 1995; Duplessis et al., 2005; Langer et al., 2008). Also in these studies the Hartig Net did not exceed the epidermal cell layer, possibly also suggesting a particularity of *in vitro* ECM. Major differences in axenic and non-axenic cultures lay in the establishment of the first fungus-root contact, inocculum quantity, the presence or absence of (contaminating) other microorganisms, water potential and root aeration and are likely to cause a specific ECM anatomy, characteristic for those conditions.

Root development during root colonization

In our study we also investigated modification of root development during ECM formation. Our results show how root development and root colonization parallel. In an early phase of interaction, LR development was stimulated. This was the case in direct as well as in indirect contacts, suggesting it to be caused rather by signalling molecules released by the fungus than by the physical contact of plant and hyphae during colonization. Different molecules that are released by ECM fungi and that may impact LR development, for instance auxin and ethylene, have been reported and may be involved in this phase (Graham and Linderman, 1980; Ho, 1987; Splivallo et al., 2009). The fact that LR stimulation is limited to the early phase of interaction (14d) implies that either the fungus specifically and only releases the LR-stimulating molecules during this phase or that the plant becomes insensitive to them with ongoing interaction. Interestingly, during colonization the fungus adopted during the early LR-stimulating phase a violet colour (of yet unknown origin) that faded at the later interaction. This strengthens that different phases during the interaction of plant and fungus can be distinguished.

Strikingly, during indirect and direct contacts a shift of LRs towards the root tip was furthermore observed. This shift was not due to an altered elongation growth of the parental adventitious roots that usually are not subjected to colonization and mycorrhiza formation. This result suggests that the fungus may interfere directly with the LR developmental program, which is based on auxin fluxes and which precisely defines the frequency and spacing of LRs (De Smet et al., 2007; Lucas et al., 2008).

Our observations have shown that root colonization was first visible at the basis of LRs and only reached the root tip at later phases. This is an interesting result and stays in contrast to what was reported by Horan and co-workers (1988) on ECM formation between *Eucalyptus* and *Paxillus involutus* or *Pisolithus microcarpus*. These authors had observed root swelling and mantle formation at the root tip during onset of root colonization in an *in vitro* sandwich culture system. Differences in the root growth rate between *Eucalyptus* and *Populus* may explain their specific colonization pattern. Our results demonstrated an increased elongation rate of young LRs that slowed down with

increasing LR age. Elongation rate slow-down is likely to be an endogenous pattern as it was also visible in control plants. Root tip colonization coincided with the root elongation decrease, suggesting that the fast growth in the beginning may not render root colonization possible, probably through a higher difficulty in adhesion of fungal hyphae on root cells. Interestingly, root elongation was slower during colonization (at 3 weeks direct contact) compared to controls but not during signal exchange only conditions (indirect contact). This result implies that root tip colonization itself provokes LR arrest and not the communication via signalling molecules. The root growth arrest was also accompanied by a change in the root cap, which started to disappear up from three weeks of contact, when hyphae began to colonize the root tip. The fact that hyphae at the root tip where necessary to provoke the decay of columella cells, suggests that this is again a result of the colonization. However, the columella stability needs to be analyzed also during ongoing indirect contacts to insure that this process is due to colonization and not only to a signal exchange. The loss of the columella may directly influence root development and be partially responsible for the decreased root growth and a fading LR stimulation after two to three weeks. The columella has been shown to be an important actor in phytohormone perception (Hahn et al., 2008). Furthermore also auxin fluxes through polar auxin transport pass through the columella (Blilou et al., 2005) and may be disturbed when these cells are absent, thereby altering root development.

Taken together our results propose that LR stimulation during the first two weeks of poplar root colonization by *L. bicolor* was the outcome of a signal exchange between both partners that acted on and altered the root-internal developmental program. Colonization started at the basis of new LRs and advanced towards the root tip from week two to four of colonization. Root elongation slow-down permitted root tip colonization and coincided with columella disappearance. The different developmental changes in the root provoked by the contact with the fungus can thus be separated in two groups: LR stimulation was an early response and colonization independent, whereas LR growth arrest and root cap decay occurred as a late response and due to colonization (to be confirmed for root cap decay). It will now be of interest to study the molecular changes in plant internal pathways that mediate root growth, LR development and LR positioning in order to understand the mechanisms that alter root development in the presence of the fungus.

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Chapter II

The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signalling

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Fungus-induced lateral root formation through auxin pathways

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The ectomycorrhizal fungus *Laccaria bicolor* stimulates lateral root formation in poplar and Arabidopsis through auxin transport and signaling

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ABSTRACT

The early phase of the interaction between tree roots and ectomycorrhizal (ECM) fungi, prior to symbiosis establishment, is accompanied by a stimulation of lateral root (LR) development. We aimed to identify gene networks that regulate LR development during the early signal exchanges between *Populus tremula x Populus alba* and the ECM fungus *Laccaria bicolor* with a focus on auxin transport and signaling pathways. Our data demonstrated that increased LR development in poplar and *Arabidopsis thaliana* interacting with *L. bicolor* is not dependent on the ability of the plant to form ectomycorrhizae. LR stimulation paralleled an increase in auxin accumulation at root apices. Blocking plant polar auxin transport with 1-naphthylphthalamic acid (NPA) inhibited LR development and auxin accumulation. An oligoarray-based transcript profile of poplar roots exposed to molecules released by *L. bicolor* revealed the differential expression of 2945 genes, including several components of polar auxin transport (*PtaPIN* and *PtaAUX* genes), auxin conjugation (*PtaGH3*) and auxin signaling (*PtaIAA*). Transcripts of *PtaPIN9*, the homolog of Arabidopsis *AtPIN2*, and several *PtaIAA*s accumulated specifically during the early interaction phase. Expression of these rapidly induced genes was repressed by NPA. Accordingly, LR stimulation upon contact with *L. bicolor* in Arabidopsis transgenic plants defective in homologs of these genes was decreased or absent. Furthermore, in Arabidopsis *pin2* the root apical auxin increase during contact with the fungus was modified. We propose a model in which fungus-induced auxin accumulation at the root apex stimulates LR formation through a mechanism involving PtaPIN9-dependent auxin redistribution together with PtaIAA-based auxin-signaling.

INTRODUCTION

Most temperate forest trees develop a mutualistic root symbiosis with ectomycorrhizal (ECM) soil fungi. During the establishment of ECM, fungal hyphae invade the root from root cap cells in- and upwards to the epidermis (Horan et al. 1988). After attachment to epidermal cells, hyphae multiply to form a series of layers that differentiate to establish a mantle structure around the root (Horan et al., 1988). An internal network of hyphae between the epidermis and root cortex cells forms the Hartig net (Blasius et al., 1986), while extraradical hyphae prospect throughout the surrounding soil and gather nutrients. Morphological observations of forming ECMs have shown that in the vicinity of the fungus the root architecture of the host plant is profoundly modified. Interaction with hyphae stimulates lateral root (LR) formation, dichotomy of the root apical meristem in conifer species, and cytodifferentiation of root cells (radial elongation, root hair decay) (Horan et al., 1988; Dexheimer and Pargney, 1991; Ditengou et al., 2000). Even though several studies have focused on LR

stimulation during ECM formation, it still remains unclear what the molecular mechanisms are that modify root development during contact with the fungus.

In the herbaceous model plant *Arabidopsis thaliana*, LR development is welldescribed (Malamy and Benfey, 1997), and the analysis of its molecular regulation is ongoing. LRs in *Arabidopsis* are derived from a subset of pericycle cells, termed pericycle founder cells, which are adjacent to the two xylem poles (reviewed in (Casimiro et al., 2003; De Smet et al., 2006)). LR initiation (LRI) is thought to go through two independent checkpoints (De Smet et al., 2007; Dubrovsky et al., 2008). First, priming of pericycle founder cells occurs in the basal meristem, which is a region at the transition between the meristem and the elongation zone of the root. Second, LRI itself- comprising cell cycle re-activation of the founder cells and subsequent further cell divisions- occurs in more proximal regions of the root and lead to the formation of a LR primordium (Malamy and Benfey, 1997). Finally, the new LR emerges after having grown through the cortex and epidermis of the parental root.

The phytohormone auxin (indole-3-acetic acid (IAA)) is considered to be one of the main triggers regulating all of the different steps of LR formation (Bainbridge et al., 2008; Ditengou et al., 2008; Laskowski et al., 2008; Nibau et al., 2008). For instance, the acquisition of founder cell identity, cell-cycle re-activation and LR emergence all correlate with and require local auxin accumulation in specific cell types (Dubrovsky et al., 2008; Fukaki and Tasaka, 2009). Recent studies have suggested that the coordination of polar auxin transport by auxin influx carriers (AUX/LAX) and auxin efflux carriers (PIN) is responsible for establishing an auxin gradient along the root with specific local maxima that regulate LR development (Bainbridge et al., 2008; Ditengou et al., 2008; Laskowski et al., 2008). In combination with polar auxin transport, local auxin biosynthesis in specific root cells was proposed to contribute to the formation of the auxin gradient (Petersson et al., 2009). Lastly, one should not neglect that the localized expression of auxin homeostasis regulating genes like IAA-amido-synthetases GH3 may significantly interfere with the apparition of auxin maxima (Brady et al., 2007) and thereby influence LR development (Nakazawa et al., 2001; Khan and Stone, 2007). GH3 IAA-amido-synthetase conjugates auxin to amino acids or sugars (Staswick et al., 2005), and thereby controls through catabolism and storage free (active) auxin levels in the cell (Hagen and Guilfoyle, 1985; Ljung et al., 2002; Staswick et al., 2005). During LRI, once an auxin maximum is formed in pericycle founder cells, auxin triggers its signaling cascade by binding to its receptors (Badescu and Napier, 2006). This activates auxin-dependent transcription factors (AUX/IAA and ARF) that act on cell-cycle regulating targets (Fukaki et al., 2005; Dreher et al., 2006) to elicit cell-cycle re-activation. The mechanism that confers founder cell identity to pericycle cells in the basal meristem is distinct from LRI. Recent results reveal its independence from the basic auxin-signaling pathway. A mechanism where the asymmetric distribution of auxin itself acts as a morphogenic trigger to prime pericycle cells was proposed (Dubrovsky et al., 2008). Together this data points out

the crucial role of auxin gradients in root development.

Recently, evidence has been reported for an ECM-fungus induced alteration of the endogenous auxin balance in the root apex of Arabidopis plants (Splivallo et al., 2009). Even though identification of key molecular factors that could explain this alteration is still lacking, earlier results on tree roots suggest an impact of the fungus on root polar auxin transport and auxin-conjugation, which could be involved in auxin-level changes. For instance, inhibitors of polar auxin transport, such as 2,3,5-triiodobenzoic acid (TIBA), restrict the stimulation of LR formation in conifer seedlings by ECM fungi (Karabaghli et al., 1998). Moreover, differential expression of a *GH3* auxin-amido-synthetase during ECM formation has been reported in *Pinus pinaster* (Charvet-Candela et al., 2002; Reddy et al., 2006) and implied that the presence of the fungus also alters auxin-homeostasis in the root via a mechanism involving enzymatic activities. Fungus-impacted polar auxin transport and auxin conjugation may thus be possible pathways responding to or causing an altered root auxin balance. How they are integrated in the perception of fungal signals and trigger then LR formation on a molecular basis during the interaction is still an enigma.

A recent study with ECM truffle fungi has suggested that fungal IAA and ethylene impact LR branching in Arabidopsis and *Cistus incanus* during the early phase of interaction (Splivallo et al., 2009). Interestingly, only a combined exogenous IAA/ethylene treatment, but not each phytohormone alone, was able to fully mimic the effect of fungal signals on root development. A multiplicity of other identified signals (auxins, alkaloids, cytokinins, flavonols, polyamines) have been found to act in either a synergistic (rutine/zeatine) or antagonistic (indole-3-acetic acid (IAA)/hypaphorine) manner during ECM formation, but to date evidence for their molecular impact on root developmental programs is lacking (Ditengou and Lapeyrie, 2000; Martin et al., 2001; Jambois et al., 2005; Martin and Nehls, 2009).

All these data show that significant knowledge on two levels is still lacking. Firstly, which fungal signaling molecules precisely influence plant root development and how they crosstalk has never been clearly demonstrated. Secondly, how fungus induced auxin level alterations are connected to root development modification is another question that needs to be addressed to complete the molecular understanding of symbiotic fungus/root interactions.

The aim of the present study was to identify gene networks inside the root, with special focus on auxin transport, conjugation and signaling, that bridge the gap between fungus-induced root endogenous auxin alteration and lateral root formation in the early phase of the *Laccaria/Populus* interaction. The availability of the genomic sequence of *P. trichocarpa* (Tuskan et al., 2006) and *L. bicolor* (Martin et al., 2008) has facilitated the molecular analysis of this interaction. Moreover, in recent years several components of the auxin signaling pathways in poplar have been identified

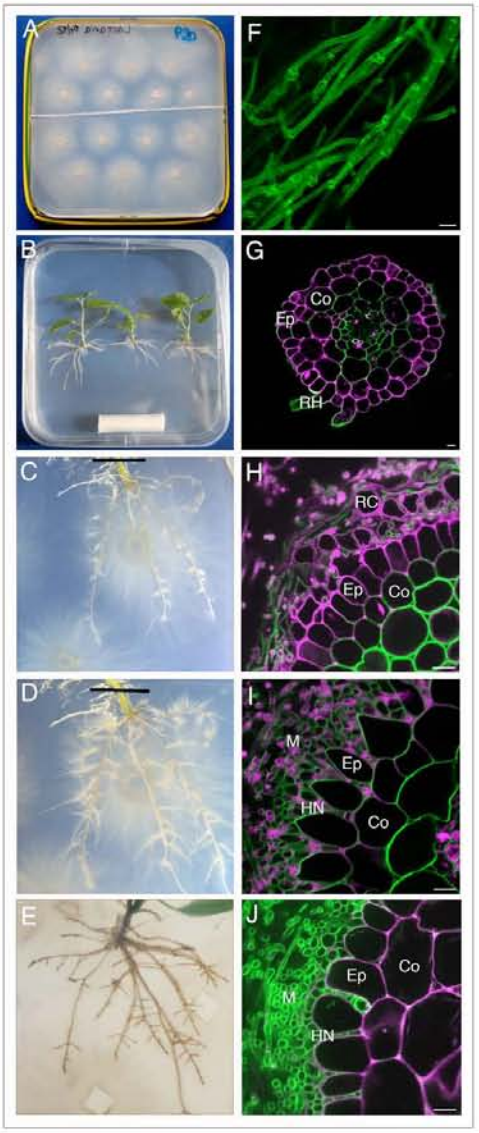

Figure 1. Time course of colonization of *P. tremula x P. alba* roots by *Laccaria bicolor* in an *in vitro* sandwich culture system. A and B, *L. bicolor* and poplar precultures, respectively. C to E, root development of poplar at 3 days of direct interaction (DODI) (C), 10 DODI (D) and 30 DODI (E). Note the increasing LR number starting at 10 DODI. Root swelling at the LR basis started at 10 DODI and LR arrest was observed at 30 DODI. F, *L. bicolor* hyphae from precultures after Uvitex staining. G, transverse root section after propidium iodide (PI) Uvitex dual staining (green : Uvitex, magenta : PI). H to J, dual fluorescent-stained transverse root sections at 3 DODI (H), 10 DODI (I) and 30 DODI (J). Root hair (RH), Root Cap cells (RC), Mantle (M), Hartig Net (HN), epidermis (Ep), cortex (Co). Note hyphae attachment at 3 DODI, mantle and Hartig Net development from 10 to 30 DODI. H, Single, magenta colored cells surrounding the epidermis are detached root cap cells (RC). Images shown are representative from a series of three experiments. Bars = 10 µm.

(Moyle et al., 2002; Schrader et al., 2003; Kalluri et al., 2007; Teichmann et al., 2008).

We characterized the symbiotic interaction between *P. tremula x P. alba* (hereafter: poplar) and *L. bicolor* at both the physiological and molecular levels, and also explored the non-mycorrhizal *A. thaliana/L. bicolor* interaction. We showed that in both species' interacting with *L. bicolor*, LR development was due to diffusible, non-host specific signaling molecules. In this context, LR induction required polar auxin transport through PtaPIN9 (highly homologous to AtPIN2*)* as well as auxin signaling through poplar Aux/IAA proteins. Based on these results, we propose a model involving these genes as important regulators during fungus-induced LR development.

RESULTS

Poplar/*L. bicolor* ectomycorrhiza development *in vitro*

An *in vitro* sandwich culture system for the generation of *P. tremula x P. alba /L. bicolor* ECMs under controlled conditions was adapted from previously described methods (Chilvers et al., 1986; Horan et al., 1988) (Fig. 1A and B). The time course of ECM development was assessed by observing root development (Fig. 1C-E) and dual-fluorescent stained transverse root sections (Fig. 1H-J). Two fluorescent markers were used, UVitex 2B for detection of fungal cell walls (green in Fig. 1) and propidium iodide for visualization of plant cell walls (magenta in Fig. 1). At three days of direct interaction (DODI), no change in root system morphology was visible (Fig. 1C). A discontinuous mycelium was attached to the root surface and the rectangular shaped epidermis cells were still tightly connected to one another (Fig. 1H). After 10 DODI, numerous LRs had developed and had a swollen appearance at their basis (Fig. 1D). At this stage, the confocal microscopic observation showed the presence of a dense mycelium sheath (mantle) surrounding the roots and the start of Hartig Net development: epidermis cells had an oval shape, were spaced and hyphae penetrated in between them (Fig. 1I). After 30 DODI, we observed numerous short LRs, swollen from their basis up to the tip (Fig. 1E). Again a Hartig Net restricted to the epidermal cell layer was visible that suggests functional symbiosis (Fig. 1J).

LR development in the presence of *L. bicolor*

Analysis of the time course of LR induction in poplar interacting with *L. bicolor* under *in vitro* conditions showed that four days of direct interaction between the symbiotic partners were sufficient to increase the LR number by three-fold compared to control plants without fungus (Fig. 2A). At 10 DODI, control plants had formed approximately five new LRs, whereas plants interacting with *L. bicolor* developed about 25 LRs. In the *in vitro* sandwich culture system we also demonstrated an indirect interaction between the partners. This was achieved by placing a cellophane membrane

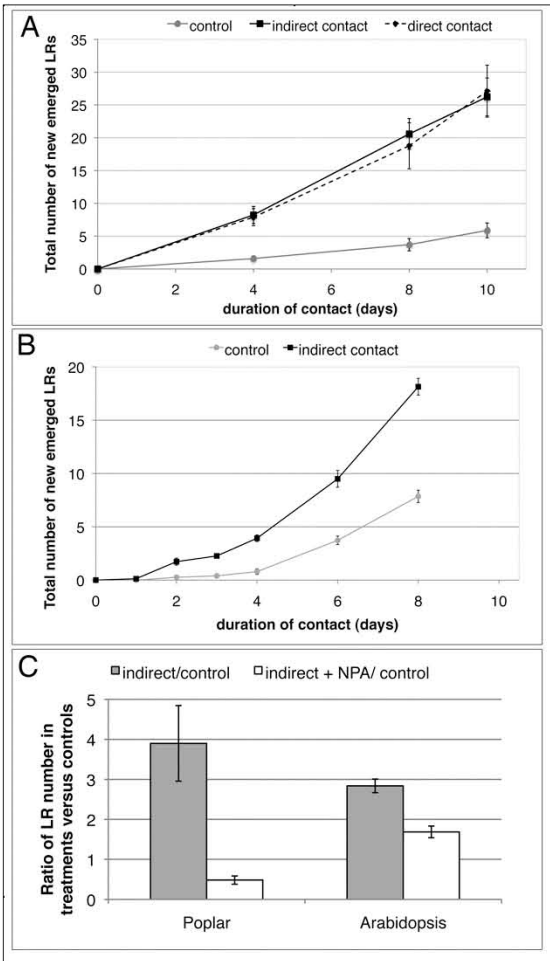

Figure 2. Time course of early lateral root (LR) development during the interaction of *L. bicolor* with mycorrhizal poplar and non-mycorrhizal *Arabidopsis thaliana*. A, Time course of LR development in *P. tremula x P. alba* in response to *L. bicolor* and B, in *A. thaliana*. Beginning at four (A) and two (B) days of respective poplar and Arabidopsis/*L. bicolor* interaction a significant (Student T-Test $p < 0.05$) LR stimulation was observed. LR stimulation was similar in indirect and direct contact in poplar. C, Effect of the polar auxin transport inhibitor NPA on LR increase in poplar (10µM NPA) after 8 DOII and Arabidopsis (5µM NPA) after 6 DOII. LR ratio of plants in indirect contact versus control plants without fungus in the presence and absence of NPA is shown. LR stimulation is significantly (Student T-Test $p < 0.01$) reduced by the NPA treatment. In each experiment and per each condition 10 to 15 individual poplar plants or 15 Arabidopsis seedlings were observed (A, B, C). Error bars indicate standard errors. If not visible they were smaller than the symbol at the data

between the two partners, which prohibits root colonization by hyphae but allows the exchange of signaling molecules between plant and fungus. Comparison of the degree of LR development in roots directly and indirectly interacting with *L. bicolor* showed that under both conditions, the number of LRs established over the induction time course was identical (Fig. 2A).

Next, we asked whether *L. bicolor* signals were able to induce LR formation in the non-mycorrhizal plant *A. thaliana*. Similarly to poplar, LR development was also induced in *Arabidopsis* during indirect contact (Fig. 2B). The time course of LR emergence was similar to that observed in poplar, with the first significant increase in LR development at 2 DOII. After 8 DOII with *L. bicolor*, *A. thaliana* seedlings had formed about 18 new LRs, whereas control plants had only formed only eight.

Taken together, the results from the colonization and LR development experiments demonstrated that LR stimulation is characteristic for the early phase of plant-fungus interaction and is thus temporally distinguished from mature ECM formation, typified by Hartig Net formation, root swelling and root growth arrest.

Inhibition of polar auxin transport during plant /*L. bicolor* interactions

The impact of polar auxin transport on fungus-induced LR stimulation was investigated. Therefore we quantified LRs formed when *L. bicolor* interacted indirectly with poplar or *A. thaliana* in the presence of the polar auxin transport inhibitor 1-naphthylphthalamic acid (NPA) at time points where we had observed a strong difference between control conditions and indirect contacts (8 and 6 DOII respectively). The NPA treatment dramatically reduced fungal LR induction in both non-mycorrhizal and mycorrhizal plant species (Fig. 2C). In poplar 10µM NPA completely inhibited LR induction by the fungus. In Arabidopsis the presence of 5µM NPA reduced LR induction by 40% compared to controls. This severe reduction of LR induction is a first argument for the involvement of polar auxin transport in LR development during the early phase of the *L. bicolor*/plant interaction.

Modification of root auxin gradients during interaction

Auxin response in roots during contact with the fungus was assessed using the synthetic *DR5* auxin inducible promotor (Ulmasov et al., 1995) fused either to a *GUS* (*A. thaliana*) or *GFP* reporter gene (*P. tremula x P. tremuloides)*. While *DR5* reports on auxin levels in *A. thaliana* (Petersson et al., 2009), it is primarily used as an auxin response promotor in poplar. GFP fluorescence intensity was observed on entire lateral root apices of *DR5:GFP* plants using confocal laser scanning microscopy. For each root the signal intensity from each 18 optical sections in a Z-stack (distance between slices was 1 µm) was measured to generate a profile of the auxin accumulation over the root apex (Fig. 3A). The result of this profiling showed that the presence of the fungus increased the total

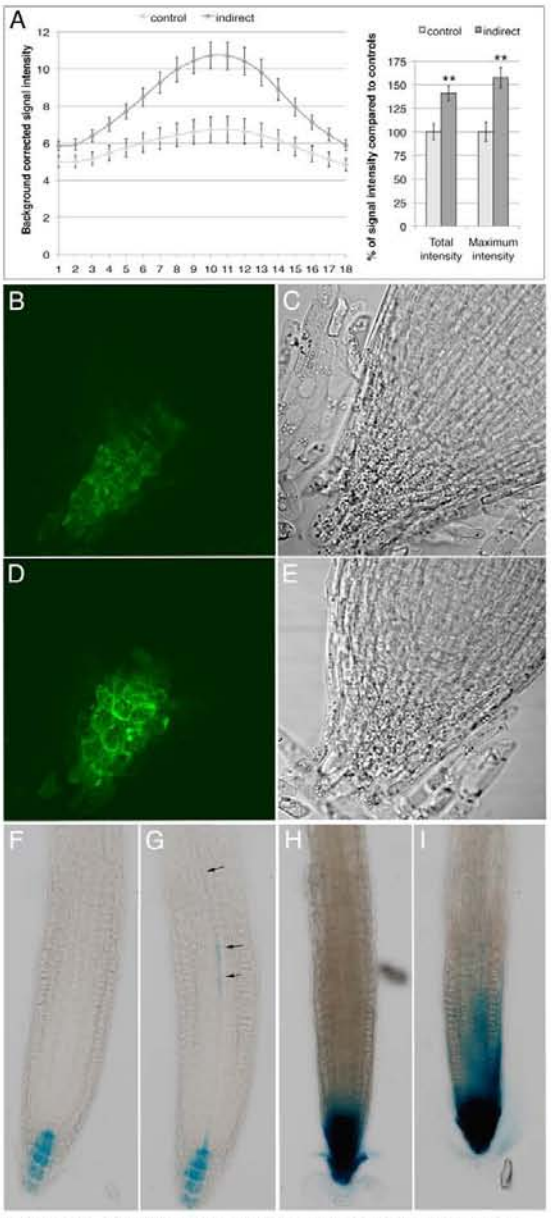

Figure 3. Modification of the auxin gradient in *P. tremula x P. tremuloïdes* and *A. thaliana* upon interaction with *L. bicolor*. A, Left: Distribution of average, background corrected GFP fluorescence intensity in the 18 optical sections of the Z-Stack through the lateral root apices of *P. tremula x P. tremuloïdes DR5:GFP* plants in control conditions or at 3 DOII. Right: Quantification of the signal intensity change as percentage of fluorescence in control root apices. Total intensity (sum of 18 sections) as well as maximum intensity (1 section, around Z-stack section 9 to 11) increased significantly. Quantification from at least 7 root apices per condition (Student T-Test, $^{**}p<0.05$). B to E, *P. tremula x P. tremuloïdes DR5:GFP* LR apex under control conditions (B, C) and at 3 DOII (D, E). F and G, *A. thaliana DR5:GUS* in control conditions (F) and at 3 DOII (G). Note the signal in provasculature at 3 DOII (arrowhead in G). Examples out of >30 biological replicates. H and I, *A. thaliana pin2 DR5:GUS* in control conditions (H) and at 3 DOII (I). A strong auxin accumulation at the root apex in control plants is observed the presence of the fungus enlarged this signal upwards. Examples out of 15 biological replicates.

fluorescence in the root apex by 42% compared to control roots. The overall profile in roots was altered as well by the fungus: a strong auxin maximum was observed in the central part of the root at 3 DOII (57% increase compared to controls) and the profile showed a higher amplitude at the apex center than in the rather equally distributed signal in control roots. No difference was observed concerning the localization of the GFP signal (Fig. 3B and D). In both conditions the Quiescent Center zone was rather weakly stained and a stronger signal was observed in columella initials. As for *A. thaliana DR5:GUS*, the presence of the fungus stimulated the appearance of a weak GUS signal in the provasculature within the basal meristem in 28 out of 32 analyzed plants (arrowheads Fig. 3G). This signal was absent in 32 out of 35 control plants (Fig. 3F). In poplar, no provascular fluorescence was observed, probably due to high tissue thickness in this region. Interestingly, the provascular signal in Arabidopsis was absent at 3 DOII when the contact was made in the presence of 1 or 10 µM NPA (Supplemental Fig. S1).

Thus *L. bicolor* stimulated LR induction is paralleled by an NPA-sensitive auxin response-increase at the root apex and in provascular tissues in Arabidopsis.

Transcript profiling of poplar roots during early interaction with *L. bicolor*

To identify which molecular processes are induced in poplar roots by signals released by the symbiont, an oligoarray-based transcript profile of poplar roots at 3 DOII was generated. This corresponds to the time point where LR induction has begun (Fig. 1 and 2). Indirect contact was chosen because it limits possible root colonization-related transcriptome changes.

Of the 39,303 genes that were represented by specific oligos on the poplar array, 26,125 (66%) were expressed in control roots. In roots interacting with *L. bicolor*, 7% (2,945) of all genes were differentially expressed (> two-fold, Student's T-Test <0.05). Among these genes, 71% (2,095) were upregulated and 29% (850) were downregulated. Of the upregulated genes, 17% (351) were induced *de novo*, as their expression could only be detected upon contact. Fifteen percent (132) of the downregulated genes were completely repressed, as their transcripts were no longer detected in roots (in Supplemental Table S1). Among the genes showing homology to known genes in databases, most were involved in cell wall-related functions, such as laccases, carbohydrate-acting hydrolases, cell wall-related kinases and phenylpropanoid metabolism enzymes (Supplemental Table S1). The second most represented gene class was transcription factors, and these included *SCARECROW (SCR)* and *PLETHORA (PLT)*. Together, these genes are known to control root apical meristem cell identity (Sabatini et al., 2003; Aida et al., 2004). PLT furthermore acts on *PIN* gene expression at the root apex (Blilou et al., 2005; Galinha et al., 2007).

In order to identify within the differentially expressed genes those which may be connected to LR initiation, we compared our results to LRI genes analyzed in Arabidopsis (Vanneste et al., 2005).

Table I. Microarray-based results of differentially expressed auxin-related genes in *P. tremula x P. alba* roots after three days of indirect interaction with the ECM fungus *L. bicolor*.

Genes are listed by gene family and within the gene family by increasing transcript accumulation. From top to bottom: auxin-responsive transcription factors (*PtaIAA*), indole-3-aceticacid-amido-synthetases (*PtaGH3*), auxin efflux carriers (*PtaPIN*), auxin influx carriers (*PtaAUX*).

Gene Name[a]	**Gene Model**[b]	**Expression in control roots**[c]	**Fold change**[d]	**p value**[e]
PtaIAA28.1	gw1.XVIII.808.1	4470	-1.9	0.0027
PtaIAA33.2	gw1.121.83.1	500	2.3	0.0001
PtaIAA3.4	estExt_fgenesh4_pm.C_LG_V0528	756	2.4	0.0001
PtaIAA3.3	fgenesh4_pm.C_LG_II000215	58	2.6	0.0026
PtaIAA19.3	estExt_fgenesh4_pm.C_LG_III0099	572	3.2	0.0004
PtaIAA34	gw1.X.53.1	nd	3.4	0.0023
PtaGH3-8	gw1.III.363.1	nd	2.8	0.0051
PtaGH3-7	fgenesh4_pg.C_LG_I000598	106	2.9	0.0151
PtaGH3-2	estExt_fgenesh4_pg.C_LG_IX0695	414	6.2	0.0000
PtaGH3-1	eugene3.02050011	2288	8.3	0.0000
PtaAUX3	estExt_fgenesh4_pg.C_LG_X1704	1029	-2.0	0.0003
PtaAUX6	grail3.0001031001	1022	2.9	0.0003
PtaPIN12	fgenesh4_pg.C_LG_XIX000547	3975	2.2	0.0204
PtaPIN2	estExt_Genewise1_v1.C_LG_XVI1213	608	2.5	0.0267
PtaPIN4	estExt_fgenesh4_pm.C_LG_V0399	171	3.0	0.0330
PtaPIN9	fgenesh4_pm.C_LG_XVIII000434	294	3.9	0.0019

[a] Gene name referring to the JGI *Populus* genome v1.1 (*PtaGH3, PtaPIN, PtaAUX*) or to Kalluri et al. (2007) (*PtaIAA*)

[b] Gene model names from JGI

[c] Background corrected expression level as units of Cy3 fluorescence on microarray. nd=not detected

[d] Ratio of expression levels in roots three days in indirect interaction with *L. bicolor* versus expression levels in control roots only covered by a cellophane membrane

[e] Student's T-Test with FDR (Benjamini-Hochberg) muliple testing correction

These authors had identified 913 LRI genes in a comparative, microarray-based approach with wildtype and auxin signaling mutant plants during chemical stimulation of LRI. A total of 46 differentially expressed poplar genes were overlapping with LRI genes in Arabidopsis (Supplemental Table S2). These contained early auxin responsive genes involved in transcriptional regulation *(AUX/IAA)* and auxin conjugation *(GH3)* as well as cell-cycle regulators. A focus was made on the regulation of members of auxin-signaling (*PtaIAA*)- and homeostasis-related (*PtaGH3*) gene families.

Six out of the 35 poplar auxin responsive transcription factors *Aux/IAA* were identified as being differentially expressed: *PtaIAA28.1*, *PtaIAA33.2*, *PtaIAA3.3*, *PtaIAA3.4*, *PtaIAA19.3* and *PtaIAA34* (names refer to (Kalluri et al., 2007)). Within these six *PtaIAA* family members, transcripts of *PtIAA28.1* were the most abundant in control roots, and *PtIAA28.1* was the only family member to be downregulated upon contact with *L. bicolor*. *PtaIAA34* was induced *de novo* in the presence of *L. bicolor*.

As for the 12 poplar *GH3* IAA amido-synthetase genes that code for proteins regulating auxin homeostasis (Hagen and Guilfoyle, 1985; Ljung et al., 2002; Staswick et al., 2005), four transcripts were induced: *PtaGH3-1* and *PtaGH3-2*, as well as *PtaGH3-7* and *PtaGH3-8*, which respectively can be considered as duplicates based on their homology to each other and to *Arabidopsis* homologs (Supplemental Fig. S2). Referring to the phylogenetic analysis, these four targets are part of Group II GH3 proteins that are active on auxin in other plant species (Staswick et al., 2005).

As the auxin gradient was affected in the plant during contact with the fungus, we also screened the microarray data for differentially expressed genes involved in polar auxin transport. Among the eight annotated members of the auxin influx carrier gene family (*PtaAUX*), two (*PtaAUX3*, *PtaAUX6*) were differentially expressed (phylogenetic tree in Supplemental Fig. S3) (Table I). Furthermore, four of the 16 members of the auxin efflux carrier gene family (Supplemental Fig. S4), *PtaPIN2*, *PtaPIN4*, *PtaPIN9* and *PtaPIN12*, were induced in roots in interaction with the fungus (Table I).

Taken together we identified 16 auxin-related genes that were differentially expressed in poplar roots during the interaction with *L. bicolor* (Table I) and that are likely to be involved in LRI and/or in modifying auxin gradients within the root. We have likely underestimated the expression ratios of several targets as transcript analysis was realized on entire root systems thereby diluting events with restricted, specific tissue localization.

Time course of auxin-related gene expression during poplar/*L. bicolor* interaction

Using quantitative real-time PCR of cDNA synthesized from total RNA of root samples collected at 1, 3, and 10 DOII as well as from four weeks-old ECM roots (30 DODI), we assessed the transcript

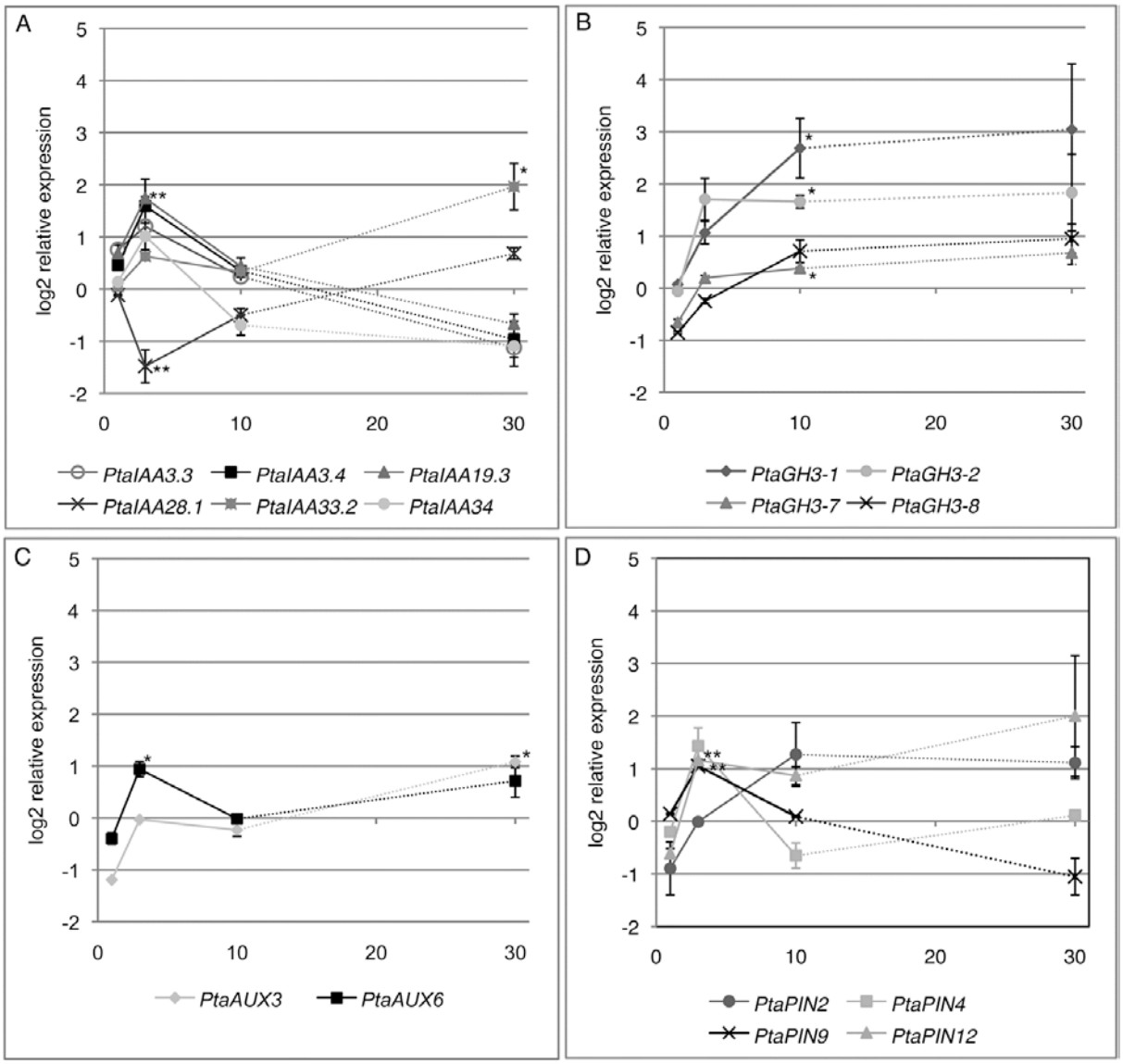

Figure 4. Expression profiles of auxin-related target genes in poplar roots at 1, 3 and 10 DOII as well as 30 DODI (horizontal axis). Log2-transformed relative expression compared to control roots is shown. A, *PtaIAA19.3* transcripts were significantly upregulated with an accumulation maximum at 3 DOII. *PtaIAA28.1* was downregulated but bottomed out at the same time point. With the exception of *PtaIAA33.2* these members of this gene family were only induced during the early phase of contact. B, *PtaGH3* genes were slowly upregulated only at 10 DOII. C, *PtaAUX6* was early induced at 3 DOII, whereas *PtaAUX3* was only induced in the late phase (30 DODI). D, PtaPIN transcript profiles differed from one another. *PtaPIN9* and *PtaPIN12* were transiently induced early (3 DOII) but *PtaPIN9* levels decreased in the late phase (30 DODI). * Student T-Test <0.05, ** Student T-Test <0.01

levels of the 16 identified auxin-related genes (Table I) over time during the poplar/*L. bicolor* interaction. Realtime PCR data from three independent biological replicates of 3 DOII were used to validate microarray data.

The expression profiles within the *PtaIAA* gene family members varied (Fig. 4A). During indirect contact *PtaIAA19.3* transcripts showed a significant increase at three DOII before leveling off back to constitutive levels. The transcript level increased 3.3 fold at 3 DOII (non-logarithmic scale). The expression profile of *PtaIAA28.1* was the inverse of the aforementioned *PtaIAA*. Its transcript level reached the lowest level (2.7-fold decrease) at three DOII and then increased slowly back to the level measured in control plants between 10 and 30 DOI. *PtaIAA33.2* displayed yet another expression profile. It was significantly upregulated up to four fold by 30 DODI and was the only late induced member of the *PtaIAA* gene family. Taken together, *PtaIAA19.3* and *PtaIAA28.1* showed transient induction, paralleling fungus-stimulated LRI, whereas *PtaIAA33.2* accumulation was activated during late symbiosis.

The *PtaGH3* gene family, involved in auxin conjugation, showed a homogeneous transcriptional pattern during the poplar/*L. bicolor* interaction (Fig. 4B). *PtaGH3-1*, *PtaGH3-2* and *PtaGH3-7* transcripts slowly accumulated, and their levels became significantly different from the controls after 10 DOII. *PtaGH3-1* was the family member displaying the highest upregulation, up to 6.4-fold higher than in control plants. By comparison, the induction of the other members, *PtaGH3-2* and *PtaGH3-7,* was 3.4-fold and 1.3-fold, respectively. Even if transcript accumulation at 30 DODI was variable between different biological replicates, a persistent profile is indicated for all four *PtaGH3* genes.

The two analyzed members of the auxin influx carrier family *PtaAUX* (Fig. 4C) showed a distinct regulation pattern. *PtaAUX6* was significantly and transiently upregulated at 3 DOII. The transcript levels of *PtaAUX3* were not altered during the early phase of the interaction, but became significantly upregulated quite late, at 30 DODI.

Members of the auxin efflux carrier family *PIN* showed a very heterogeneous regulation profile (Fig. 4D). Only *PtaPIN9* and *PtaPIN12* were significantly elevated by 2.0- and 2.2-fold, respectively, at 3 DOII. Subsequently, *PtaPIN9* levels decreased rapidly and reached constitutive levels at 10 DOII. *PtaPIN12* levels varied between biological replicates at further time points but indicated a persisting induction up to 30 DODI.

In summary, *L. bicolor* caused the transient induction of a series of genes of the auxin signaling and polar auxin transport family, including *PtaIAA19.3, PtaIAA28.1, PtaAUX6* and *PtaPIN9*. Members of the IAA-amido-synthetases family *PtaGH3-1, PtaGH3-2, PtaGH3-7* accumulated slowly and

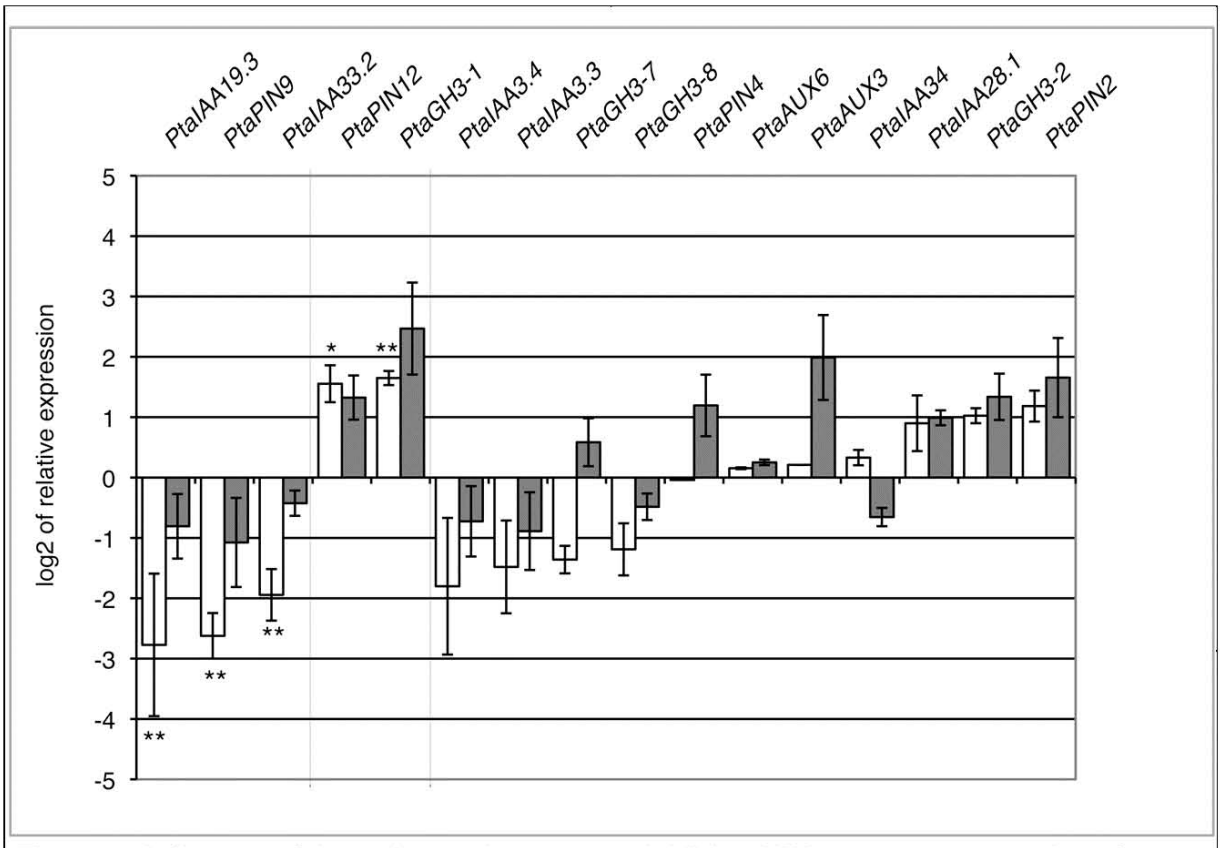

Figure 5. Influence of the polar auxin transport inhibitor NPA on gene expression after three DOII (white bars) or ten DOII (grey bars) of poplar with *L.bicolor*. Ratios of the transcript levels in roots in the presence versus the absence of NPA, during indirect interaction with *L. bicolor* are presented. The graph is separated into three parts: the left part presents genes whose fungal induction or repression was significantly abolished by NPA (in order of magnitude referring to 3 DOII). A drastic reduction in *PtaIAA19.3*, *PtaPIN9* and *PtaIAA33.2* transcripts by the presence of NPA at three DOII was observed. The middle part presents genes whose fungal-induction was significantly increased by the presence of NPA. Note the super-induction of *PtaPIN12* and *PtaGH3-1*. The right part refers to genes whose expression was not statistically effected by NPA. *Student T-Test <0.05, **Student T-Test <0.01

their levels remained elevated throughout the contact. Lastly *PtaAUX3* and *PtaIAA33.2* were specifically and only induced at late stages of the interaction.

NPA effect on gene expression during poplar/*L. bicolor* interaction

We analyzed how the polar auxin transport inhibitor NPA, which inhibited LR stimulation by the fungus (Fig. 2C), influenced the expression of *L. bicolor*-regulated auxin-related genes. We generated cDNAs from poplar roots at 3 or 10 DOII in the presence or absence of NPA and subjected them to quantitative real-time PCR.

The expression profiles of certain members of the *PtaIAA* family were inverted by the presence of NPA (Fig. 5): The presence of NPA during the contact with *L. bicolor* abolished fungus-induced transcript accumulation of *PtaIAA19.3, PtaPIN9* and *PtaIAA33.2*. A repression of 2.0 (*PtaIAA19.3*), 2.0 (*PtaPIN9*) and 2.5 (*PtaIAA33.2*) times compared to controls without fungus and NPA was observed instead of the before observed significant induction of 3.3 and 2.0 times for *PtaIAA19.3* and *PtaPIN9* respectively (Fig. 4 and 5). In contrast, NPA-based inhibition of auxin polar transport amplified by three fold the induction of *PtaPIN12* and *PtaGH3-1* over levels induced by the fungus alone thus leading to transcript levels respectively 6 and 33 fold higher than in control plants without fungus and NPA. Interestingly, for all five mentioned genes, NPA impacted transcript levels at three days of poplar/*L. bicolor* interaction, but at later stages (10 days) its effects on plants in interaction with the fungus were no longer statistically relevant. The differential expression upon contact with the fungus of all other gene targets was not significantly influenced by the presence of NPA, neither at 3 nor at 10 DOII.

These results indicate that the repression of fungus-induced LR induction by NPA is mirrored by dramatic changes in transcript abundance, especially that of early, transiently regulated auxin signaling and transport genes such as *PtaPIN9* and *PtaIAA19.3*.

LR development in *A. thaliana* auxin mutants during interaction with *L. bicolor*

To gain additional insights into the crucial role of auxin transport and signaling during fungus-induced LR development, we assessed LR development in response to *L. bicolor* in different Arabidopsis mutants altered in auxin perception (*tir1afb1,2,3*), signaling (*slr1* (IAA14), influx (*aux1*) and efflux (*pin2, pin2,3,4,7*). The Arabidopsis mutants chosen were either those known to have an important LR phenotype (reduction or absence of LR development in *slr1* and *tir1afb1,2,3*) or those genes most closely related to genes in poplar that were found to be regulated (*pin2* (*PtaPIN9*), *aux1* (*PtaAUX*3)) during *L. bicolor*-induced LR development. The effect of *L. bicolor* on plants defective in PIN3 or additionally also PIN4 and PIN7 were examined because of their role

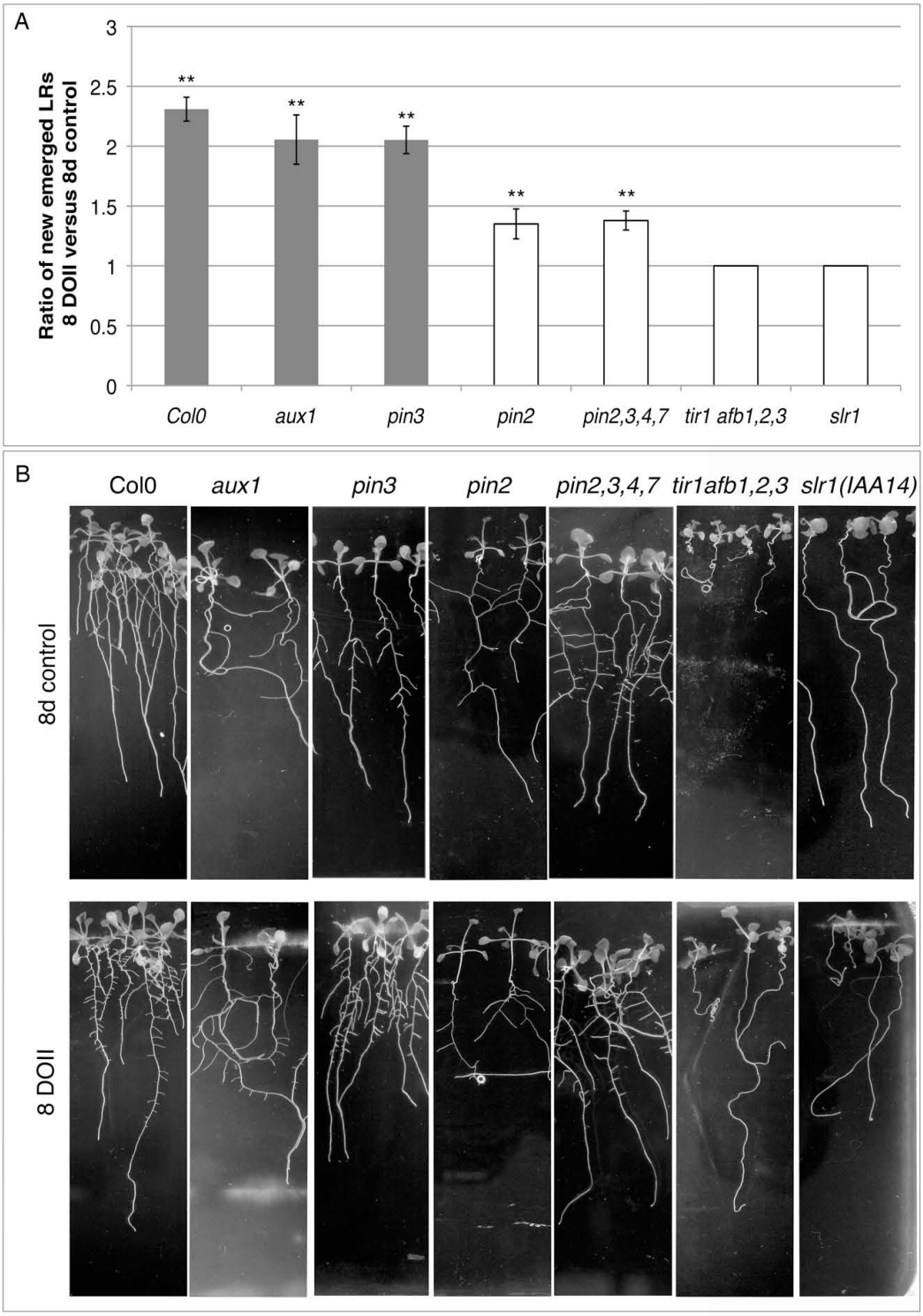

Figure 6. LR development in *Arabidopsis thaliana* auxin mutants in the presence of *L. bicolor* after 8 DOII. A, Ratio of LR number that developed in plants in contact with *L. bicolor* versus control plants without fungus. Significant LR increases are marked by asterisks (**Student T-Test, $p<0.01$). Ratios in mutants that differed significantly from Col0 are represented as white bars. LR development was stimulated by *L. bicolor* in Col0, *pin3* and *aux1* to a similar extent and significantly less in *pin2* and *pin2,3,4,7*. The quadruple auxin receptor mutant *tir1 afb1,2,3* and the IAA14 gain of function mutant *slr1* were completely insensitive to *L. bicolor* in terms of LR stimulation. B, root development of control plants and plants after 8 DOII. Note the absence of LRs in the *slr1* and *tir1afb1,2,3* negative control plants and at 8 DOII. Per treatment and mutant line 10 to 15 biological replicates were analyzed.

together with PIN2, in the auxin reflux loop at the root apex (Blilou et al., 2005). At five days after germination mutants were placed into contact with *L. bicolor* and LR development was observed after eight DOII (Fig. 6). The auxin perception quadruple mutant *tir1afb1,2,3* and the IAA14 protein-stabilized mutant *slr1*, which both did not form LRs under control conditions (Fig. 6B), also did not develop LRs in response to *L. bicolor* (Fig. 6A and B). As for auxin transport mutants, we observed that in the presence of *L. bicolor*, *aux1* was still able to develop LRs, as was *pin3*. The magnitude of LR stimulation in both mutants was comparable to wildtype. In both the single *pin2* mutant as well as the quadruple *pin2,3,4,7* mutants a significant increase in LR during contact with the fungus was observed. LR stimulation in these mutants was 1.37 times higher as compared to controls of the same lines in absence of fungus, whereas wildtype plants developed up to 2.4 times more roots during contact. LR stimulation in the quadruple mutant *pin2,3,4,7* was not further inhibited than the single *pin2* mutant. We further analyzed auxin distribution in the *pin2* mutant harboring the *DR5::GUS* reporter (Fig. 3H and I). Whereas in wildtype plants the presence of the fungus stimulated auxin accumulation in specific cells of provascular tissues, in *pin2* a strong and diffuse increase of DR5 directed GUS activity was observed in the epidermis and lateral root cap and the provascular signal was visible only in 2 out of 13 analyzed plants at 3 DOII.

Together these data imply a prominent role of auxin transport through AtPIN2 in fungus-regulated auxin gradient modification and LR initiation.

DISCUSSION

Although LR stimulation during ECM formation is a recognized phenomenon, the molecular mechanisms that are activated in roots by the presence of the fungus and that regulate LR stimulation are so far unknown. Here we investigated the early interaction of *L. bicolor* with roots of a mycorrhizal and a non-mycorrhizal plant. Our molecular and functional analyses revealed that auxin transport and signaling are key mechanisms regulating LR development in response to fungal signaling molecules.

In interaction with *L. bicolor*, the early response of poplar resulted in an increase in LRs paralleled by an enhanced auxin response at the root apex, as revealed by an increase in *DR5*-driven *GFP* expression. Indirect contact experiments showed that these modifications did not require physical contact between hyphae and root cells, suggesting a role for diffusible signaling molecules. Using the non-mycorrhizal plant *Arabidopsis thaliana*, we further demonstrated that these diffusible signaling molecules induced LRs and root apex auxin-response increase in a host-plant independent manner and that both did not rely on the mycorrhizal capacity of the plant partner. Equivalent results have been reported recently for the truffle/Arabidopsis and truffle/*C. incanus* interaction (Splivallo et

al., 2009). Thus LR-inducing signaling molecules from various fungi have rather broad functions in plant development, just as phytohormones would be expected to have.

Auxin homeostasis is altered in the root during interaction with *L. bicolor*

The increase in auxin accumulation at the root apex during contact with the ECM fungus has only recently been reported by Splivallo et al. (2009) and our data confirm and extent this finding to another ECM fungus and to poplar as a mycorrhizal host plants. Two hypothetical scenarios can explain this auxin accumulation. First, auxin secreted by the ECM fungus may be taken up into the root and accumulate in the tip. The root tip is usually the first part of the root colonized by the fungus (Horan et al., 1988; Splivallo et al., 2009), which would explain this local auxin increase at the apex. Anyhow, Splivallo et al. (2009) have shown that exogenous auxin alone was not able to mimic the effect of the fungus, which also suggests that fungal auxin alone would not be the sufficient to induce the observed DR5 signal increase. For instance, Splivallo and co-workers (2009) have suggested a combined action of fungal auxin and ethylene on the root. As ethylene is known to impact endogenous auxin biosynthesis in roots (Stepanova et al., 2007; Stepanova et al., 2008), a second scenario may include an activation of endogenous auxin biosynthesis at the root apex by fungal ethylene leading to the observed auxin accumulation. A recent study had shown that artificial induction of auxin biosynthesis at the Quiescent Center leads to enhanced *DR5::GFP* expression first in the columella region, then in the lateral root cap and finally also in the epidermis and provascular strands (Blilou et al., 2005). Even if a lot weaker, results from Splivallo et al. (2009) and our data indicate an increase of the signal at the same sites. Auxin measurements will be necessary to confirm that the increased auxin response is actually due to an increased auxin quantity (Petersson et al., 2009), that might be caused by activation of auxin biosynthesis at the root apex.

Results from the microarray based transcript profile have shown an increase in *PtaPIN12* and *PtaGH3* transcripts, whose Arabidopsis homologs are thought to be involved in auxin homeostasis (Staswick et al., 2005; Mravec et al., 2009). AtPIN5 (homolog of PtaPIN12) has been suggested to import auxin into the ER and thereby to decrease free auxin levels in the cell. Its overexpression was accompanied by an increase in IAA-conjugates with amino acids, suggesting a crosstalk with IAA-amido-synthetases of the GH3 family (Mravec et al., 2009). We found strong induction of *PtaGH3-1* and less strong induction of *PtaPIN12* during the combined NPA/fungus treatment, which coincided with a strong decrease of the DR5:GUS signal at the Arabidopsis root apex. Together these results suggest that PtaGH3-1 and PtaPIN12 actively decrease the auxin maximum in the root apex. We hypothesize that both genes are involved in tuning auxin homeostasis during fungus induced auxin accumulation in the root. Their impact may also explain why only a week difference in auxin

accumulation is observed at the root apex, especially as certain AtGH3 are expressed in these tissues (Brady et al., 2007). It will be necessary to investigate in more detail the spatial and temporal aspects of auxin accumulation in the root during interaction with the fungus and to connect results to protein localization of auxin-homeostasis regulators.

Polar auxin transport regulates LR induction during the root/*L. bicolor* interaction

Expression analysis of polar auxin transporters revealed that the expression of *PtaPIN9*, which was induced during the early root/fungus interaction, was negatively impacted by the presence of the polar auxin transport inhibitor NPA. Furthermore, the reduction in *PtaPIN9* transcripts paralleled the absence of LR stimulation. These results suggest a role of PtaPIN9 during fungus-induced LR induction. PtaPIN9 is the homolog of Arabidopsis AtPIN2, a protein involved in basipetal auxin transport from the root apex upwards to the elongation zone and in creating an auxin reflux loop at the root apex (Muller et al., 1998; Blilou et al., 2005). Interestingly, *L. bicolor* induced LR stimulation was dramatically decreased in *pin2* but not in *pin3* transgenic Arabidopsis plants. Furthermore, the absence of PIN3, PIN4 and PIN7 in the quadruple mutant *pin2,3,4,7* did not further decrease LR stimulation compared to single *pin2* mutants. This suggests that specifically AtPIN2 dependent polar auxin transport is required for fungus induced LR stimulation. *AtPIN2* transcription has been shown to be positively influenced by auxin and by PLT genes (Sieberer et al., 2000; Benkova et al., 2003; Blilou et al., 2005; Vieten et al., 2005). If we assume functional homology between *AtPIN2* and *PtPIN9*, the increasing auxin accumulation at the root apex, together with the *PLT* increase may impact *PtaPIN9* transcription. Whether this leads to an increase in PtaPIN9 protein and also to a change in localization still needs to be confirmed, as the exact localization is crucial for directing the auxin flow (Laskowski et al., 2008). Nonetheless, we hypothesize that a fungus-induced increased amount of PtaPIN9 protein, localized to apical and lateral cell membranes in the epidermis and the cortex leads to higher accumulation of auxin at the pericycle within the elongation zone/differentiation zone in the root, a mechanism thought to prime a larger region for lateral root induction (Laskowski et al., 2008). When Blilou and co-workers (2005) expressed a bacterial auxin biosynthesis gene at the quiescent center of Arabidopsis roots either in a *pin2* mutant background or in NPA treated wildtype plants, the induction of the DR5:GFP signal was, in contrast to a wildtype background without NPA, absent or only weak in provascular strands. Accordingly, in our study during Arabidopsis/fungus contact, the inhibition of auxin polar transport in general through NPA or specifically in *pin2* lead to the absence of the provascular auxin signal and to the absence of LR stimulation. Nevertheless, in *pin2* a strong and diffuse auxin accumulation was observed at the root apex during contact (Fig. 3H and I), that was absent in control plants, implying a function of AtPIN2 in efficiently redistributing excess auxin which accumulates during interaction with the fungus. Furthermore, it has also been reported that in the Arabidopsis *wav6-52* mutant, which has an increased amount of PIN2 protein, a strong DR5:GFP signal extends into the root

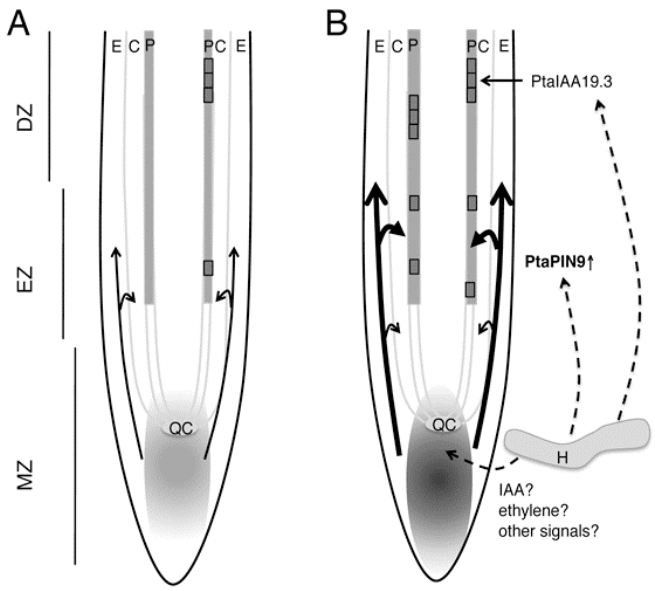

Figure 7. Hypothetical model of the molecular mechanism underlying fungus induced LR development in poplar. A, PtaPIN9 directed basipetal polar auxin transport (arrowhead) before contact with the fungus. The auxin maximum around the Quiescent center is indicated by a grey shadow. Pericycle founder cells (single grey box) are primed for LRI in the elongation zone (EZ) and LR are initiated through subsequent cell divisions (multiple grey boxes) in the differentiation zone (DZ). B, The presence of the fungus stimulates auxin accumulation at the root apex (grey shadow) through an unknown mechanism. The increased auxin level and/or other fungal signals stimulate PtaPIN9 expression. PtaPIN9 protein enhances basipetal auxin transport (bold arrowheads) that hence primes more pericycles cells for LRI. LRI occurs through a PtaIAA19.3 dependent signaling mechanism in the differentiation zone. Whether fungal signals act directly on PtaIAA19.3 expression needs to be analyzed. Epidermis (E), Cortex (C), Pericycle (P), Quiescent Center (QC), Hyphae (H).

elongation zone (Abas et al., 2006). Together, our data compared to the literature strengthen the hypothesis that provascular auxin accumulation and LR induction may result from an apical/lateral auxin transport related to AtPIN2 (PtaPIN9). Immunolocalization of PtaPIN9 in poplar and quantification of PtaPIN9 induction in these specific tissues will allow confirming and deepening the impact of PtaPIN9 during fungus/root crosstalk.

Auxin signaling regulates LR induction during the root/*L. bicolor* interaction

Our transcript profiling analyses revealed a differential expression of early auxin-responsive genes involved in auxin signaling (*PtaIAA*). Quantitative real-time PCR analysis of these differentially-expressed targets at various time-points during the poplar/*L. bicolor* interaction, identified genes, such as *PtaIAA19.3* and *PtaIAA28.1,* whose transcript levels were modified early, but only transiently. When defective or absent, homologs of the *Arabidopsis* auxin transcription regulators *PtaIAA19.3* and *PtaIAA28.1* are known to cause a strong LR phenotype (Tian and Reed, 1999; Rogg et al., 2001; Tatematsu et al., 2004; Muto et al., 2007). Strikingly, when NPA was applied during the poplar/*L. bicolor* interaction, *PtaIAA19.3* transcript accumulation was repressed in the presence of the fungus and no LR stimulation was observed. This suggests a connection between auxin signaling via *PtaIAA19.3* and fungus induced LR development. Experiments with Arabidopsis *slr1* (a stabilized form of AtIAA14 conferring a gain of function mutation), which is blocked in auxin signaling (Fukaki et al., 2002), confirmed that the classical auxin-signaling pathway is required for fungus-induced LR development in plants. In poplar *PtaIAA19.3* may be a key actor in the basic auxin-signaling pathway in roots during LRI.

CONCLUSION

Our data suggested a model (Fig. 7) in which undefined fungal signals cause an auxin accumulation at the root apex and increase the apical/lateral polar auxin transport through PtaPIN9. Excess auxin that accumulates during contact is specifically transported to provascular pericycle cells, which are primed to LR founder cells. We assume that a stronger auxin accumulation in these tissues primes a higher number of cells. Priming of founder cells for LRI has been suggested to be independent of AUX/IAA signaling. Anyhow, LRI in the differentiation zone involved auxin accumulation and auxin-signaling. Therefore we propose that auxin signaling through PtaIAA19.3 in the differentiation zone is part of LRI downstream of acquirement of founder cell specification in the basal meristem (meristem/elongation zone). (Fig. 7).

MATERIALS AND METHODS

Plant and fungal material and growth conditions

Experiments were performed with the hybrid *Populus tremula x Populus alba* (INRA clone 717-1-B4) and the transgenic hybrid *Populus tremula x Populus tremuloides DR5:GFP*. Plants were micropropagated *in vitro* and grown on 1/2 MS medium (Murashige and Skoog, 1962) in glass culture tubes under a 16 h photoperiod at 24°C in a growth chamber.

The dikaryotic vegetative mycelium of the ECM fungus *Laccaria bicolor* strain S238N (Maire P.D. Orton) was maintained at 25°C on modified Pachlewski medium P5 (Deveau et al., 2007).

Seeds of *Arabidopsis thaliana* (L.) Heynh. ectoype Columbia (Col-0), *aux1*-t (also known as *aux1-100* (Bennett et al., 1996)), *pin2/eir1-1* (Roman et al., 1995), *pin2 in DR5:GUS* (Sabatini et al., 1999), *pin3* (*salk_005544*) and *pin2,3,4,7* (http://signal.salk.edu/cgi-bin/tdnaexpress/) (Blilou et al., 2005), *slr1* (Fukaki et al., 2002), *tir1afb1,2,3* (Gray et al., 1999; Dharmasiri et al., 2005) were surface-sterilized and sown on solid *Arabidopsis* medium (2.3 g L^{-1} MS salt, 1% sucrose, 1.3% agar-agar (pH 6.0 adjusted with KOH) and 1g L^{-1} MES sodium salt. After stratification for one to two days at 4°C, seeds were germinated under a long-day period (16 h light, 8 h darkness) at 21°C.

Co-culturing in a sandwich culture system

For mycorrhiza formation on *in vitro* poplar (*P. tremula x P. alba*) by *L. bicolor,* we modified an existing sandwich co-culture system initially developed for the *Eucalyptus*/*Pisolithus* interaction (Chilvers et al., 1986; Burgess et al., 1996) (Fig. 1). Free-living mycelium of *L. bicolor* S238N was grown for 10 days on cellophane-covered agar (12g L^{-1}) plates containing sugar-reduced Pachlewski medium P20 (Deveau et al., 2007). In parallel, plant material was prepared. In order to synchronize rhizogenesis, stem cuttings from *in vitro* poplar plants were pre-cultured on one-half MS medium containing 2 mg L^{-1} indole-butyric-acid (IBA) for 7 days. Rooted cuttings were then transferred to vertically arranged 12 x 12 cm square Petri dishes half covered with a cellophane membrane and cultured for three weeks under a 16 h/day light photoperiod at 24°C.

For co-cultures, plants were transferred to 12x12 cm Petri dishes containing solidified (12 g L^{-1} agar) low-carbon Paschlewski medium (P20), pH 5.8, buffered with 1g L^{-1} MES sodium salt covered by a 6x12 cm cellophane membrane. A mycelium-covered cellophane membrane was placed fungus-side down (direct interaction) or fungus-side up (indirect interaction) on the roots. Petri dishes were closed with Parafilm on the upper and lower side and with band-aid (insuring high gaz-permeability) on both sides. Cultures were arranged vertically and the lower part of the dish was covered with a small black plastic bag to prevent light from reaching the fungus and roots. The sandwich cultures were kept in the same conditions as the poplar plant cultures. When Arabidopsis was put into contact

with *L. bicolor*, mycelium-covered cellophane membranes were directly laid on the plant roots grown on MS medium (with 1 g L^{-1} MES sodium salt). The medium used for NPA treatment and mutant/*L. bicolor* contact contained 1g L^{-1} MES sodium salt. Control poplar and Arabidopsis plants were covered with a cellophane membrane without fungal mycelium.

Observation of root development

For LR quantification, 10 to 15 individual poplar plants (3 per Petri dish) or 10 to 15 Arabidopsis seedlings in the respective conditions were observed every two to four days using a Discovery V.8 stereo microscope (ZEISS, Germany). LRs were counted and pictures were taken. To determine the extent of root colonization by *L. bicolor* we observed root morphology every ten days. Samples were taken for sectioning at each time point.

Microscopic observation

In order to ascertain the formation of the intra-radicular Hartig net, we subjected root sections to propidium iodide/UVitex double staining for plant and fungal structures, respectively. One-centimeter root tips were fixed in 4% (w/v) *para*-formaldehyde in phosphate buffered saline (PBS) (pH 7) overnight at 4°C. Roots were washed in PBS and embedded in 6% (w/v) agarose. Thirty µm transversal sections were prepared using a vibratome (LEICA, Germany). Sections were stained in 1% (w/v) UVitex 2B (Polyscience) in PBS for 2 min, washed and then counter-stained with propidium iodide (1:100 dilution) (Sigma-Aldrich) (Moldenhauer et al., 2006; Xu et al., 2006). For DR5:GFP observation, fresh 2 cm root tips from *P. tremula x P. tremuloides DR5:GFP* were mounted in Slowfade Gold Antifade Reagent (Molecular Probes) and observed immediately. All samples were observed with a Radiance 2100 Rainbow confocal scanning laser microscope (NIKON-BIORAD) equipped with an Apochromat X60 (NA 1.4) oil objective. A wavelength of 405 nm was used for Uvitex 2B excitation and emission was detected between 500 to 560 nm. Propidium iodide was excited at 514 nm with an argon laser line and the emitted fluorescence was detected above 550 nm. GFP was excited using the 488nm argon laser line in conjunction with a 505-530 band-pass filter. Settings (laser intensity, gain, offset, magnification) for DR5:GFP observations were maintained equally between all samples. Histological detection of β-Glucoronidase (GUS) was performed according to (Scarpella et al., 2004). *DR5:GUS* plants were stained for 90 minutes, pin2/DR5:GUS plants for 120min at 37°C. Samples were mounted in 50% (v/v) glycerole and observed using a Zeiss Axiovert 200M MOT (Carl Zeiss MicroImaging, Germany).

RNA extraction and cDNA synthesis

At each time-point of quantitative Realtime-PCR analysis three pools of six independent root systems for each control roots and fungal treatments were harvested and frozen in liquid nitrogen. For microarray analysis three additional pools of six root systems for each controls and indirect interaction were harvested from a second experimental series. Total RNA was extracted from these samples using the RNAeasy kit (QIAGEN) as per manufacturer's instructions. An in-column digestion step with DNAse I (QIAGEN) was part of the extraction. RNA quality was verified by Experion Standardsens Capillar gels (BIORAD). cDNA for NimbleGen microarrays was synthesized using Smart cDNA Synthesis kit (CLONTECH) containing an amplification step on the cDNA level (Duplessis et al., 2005). RNA for real-time PCR was additionally subjected to a second DNA digestion step using DNAfree (AMBION) before synthesizing cDNA from 250ng total RNA using an iScript kit (BIORAD).

NimbleGen Microarray transcript profiling

The *Populus* whole-genome expression array version 2.0 (S. DiFazio, A. Brunner, P. Dharmawardhana, and K. Munn, unpublished data) manufactured by NimbleGen Systems Limited (Madison, WI) contains in duplicates three independent, non-identical, 60-mer probes per whole gene model plus control probes and labeling controls. Included in the microarray are 65,965 probe sets corresponding to 55,970 gene models predicted on the *P. trichocarpa* genome sequence version 1.0 and 9,995 aspen cDNA sequences (*Populus tremula, Populus tremuloides*, and *P. tremula x P. tremuloides*). The Populus version 2.0 oligoarray is fully described in the platform Gene Expression Omnibus (GEO) at NCBI (http://www.ncbi.nlm.nih.gov/geo). NimbleGen whole genome microarray analyses were performed in three biological replicates (independent from quantitative Realtime-PCR samples) with a technical replicate on each array as per manufacturers instructions. Expression data were processed in the following way: to assure a high specificity all independent 60mer oligos for the 55,970 genes were blasted against the *Populus* genome v1.1 available on http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html and only probes with less than 10% homology to other gene models than the gene model they were designed for were retained for the further analysis. Due to this stringent filtering about 30% of the genes (16,667) were excluded since all three independent oligos failed the given specificity. Fluorescence data were normalized between all different arrays using ARRAYSTAR software (DNASTAR). Average expression levels from all three biological repetitions were calculated for each gene from the specific independent probes and were used for further analyses. A Student's T-Test with FDR (Benjamini-Hochberg) muliple testing correction was applied on the data using ARRAYSTAR software (DNASTAR). Transcripts with a significant p-value (<0.05) and more than 2.0 change in transcript level were considered as significantly differentially expressed in roots in contact with *L. bicolor* compared to control roots. The signal to

noise threshold (background signal) was estimated as described in Martin *et al.* (2008). The complete expression dataset is available as series (accession number GSE16662) at the Gene Expression Omnibus at NCBI (http://www.ncbi.nlm.nih.gov/geo/). Expression data for all genes that were represented at least by one specific 60mer oligo on the array are also available in Supplemental Table S1.

Quantitative Real-time PCR

Specific primer sequences can be found in Supplemental Table S2. We used ubiquitin ((Kohler et al., 2004), *P. trichocarpa* gene model estExt_Genewise1_v1.C_LG_XV0407; GenBank ID CA825222) and a putative protein as reference genes ((Gutierrez et al., 2008), *P. trichocarpa* gene model estExt_fgenesh4_pm.C_LG_IX0344). Real-time PCR was performed using a Chromo4 Light Cycler and OpticonMonitor Software. Real-time PCR analyses were performed in three biological replicates (independent from microarray samples) with a technical replicate for each reaction. PCR reactions were realized with a technical replicate for each cDNA from three biological repetitions using SYBRGreen Supermix following the manufacturer's instructions (BIORAD). Fold changes in gene expression between treated and control roots were based on ΔΔCt calculations according to (Pfaffl, 2001). The means of each of the three ΔΔCt values were presented as histograms. ΔΔCt values calculated with either of the reference genes were similar. Only ubiquitin-normalized data is presented. For each mean ΔΔCt significance was calculated using Student's T-Test.

Phylogenetic trees

Phylogenetic trees based on entire protein sequence alignments were constructed using MEGAlign (ClustalX Alignment, Pairwise deletion). A neighbor-joining algorithm (pairwise deletion, p-distance) with 5000 bootstrap repetitions was chosen to establish the phylogenetic trees.

Image analysis

For DR5:GFP fluorescence quantification ImageJ software with the Stack-Measure Macro was used. Background intensity was equivalent for all images and was subtracted for further quantification. Image assembly of all figure panels was realized in Adobe Photoshop.

SUPPLEMENTAL MATERIAL

Supplemental Figure S1. Effect of NPA on auxin accumulation in *AtDR5:GUS* transgenic plant apices during contact with *L. bicolor*.

Supplemental Figure S2. Neighbor-Joining cladogram of the eight members of the *Populus* auxin influx carrier protein family AUX/LAX and their four and ten homologous protein sequences in *Arabidopsis thaliana* and *Oryza Sativa*.

Supplemental Figure S3. Neighbor-joining cladogram of the 16 members of the *Populus* auxin efflux carrier protein family PIN and their eight and thirteen homologous protein sequences in *Arabidopsis thaliana* and *Oryza Sativa* respectively.

Supplemental Figure S4. Neighbor-Joining cladogram of the 12 members of the *Populus* IAA-amido-synthetase protein family GH3 and their 19 and 12 homologous protein sequences in *Arabidopsis thaliana* and *Oryza sativa* respectively.

Supplemental Table S1. Gene expression data of *P. tremula x P.alba* roots after three days of indirect interaction with *L. bicolor*.

Supplemental Table S2: List of Arabidopsis LRI genes (Vanneste et al., 2005) and expression data from their poplar homologs in control roots and roots after three days of indirect interaction *L. bicolor*).

Supplemental Table S2. Specific primers for auxin-related target genes in *P. tremula x P. alba* used in quantitative Realtime PCR.

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Supplemental Data

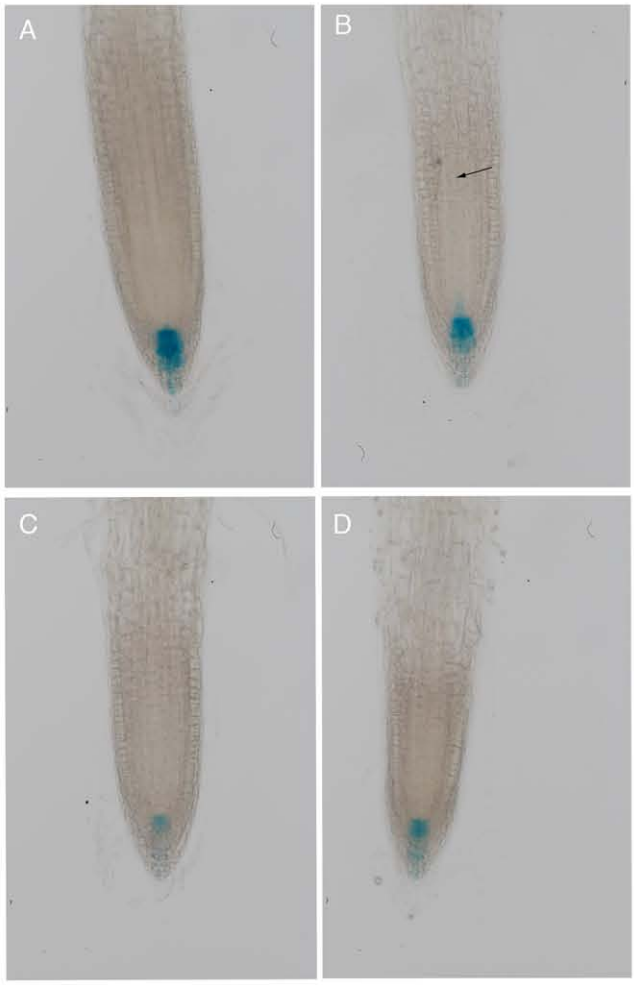

Figure S1. Effect of NPA on auxin accumulation in transgenic Arabidopsis *DR5:GUS* apices during contact with *L. bicolor*. A, 1µM NPA during 3 days. B, 1µM NPA and 3 DOII. C, 10 µM NPA during 3 days. D, 10 µM NPA and 3 DOII. Increasing NPA concentrations inhibit formation of a proper auxin maximum at the root apex in control plants. Furthermore, auxin accumulation in provasculature during contact with the fungus was strongly decreased or absent in NPA treated plants. Examples out of 15 biological replicates per condition.

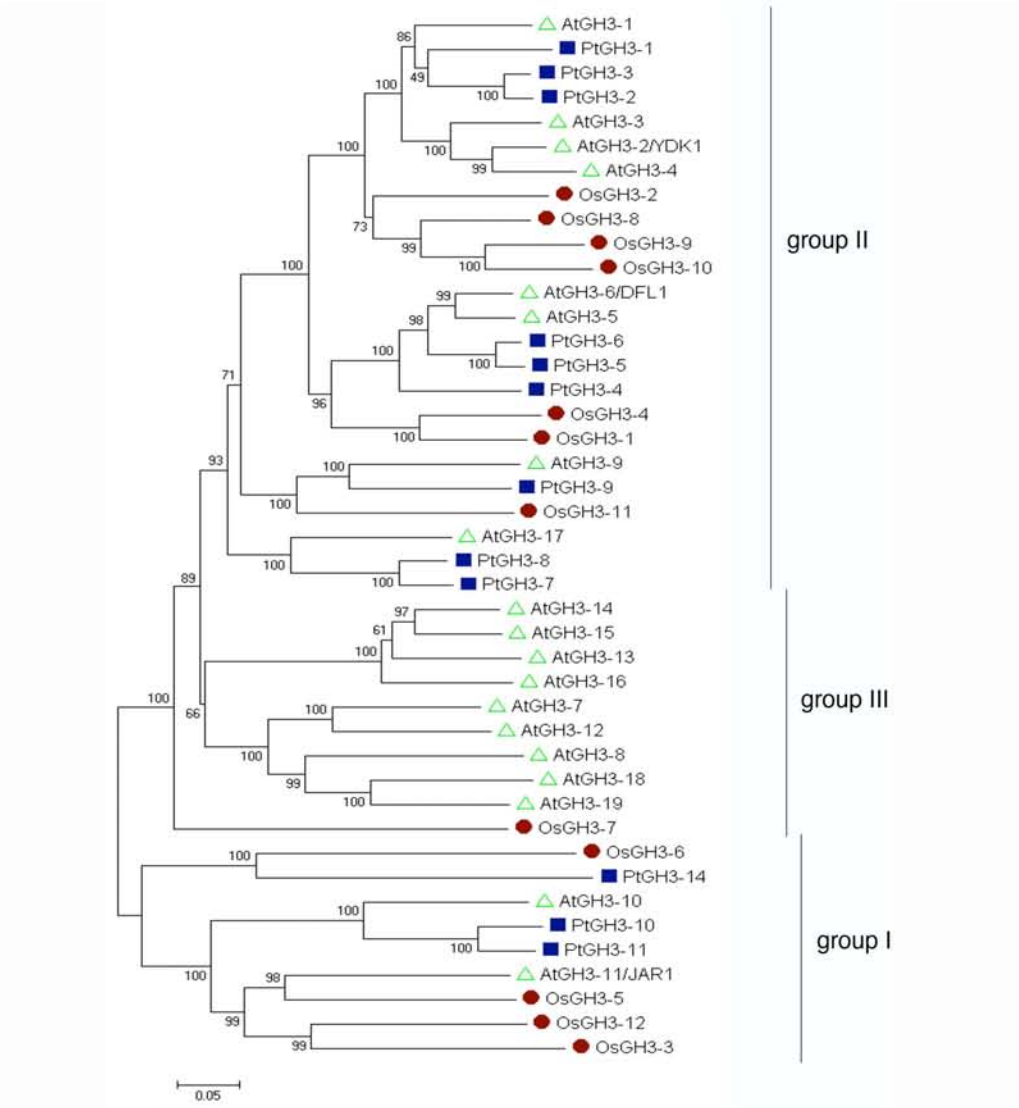

Figure S2. Neighbor-Joining cladogram established with entire protein sequences of the 12 members of the *Populus* IAA-amido-synthetase protein family GH3 (blue squares) and their 19 and 12 homologous protein sequences in *Arabidopsis thaliana* (green triangles) and *Oryza sativa* (red points) respectively. Subgroups refer to Staswick et al., 2005 and Jain et al., 2006. Note that no members of the *Populus* GH3 protein family are present in group III.

Poplar sequences from http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html PtGH3-1 (eugene3.02050011), PtGH3-2 (estExt_fgenesh4_pg.C_LG_IX0695), PtGH3-3 (eugene3.00012406), PtGH3-4 (fgenesh4_pg.C_LG_XIII000498), PtGH3-5 (eugene3.00111054), PtGH3-6 (eugene3.00130808), PtGH3-7 (fgenesh4_pg.C_LG_I000598), PtGH3-8 (gw1.III.363.1), PtGH3-9 (eugene3.00021734), PtGH3-10 (estExt_Genewise1_v1.C_LG_XIII3413), PtGH3-11 (grail3.0120002401), PtGH3-14 (eugene3.00140831), Arabidopsis sequences from http://www.arabidopsis.org/ AtGH3-1 (At2g14960), AtGH3-2/YDK1 (At4g37390), AtGH3-3 (At2g23170), AtGH3-4 (At1g59500), AtGH3-5 (At4g27260), AtGH3-6/DFL1 (At5g54510), AtGH3-7 (At1g23160), AtGH3-8 (At5g51470), AtGH3-9 (At2g47750), AtGH3-10 (AT4g03400), AtGH3-11/JAR1 (At2g46370), AtGH3-12 (At5g13320), AtGH3-13 (At5g13350), AtGH3-14 (At5g13360), AtGH3-15 (At5g13370), AtGH3-16 (AT5G13380), AtGH3-17 (At1G28130.1), AtGH3-18 (At1g48660), AtGH3-19 (At1g48670), Rice sequences from http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/ OsGH3-1 (AK063368), OsGH3-2 (LOC_Os01g55940), OsGH3-3 (AK072125), OsGH3-4 (AK101932), OsGH3-5 (AK071721), OsGH3-6 (AK106538), OsGH3-7 (AK099376), OsGH3-8 (AK101193), OsGH3-9 (AK106839), OsGH3-10 (LOC_Os07g38860), OsGH3-11 (LOC_Os07g47490), OsGH3-12 (LOC_Os11g08340).

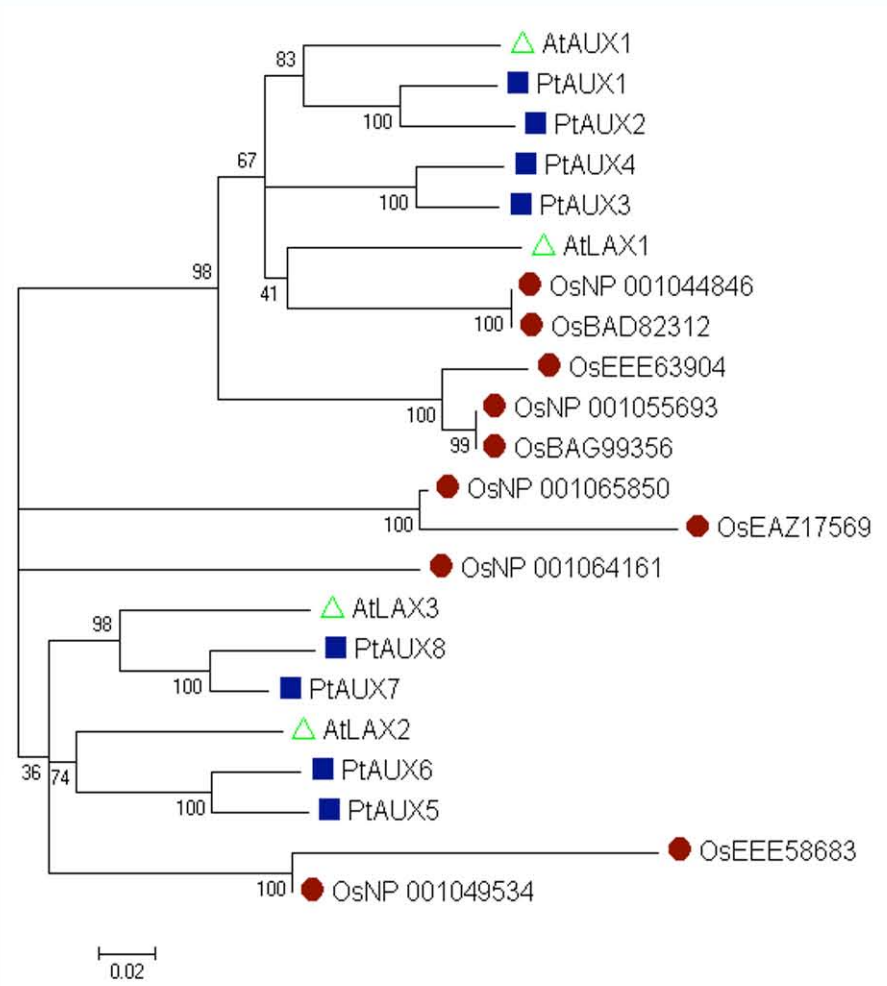

Figure S3. Neighbor-Joining cladogram established with entire protein sequences of the eight members of the *Populus* auxin influx carrier protein family AUX/LAX (blue squares) and their four homologous protein sequences in *Arabidopsis thaliana* (green triangles) and ten *Oryza Sativa* homologous (red circles). *Populus* sequences from http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html PtAUX1 (grail3.0023028402), PtAUX2 (eugene3.00161081), PtAUX3 (estExt_fgenesh4_pg.C_LG_X1704), PtAUX4 (estExt_Genewise1_v1.C_LG_VIII1679), PtAUX5 (estExt_fgenesh4_pg.C_LG_IV1437),PtAUX6 (grail3.0001031001), PtAUX7 (estExt_fgenesh4_pg.C_LG_V0933), PtAUX8 (grail3.0003074001), Arabidopsis sequences from http://www.arabidopsis.org/ AtAUX1 (AT2G38120.1), AtLAX1 (AT5G01240.1), AtLAX2 (AT2G21050.1), AtLAX3 (AT1G77690.1). Rice sequences were identified through protein Blast analysis. NCBI accession numbers for rice proteins are given in the cladogram.

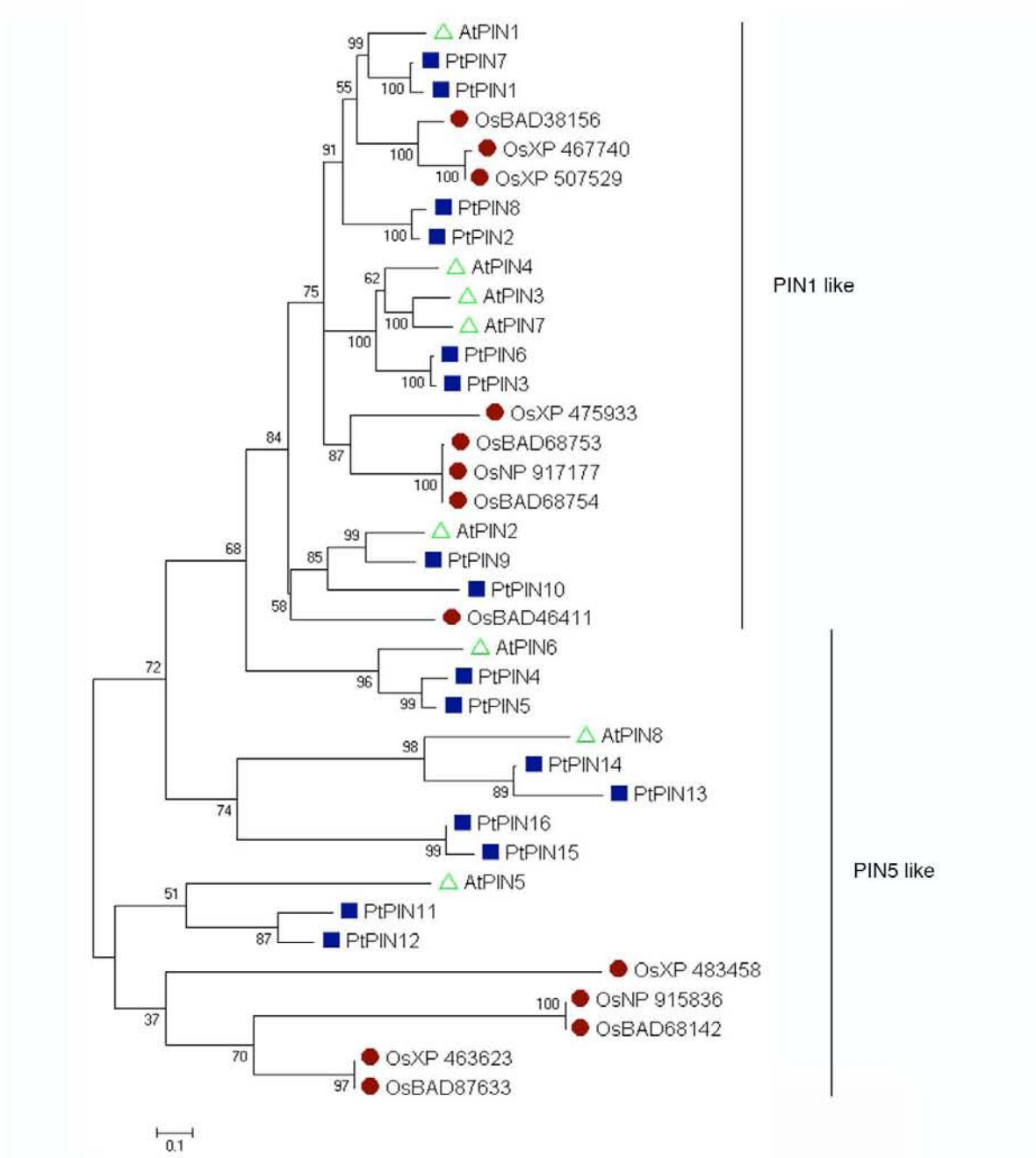

Figure S4.Neighbor-Joining cladogram established with sequences of the hydrophilic central loop of the 16 members of the *Populus* auxin efflux carrier protein family PIN (blue squares) and their eight homologous protein sequences in *Arabidopsis thaliana* (green triangles) as well as thirteen *Oryza sativa* homologs (red circles). Subgroups in the cladogram refer to Mravec et al. 2009.

Arabidopsis sequences from http://www.arabidopsis.org/ AtPIN1 (AT1G73590.1), AtPIN2 (AT5G57090.1), AtPIN3 (AT1G70940.1), AtPIN4 (AT2G01420.1), AtPIN5 (AT5G16530.1), AtPIN6 (AT1G77110.1), AtPIN7 (AT1G23080.1), AtPIN8 (AT5G15100.1), Poplar sequences from http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html PtPIN1 (estExt_fgenesh4_pg.C_LG_XV0366), PtPIN2 (estExt_Genewise1_v1.C_LG_XVI1213), PtPIN3 (gw1.X.6584.1), PtPIN4 (estExt_fgenesh4_pm.C_LG_V0399), PtPIN5 (fgenesh4_pm.C_LG_II000334), PtPIN6 (fgenesh4_pm.C_LG_VIII000556), PtPIN7 (estExt_Genewise1_v1.C_LG_XII1068), PtPIN8 (eugene3.00060333), PtPIN9 (fgenesh4_pm.C_LG_XVIII000434), PtPIN10 (fgenesh4_pm.C_LG_I000524), PtPIN11 (estExt_fgenesh4_pg.C_870067), PtPIN12 (fgenesh4_pg.C_LG_XIX000547), PtPIN13 (fgenesh4_pg.C_LG_IV001142), PtPIN14 (gw1.XVII.929.1), PtPIN15 (fgenesh4_pg.C_LG_XIV000875), PtPIN16 (gw1.5147.2.1).
Oryza Sativa sequences given as NCBI accession numbers were identified by Mravec et al., 2009. Hydrophilic central loops of all sequences were extracted after prediction using http://phobius.sbc.su.se/cgi-bin/predict.pl

Table S1. *Gene expression data of* P. tremula x P.alba *roots after three days of indirect interaction with* L. bicolor *(as separate Excel file).*

Table S2: *List of Arabidopsis LRI genes (Vanneste et al., 2005) and expression data from their poplar homologs in control roots and roots after three days of indirect interaction* L. bicolor *(as separate Excel file).*

Table S3. *Specific primers for auxin-related target genes in* P. tremula x P. alba *used in quantitative Realtime PCR.*

Gene Name[a]	Forward Primer (5')	Reverse Primer (3')	Product size (bp)
PtaIAA28	TGCAGCTCGAAGAGAAACTG	GAACATCCCCAACAAGAATCC	146
PtaIAA33.2	GGAGGGTCGACCGCATCA	AATTGCCCTTCGCTGGCAGTAT	190
PtaIAA3.4	CAGAGGAGTCCAGGTCCAA	GGCACTGTCTCTTTCACCATT	71
PtaIAA3.3	AAGCGATGAGCCAGAGAAAC	TAGAGCTGCCCTTGGACCTA	98
PtaIAA19.3	TTGAACTCGGGAGAACTTGACT	TGATGGTGAATCGTCTGTTCTT	106
PtaIAA34	ATTGTGACGAGGAGGCAGAG	AGCCTTAACCCAGATGCAGA	178
PtaGH3-8	GCATGTCAAATACAGCGACAG	CCTTACTAGAATCGCCGTTGAG	97
PtaGH3-7	GTCCAGTGCAACGGTGTTAG	GTAGAAGCCGGTCACCATGAG	168
PtaGH3-2	CTGCTGGGATCAGTCAGGAT	CGTTTGTAACTCGGCCTCAT	236
PtaGH3-1	GCCCCAACGAGACAATTCTA	CCAGTTGAGTTGAAGGAATCG	152
PtaPIN12	GTGGCACTGATGTTGGGGTATGG	GGCATCGGCGCCAATAAATAGGTA	174
PtaPIN2	GTTAATGGCAAGAATGCGAGTCCAAG	CAAGCTTAGAAAGCACTGGACCATC	397
PtaPIN4	CTGGTTGCGTATGGATCAGTGAAAT	AGAGCAGAACAAGGGCTAAGACTTTCG	196
PtaPIN9	CAGGCTGATGCTGAGATTGGAGA	GTTTCGGACTTGCGGCCTTGCTA	256
PtaAUX3	GTCCAATCAGAAACAAGGAGAGG	TTAAGCCTAAAACCGGATTCATC	105
PtaAUX6	AGGTTCTTGATGGGCTCCTT	CCCCGAAGATGTAAGTCCAA	165
PtaUbiquitin	GCAGGGAAACAGTGAGGAAGG	TGGACTCACGAGGACAG	152
PtaPutative Protein	GCTGCACTTGCATCAAAAGA	GCAACTTGGCATGACTCTCA	119

[a] Gene name referring to the JGI *Populus* genome v1.1 (*PtaGH3, PtaPIN, PtaAUX*) or to Kalluri et al. (2007) (*PtaIAA*)

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Chapter III

Defence-related signalling in the early interaction between the ectomycorrhizal fungus *L. bicolor* and roots of poplar and Arabidopsis: new perspectives on fungus-induced lateral root stimulation

- Article in preparation -

Defence-related signalling in the early interaction between the ectomycorrhizal fungus *L. bicolor* and roots of poplar and Arabidopsis: new perspectives on fungus-induced lateral root stimulation

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Abstract

LR stimulation during early signal exchange between roots and ectomycorrhizal (ECM) fungi, has recently been shown to be achieved by modulation of auxin gradients through altered polar auxin transport (PAT) and through activation of auxin signalling pathways in the root. However, it remains unclear how the presence of the fungus impacts auxin pathways in the root. Here, we show evidences for a scenario challenging the commonly accepted effect of auxin or ethylene secreted by the fungus on LR stimulation. Indeed, LR induction in *A. thaliana* either through exogenously applied auxin or in the presence of *L. bicolor* followed different pattern concerning time course and extent. Application of auxin concentrations, 50 times higher than those assumed to be secreted by the fungus together with the fungus did not cover nor interfere with fungal LR stimulation. Volatiles compounds released by the fungus completely mimicked LR induction observed upon growth conditions that permitted only exchange of volatile but not soluble molecules. Interestingly, Arabidopsis ethylene insensitive mutant *ein2-1* still showed LR stimulation when exposed to fungal volatiles. In order to identify signalling pathways, that are activated during root/fungus contact and that might interact with root auxin pathways, we combined molecular analysis, drug feeding experiments and microarray data analysis of poplar roots during contact with *L. bicolor*. Our data showed that the presence of the fungus induced stress-related responses in the plant. The redox balance (GSH:GSSG) in the roots indicated oxidative stress and microarray data gave hints on activation of the stress-related jasmonic acid and ethylene pathways in roots. Lastly, we showed that inhibition of brassinosteroid biosynthesis, which is also involved in stress responses, negatively impacted auxin accumulation and LR stimulation. These results suggest that stress-signalling cascades upstream of auxin pathways may be the trigger of fungus induced LR stimulation in plants during ECM .

Introduction

Interactions of tree roots with symbiotic, ectomycorrhizal soil fungi lead to profound modification of root development, even before the formation of a functional (nutrient exchanging) symbiosis. Among the first responses of the plant to the presence of the fungus is the stimulation of lateral root (LR) development (Smith and Read, 2008). From extensive studies on Arabidopsis, we know that LR development is mediated by the phytohormone auxin (indol-3-acetic acid (IAA)). Polar auxin transport (PAT) through auxin influx carriers (AUX/LAX) and auxin efflux carriers (PIN) but also localized auxin biosynthesis, conjugation and degradation through various enzymes leads to the formation of discrete auxin maxima within root tissues (Petersson et al., 2009; Vanneste and Friml, 2009). Where auxin accumulates, it binds to its receptors and thereafter activats the transcription transcription of auxin responsive genes (Badescu and Napier, 2006). Those latter elicit responses

such as cell division and differentiation, thereby tuning meristem maintenance, root growth and lateral root initiation. Analysing the plant/ECM fungus interaction, we recently investigated (Felten et al. (in press) at a molecular level whether modifications in the expression of key players within the auxin pathway were detectable and could be related to stimulation of LR development. This study was carried out in parallel on both *Arabidopsis thaliana* and *Populus tremula x Populus alba* roots during early signal exchange (without colonization) with the ectomycorrhizal basidiomycete *Laccaria bicolor*. Our previous results had already shown that signals released by the fungus positively impacted auxin responses at the root apex and stimulated transcription of PAT- (*PtaPIN9*) and IAA signalling-related genes (*PtaIAA19.3*). The inhibition of these target genes correlated well with the absence of LR stimulation, however it remains unclear how a fungus not even in physical contact with the root interferes with auxin pathways and stimulates LR development.

Although several studies have stressed the importance of auxin and ethylene produced by ECM fungi as the major regulators of LR induction during plant/fungus interaction (Tranvan et al., 2000; Splivallo et al., 2009), there are however some reports putting their role into question (Karabaghli-Degron et al., 1998). For instance, Karabaghli-Degron and co-workers (1998) showed that the ECM fungus *L. bicolor* induced LRs when in contact with Norway spruce roots, even though it secreted only 10nM IAA. They could mimic LR induction, only with IAA concentrations as high as 100µM in order to reach LR stimulation comparable to the presence of the fungus. This contested the fact that fungal IAA would be sufficient to provoke LR stimulation in the plant. Unfortunately, none of these studies addressed questions about how fungal auxin penetrates host roots, nor could a fungus genetically or chemically impaired in auxin or ethylene synthesis be used to elucidate their impact on LR development

Another possibility that has never been addressed would be that LR induction occurs by a rather broad, unspecific mechanism due to a stress reaction activated in the plant by the presence of the fungus. The activation of stress/defence-responses in plant roots upon interaction with ECM fungi has been demonstrated at a transcriptional level (Duplessis et al., 2005; Le Quere et al., 2005; Sebastiana et al., 2009) as well as the production of reactive oxygen species (ROS) (Salzer et al., 1997; Gafur et al., 2004; Baptista et al., 2007). It has been proposed that this stress response may be similar to the hypersensitivity response (HR) observed during plant/pathogen interactions (Salzer et al., 1997). In contrast to the latter, during ECM symbiotic interactions the HR response would be rapidly and actively repressed to permit the interaction of both partners and symbiosis formation (Salzer et al., 1997; Tagu et al., 2003). Plant internal messengers that get rapidly induced and mediate defence response are for instance ET, jasmonic acid (further called jasmonates or JAs) and ROS (Pozo et al., 2005; Fujita et al., 2006; Torres et al., 2006; van Loon et al., 2006). Brassinosteroids (BR) also have been suggested to be involved in stress-reactions and disease resistance in tobacco and rice (Müssig et al., 2000; Nakashita et al., 2003). Studies, mainly in

Arabidopsis, have revealed cross-points, where these signals converge into auxin pathways (Nakamura et al., 2003; Benkova and Hejatko, 2009; Fukaki and Tasaka, 2009; Sun et al., 2009), impacting PAT, IAA homeostasis (biosynthesis or catabolism) and IAA signalling and ultimately, interfering with root development. Cross-talk possibilities of IAA and ET are numerous (Stepanova et al., 2007). So ET has been reported to influence PAT in the root via AtPIN1, 2, 4 and AUX1, as well as auxin signalling (Ruzicka et al., 2007) and also activates IAA biosynthesis by activating a tryptophan amino transferase *TAA1* (Ivanchenko et al., 2008; Stepanova et al., 2008). Jasmonates can as well influence PAT in roots by negatively interfering with protein levels of auxin efflux carriers AtPIN1 and AtPIN2 (Sun et al., 2009). The latter study has also revealed that jasmonates stimulate anthranilate synthase *ASA1* and leads to an increased auxin response as visualized by the *pDR5:GUS* reporter. Lastly, there are also connections between BRs and the control of PAT (Li et al., 2005) as well as auxin signalling (Nakamura et al., 2003). These inter-hormonal cross-talks open a vast spectrum of possibilities of how a stress-response regulated by ET, JA, BRs and ROS might impact root development through auxin pathways.

In the present study we aimed on clarifying whether fungal auxin could really be account for LR stimulation during root/ECM fungus crosstalk and if not, proposing perspectives for new pathways. We conducted our study based on the interaction of *L. bicolor* with roots of *A. thaliana* or *P. tremula x P. alba*. Both have been shown before to respond with LR stimulation in the presence of the fungus (Felten et al. (in press). We first assessed whether fungus-induced LR stimulation during Arabidopsis/*L. bicolor* interaction was due to IAA released by the fungus. As this was not the case, we aimed next on uncovering whether other pathways, such as defence-responses, were activated during root/ECM fungus interaction and how these may be connected to auxin triggered LR induction. In this objective, we found that (i) a change in the redox balance in roots (GSSG:GSH) indicated the presence of oxidative stress, (ii) biosynthesis of the stress-related phytohormone brassinosteroids was required for LR induction and (iii) genes coding enzymes in ET and JA biosynthesis, two other stress-related phytohormones, were upregulated in roots during interaction with the fungus. Lastly we tested whether volatiles of *L. bicolor* may account for LR induction in Arabidopsis plants. Those may be containing as well ethylene and jasmonates that would trigger stress-responses and LR development in plants. Together our data give hints on the activation of stress-related pathways overlapping with LR stimulation during ECM fungus/root crosstalk. They furthermore point out cross-talks between stress-responses and auxin pathways and propose targets for further detailed investigation of the molecular mechanism of LR induction during fungus/root interaction.

Material and Methods

Plant and fungal material

Experiments were performed with *Arabidopsis thaliana* (L.) Heynh. ectoype Columbia (Col-0), *A. thaliana pDR5:GUS* (Ulmasov et al., 1997), *A. thaliana ein2-1* (Guzman and Ecker, 1990) and the hybrid *Populus tremula x Populus alba* (clone INRA 717-1-B4). Seeds of Arabidopsis plants were surface-sterilized and sown on solid Arabidopsis medium (2.3 g L^{-1} MS salt, 1% (w/v) sucrose, 0.1% (w/v) MES sodium salt, 1.3% (w/v) agar-agar, pH 6.0 adjusted with KOH). After stratification for two days at 4°C, seeds were germinated under a long-day photoperiod (16 h light, 8 h darkness) at 21°C. Poplar plants were micro-propagated *in vitro* and grown on half MS medium (Murashige and Skoog, 1962) in glass culture tubes under a 16 h photoperiod at 24°C in a growth chamber as described in Felten et al. (in press). The dikaryotic vegetative mycelium of the ECM fungus *Laccaria bicolor* strain S238N (Maire P.D. Orton) was maintained at 25°C on modified Pachlewski medium P5 (Deveau et al., 2007).

Exposition of *A. thaliana* seedlings to *L. bicolor* volatiles

For all experiments *Arabidopsis thaliana* seedlings were germinated on the above described Arabidopsis medium and transferred to the respective plates for co-culturing at 5 dag. *L. bicolor* mycelium was pre-grown on sugar reduced Pachlewski medium (P20) on cellophane membranes (Felten et al., in press). For exposition of seedlings to volatiles 9cm round Petri dishes were used either with a two or three-compartment structure or non-compartmented squared 12x12cm Petri dishes. In compartmented plates one compartment was filled with Arabidopsis medium, a second with sugar reduced Pachlewski medium. For volatile oxidation in the three-compartmented plate, the third compartment was filled with sterilized cotton, which, depending on the conditions was or not rolled in $KMnO_4$ powder beforehand. $KMnO_4$ was used because it is a very strong oxidizing reagent. To each of the Arabidopsis medium containing compartments, five Arabidopsis seedlings were transferred, to the P20 medium compartments a piece of cellophane membrane (control conditions) or three (2-compartmented plate) or two (3-compartmented plate) 10 days old fungal colonies on cellophane membrane were added. Petri dishes were closed with Nescofilm™. Squared plates without separated compartments were filled with Arabidopsis medium and after solidification, 1cm Agar band was cut in the middle of the plate and discarded. Five seedlings of each, wildtype and *ein2-1,* were transferred to each plate and, on the second half of the plate, six fungal colonies on cellophane membrane were applied. Control plants contained cellophane membranes without mycelium. Plates were closed with Nescofilm™ and for certain plates wholes were cut into the Nescofilm™ to reduce volatile accumulation in the plate. All plates were cultured vertically at 21 or 24°C (for a given experiment, experimental conditions were the same for all samples) under a 16h photoperiod. LR development was measured every two days on 50 seedlings per conditions in the

two-compartmented plate experiment and on 13 to 20 seedlings per condition in the three-compartmented plate experiment. In squared plates LR development was recorded after six days of exposition, on five seedlings per condition and line (wt, *ein2-1*).

Auxin and brassinazole treatments during co-culturing

IAA was dissolved in drops of ethanol and the solution was completed with ultrapure water to a final IAA concentration of 10mM. Brassinazole was dissolved in DMSO to a final concentration of 1mM. Stock solutions were sterile filtered. IAA or brassinazole were incorporated to the desired concentrations into Arabidopsis medium (described above) after autoclaving. At five dag, rooted Arabidopsis seedlings were transferred onto plates (10-15 seedlings/plate) supplemented with either IAA or brassinazole or without any hormones or inhibitors (controls). A cellophane membrane with or without (for controls) 10 days old *L. bicolor* mycelium was laid (upside-up for membranes with mycelium) on the roots of the seedlings.

Lateral root quantification

For lateral root quantification, Petri dishes were observed every 24 hrs using a Discovery V.8 stereo microscope (ZEISS, Germany) and emerged LRs were counted. Images of plants were taken directly with a camera (Axiocam MRc, ZEISS, Germany) on the stereo microscope.

GSH and GSSH quantification in poplar roots

HCl soluble low-molecular weight thiols (GSH, GSSG) were determined as reduced monobromobimane derivatives on a reversed-phase HPLC by fluorescence detection. For each of three independent biological replicates per conditions, six root systems were pooled, frozen in liquid nitrogen and disrupted using a TissueLyser (Qiagen). Per sample, 100mg of frozen tissue powder were added to 50mg polyvinylpyrrolidone (PVP) in 750µL 0.1N HCl, vortexed and kept on ice. For standards 0, 5, 10, 15, 20 and 30µl of 1mM GSH were treated similarly as tissue samples and thus added to PVP in HCl. Samples and standards were centrifuged for 30min at 14,000rpm. Of each sample or standard, 120µL of the supernatant were added to 180µL 2-(N-Cyclohexylamino)ethanesulphonic acid (CHES-buffer, 200mM, pH 9.3). Mixtures for GSH standard and tissue samples were prepared twice, in order to analyze total GSH plus GSSG levels as well as GSSG levels only. For the latter, GSH was blocked by adding 30µL of 5mg/mL (w/v) N-ethylmaleimide (NEM) followed by 10min incubation at RT. Then, 30µL of 15mM dithiothreitol (DTT) stock solution was added to all samples and the reduction-reaction was carried out at room temperature for 1h. For conjugation, 20µL of 30mM monobromobimane derivative stock solution in acetonitril were added to each sample followed by incubation for 5 min in the dark. Samples were completed to 600µL with 10% (v/v) acetic acid to stabilize derivatives. After spin down of eventual solid residues, samples were subjected to HPLC analysis (Shimadzu Corp, Kyoto, Japan) according

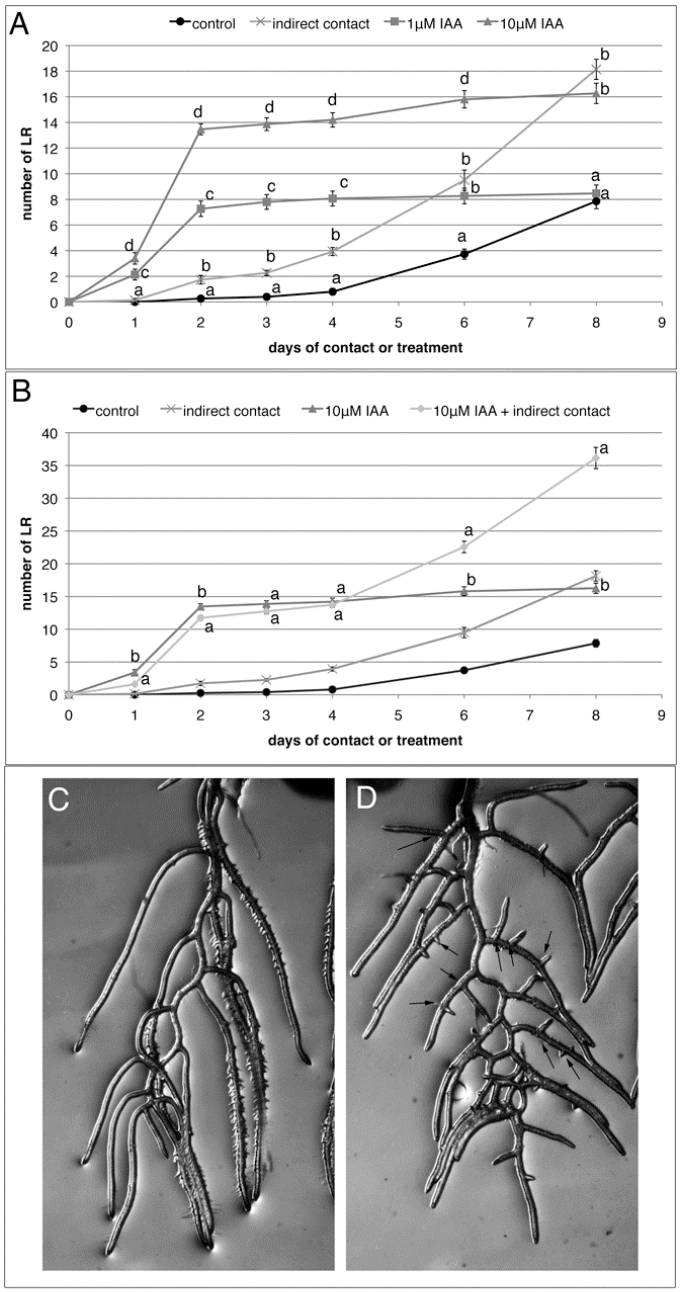

Fig. 1 Comparison of LR stimulation in *A. thaliana* in contact with *L. bicolor* and exogenous IAA treatment. **A** LR development in controls (covered with a cellophane membrane), indirect contacts with the fungus and 1 or 10µM IAA treatment (covered by a cellophane membrane) of 5dag *A. thaliana* seedlings. IAA rapidly stimulated LR development but let after two days to a plateau whereas LR stimulation through *L. bicolor* is slower but persists over the first ten days. **B** LR development in a combined indirect contact plus 10µM IAA treatment. During the first four days LR development followed a tendency similar to IAA treatment alone (rapid increase until day two followed by a plateau). After four days LR development increased linear, paralleling the slope of LR induction in indirect contacts without IAA. Different letters indicate significant difference (Student T-Test, $p<0.05$) between the respective conditions at each time-point (A,B). For each condition 15 to 25 independent seedlings were observed. **C** LR development after 8 days of 10µM IAA treatment. **D** LR development after 8 days indirect contact in the presence of 10µM IAA. Note the high number of secondary degree LRs in D (arrows) that are absent in C.

to (Herschbach et al., 2002). The amount of GSH measured after reaction with NEM referred as the GSSG content. After subtraction from the total GSH pool measured after reduction with DTT in the samples without NEM, the difference corresponded to the amount of GSH present in the sample.

GUS staining

GUS staining for detection of β-Glucuronidase activity in Arabidopsis *pDR5:GUS* seedlings after 10 days of indirect interaction with *L. bicolor* was performed according to (Scarpella et al., 2004) during 90 min at 37°C. Seedlings were mounted in 50% glycerol and observed using a Zeiss Axiovert 200M MOT (Carl Zeiss MicroImaging, Germany).

Analysis of microarray data

The generation of the NimbleGen whole genome-microarray data from poplar roots at three days of indirect interaction (colonization prohibited, signal exchange allowed) with *L. bicolor* under *in vitro* conditions is described in Felten et al. (in preparation). For identification of differentially expressed genes related to the mentioned stress induced hormone pathways, *A. thaliana* protein sequences of key actors in these pathways, obtained from published literature, were blasted (tBLASTN) against transcripts of *Populus trichocarpa* on http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html. Neighbor-joining cladograms (pair-wise deletion, p-distance, 5000 bootstrap repetitions) were constructed after alignment in MEGA. For large gene-families, in which only specific members had been identified to be involved in the given signalling pathways (e.g. AtWRKY70, MYC2), poplar protein sequences that were recovered by tBLASTN with Arabidopsis proteins were counter-blasted against *Arabidopsis thaliana* proteins on http://www.arabidopsis.org/Blast/index.jsp to insure highest homology to the protein of interest and not to other members of the respective gene families. For analysis of differentially expressed genes in the 212 member EREBP/AP2 domain family in poplar, targets in poplar were obtained from the Database of Poplar Transcription Factors http://dptf.cbi.pku.edu.cn/.

Results

Exogenous applied auxin and ECM fungal signals have additive effects on LR development

The effect of exogenous IAA treatment (1 and 10µM) on LR stimulation in 5 dag Arabidopsis was compared to the LR-stimulatory effect of *L. bicolor*. Both concentrations of IAA let to a rapid induction of LRs already detectable after 1 day (Fig. 1A). At this time-point no emerged LR was visible on control plants whereas IAA treated seedlings had developed, respectively 2.1 and 3.4 LRs per plant on 1 and 10µM IAA. The rapid increase of LRs in the presence of exogenous IAA lasted up to two days before reaching a plateau. At this time point, plants had formed an average of 7.3 and 13.9 LRs respectively compared to 0.3 LRs in control plants. Unlike IAA treatments, LR induction

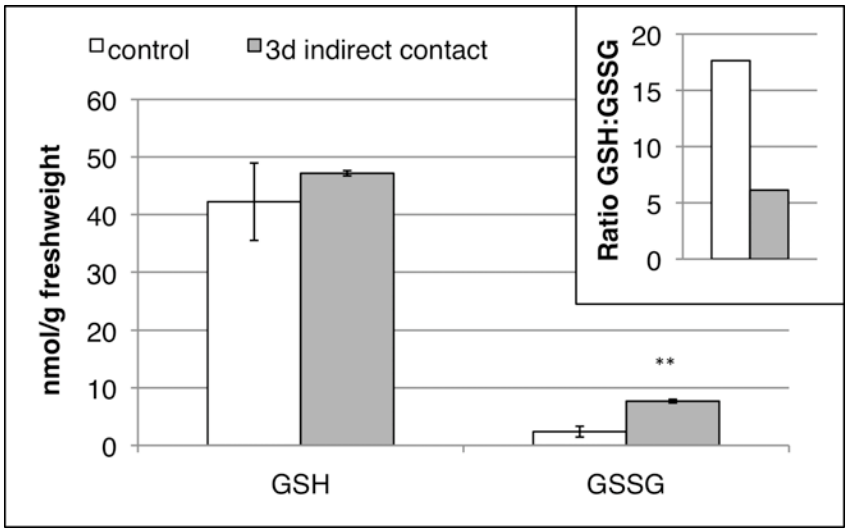

Fig. 2 Variation of thiols in poplar roots at three days of indirect interaction with *L. bicolor*. GSH levels were not significantly altered. GSSG levels increased during contact. This changed the GSH:GSSG ratio from 17 in control roots to 6 during contact. ** Student T-Test, $p<0.01$. Analyzes were realized on three biological replicates (each out of six pooled root systems) for each condition.

by *L. bicolor* followed a different pattern. The LR number increased more slowly but continuously, with the first significant difference being observable at two days (1.7 LRs compared to 0.3 in control conditions). At six days the LR number on plants in contact with the fungus had reached similar values as plants treated with 1µM IAA (9.5 and 8.3 respectively) and at eight days the number of LRs in the fungal treatment exceeded LRs stimulated by 10µM exogenous IAA (18.2 compared to 16.3, (7.9 in controls)). Exogenous IAA treatments caused rapid (after one day) and enduring arrest of primary root growth in Arabidopsis, whereas the sole application of the fungus did not interfere with root elongation up to eight days (Supplemental Fig. S1).

L. bicolor had been suggested to secrete IAA, but the amount is rather low (about 10nM in four week old liquid *L. bicolor* cultures) (Karabaghli-Degron et al., 1998). We tested whether an addition of 10µM exogenous IAA in addition to the fungus, would result in a different time course of LR induction than observed with exogenous IAA or the fungus alone. Interestingly, previously observed effects of both exogenous IAA and fungus on LR development were additive (Fig. 1B). In a first phase, from day zero to day four, LR increase followed the pattern of the sole 10µM IAA treatment. From day four, the LR number continued to increase but did not reach a plateau and thereby paralleled with the slope observed in case of fungal treatment. At six days, significantly more LRs had developed in combined IAA plus fungus treatment compared to the IAA treatment alone (22.6 compared to 15.8). At eight days in the combined treatment 36.14 LRs had formed compared to respectively 16.3 and 18.1 LRs with 10µM IAA or fungus alone. LRs developing in the combined IAA plus fungus treatment emerged as secondary order LRs from those which developed during the first four days under the influence of the exogenous IAA treatment (Fig. 1C and D). Together these data show that LR stimulation by *L. bicolor* was neither mimicked nor masked by an IAA treatment. For these reasons we suggest that fungal IAA is not (or at least not the only) inducer of LR development during plant/fungus contact.

Modification of the redox balance in poplar roots during contact with *L. bicolor*

We analyzed whether a stress response occurred in poplar roots upon contact with *L. bicolor,* by assessing changes in the redox balance. The amount of reduced (GSH) and oxidized (GSSG) was quantified based on a HPLC technique in three biological replicates, each out of six pooled root systems, respectively for control roots without fungus and roots at three days of indirect interaction with *L. bicolor*. Levels and ratios of the reduced and oxidized forms of glutathione can be used as an indicator of a tissue's overall redox status (Schafer and Buettner, 2001). We observed a 3.2 fold increase in GSSG amounts but none in the GSH levels in roots upon contact with the fungus (Fig. 2). This led to a decrease of the GSH:GSSG ratio, from 17:1 in control roots to 6:1 in roots in contact with *L. bicolor*. The decrease of the GSH:GSSG ratio indicates the presence of oxidative stress in the root when in contact with the fungus.

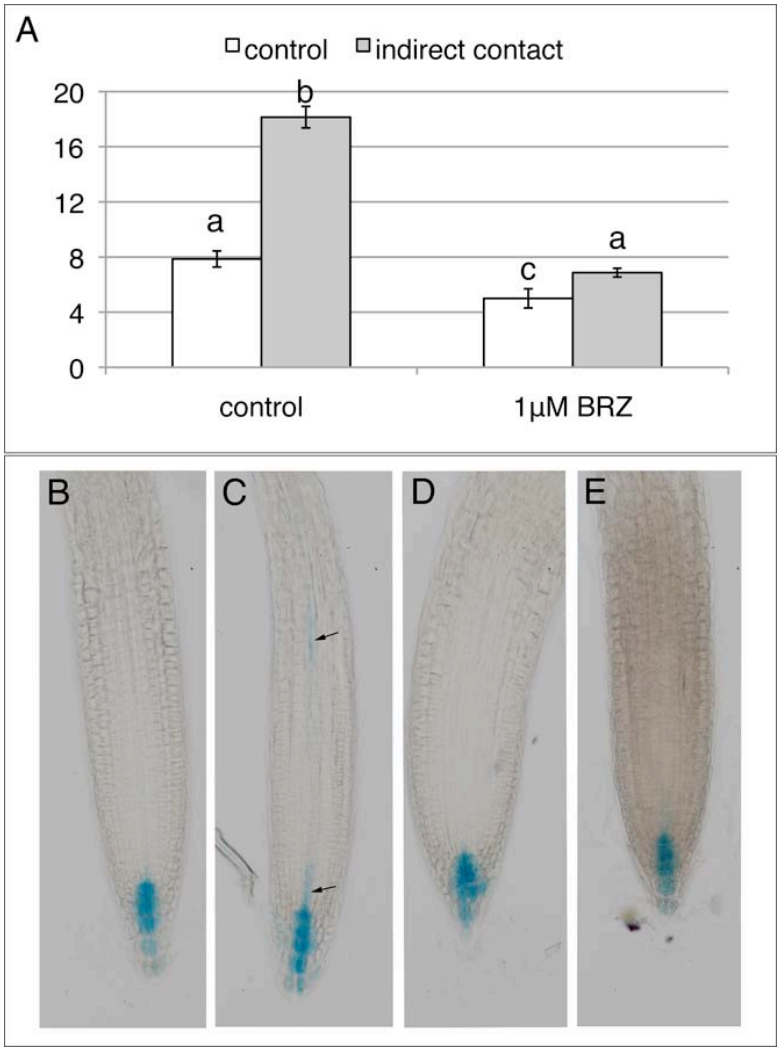

Fig. 3 Effect of brassinosteroid synthesis inhibitor brassinazole (BRZ) on LR stimulation and primary root auxin gradient in *A. thaliana* after 8 days of indirect interaction with *L. bicolor*. **A** The presence of 1µM BRZ lowered LR stimulation by *L. bicolor*. Different letters indicate significant difference (Student T-Test, $p<0.05$). **B-E** *pDR5:GUS* signal in primary roots in control conditions. **C** after 8 days of indirect interaction with *L. bicolor*. **D** *pDR5:GUS* signal in primary roots in the presence of 1µM BRZ and **E** in the presence of 1µM BRZ during indirect contact with *L. bicolor*. Arrows indicate GUS signal appearing in the basal meristem upon contact with the fungus (C).

Importance of brassinosteroids during *L. bicolor* induced LR development in Arabidopsis

Brassinosteroids have been reported to be involved in redox stress, as they induce peroxide accumulation (Xia et al., 2009). The change in roots towards a more oxidative milieu, most probably caused by increased peroxide levels, could involve brassinosteroids in the early plant/fungus interaction. To elucidate this point, we explored whether the brassinosteroid biosynthesis inhibitor brassinazole (BRZ) (Asami et al., 2000) would interfere with fungus induced LR stimulation and with fungal-induced modification of auxin responses (Felten et al., in press) at the primary root apex of *A. thaliana* plants. Blocking brassinosteroid biosynthesis with 1µM BRZ reduced LR stimulation by the fungus. After eight days of co-culturing without the inhibitor, LR number in controls was stimulated by a factor of 2.3, whereas in the presence of BRZ, this stimulation was only of 1.3 times compared to plants growing in presence of the inhibitor but without fungus (Fig. 3A). Using auxin marker *pDR5:GUS* plants (Ulmasov et al., 1997; Ottenschlager et al., 2003) we tested whether BRZ would interfere with the characteristic auxin response in the stele at the basal meristem in the root upon contact (Fig. 3B, C and Felten et al., in press). In accordance with results obtained previously with another drug that also abolished fungal LR stimulation such as NPA (Felten et al., in press), BRZ inhibited the appearance of the auxin response in the basal meristem. These results imply that brassinosteroids are required during the early interaction of roots with *L. bicolor*.

Microarray analyses reveal activation of various signalling pathways during contact

Microarray data originating from the early signal exchange (three days indirect contact) between the mycorrhizal host-plant *P. tremula x P. alba* and *L. bicolor* (Felten et al., in press) was screened for the activation of additional stress-responsive targets such as genes involved in ET and JA pathways, which may link stress responses and LR stimulation. The time point when material (entire root systems) was harvested for microarrays referred to the time point of fungal signal-perception and LR initiation in poplar roots during the early signal exchange with *L. bicolor*.

Ethylene biosynthesis

ET is derived from the amino acid methionine, after conversion to *S*-adenosylmethionine. The latter is the substrate for ACC synthase (ACS) that generates 1-aminocyclopropane-1-carboxylic acid (ACC) that is then converted in a last step to ET by ACC oxidase (ACO) (Argueso et al., 2007). There are 12 genes annotated as ACS in Arabidopsis and 8 in poplar (phylogenetic tree in Fig. S2). Within the poplar *ACS* family, *PtxaACS2* was the only member being upregulated, by 2.8 times, during root/fungus contact (Table I and Supplemental table S1). *PtxaACS2* is homolog to *AtACS1*, *AtACS2* and *AtACS3*. Concerning ACOs in poplar, eight proteins were annotated in the JGI database and they show homology to six sequences in Arabidopsis, namely three annotated AtACOs, two putative ACOs and one Oxoglutarat synthase (Fig. S3). Two of the eight *PtaACOs* were upregulated

in roots during contact with *L. bicolor*: *PtxaACO2* by 3 fold and *PtxaACO5* by 3.7 fold. *PtxaACO2* is closely related to *AtACO2*, 4 and a putative *AtACO* and *PtxaACO5* to *AtACO1*. These results suggest that the early step of poplar/*Laccaria bicolor* interaction is accompanied by a change in ET biosynthesis.

Jasmonic acid biosynthesis

Biosynthesis of jasmonates starts in the phospholipid pathway and involves several enzymes. Key actors in the processing of lipids into jasmonic acid are lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC) and OPDA reductase (OPR) (Wasternack, 2007). Three cycles of β-oxidation follow the action of OPR before jasmonic acid is generated. Except for Arabidopsis AOS, multigene families encode the three other enzymes. In poplar, 21 proteins with homology to six Arabidopsis LOX proteins were identified (Fig. S4), eight proteins homolog to one AtAOS (Fig. S5), three poplar homologs to four AtAOCs (Fig. S6), and twelve sequences had similarity to six AtOPRs (Fig. S7). Microarray data showed upregulation of one poplar homolog within each of the AOS, AOC and OPR family, but no differential expression of poplar LOX homologs. Among the eigh AOS homologs *AtAOS* (*eugene3.00090572*, annotated as *PtCYP74C8*) showed a 5.8 fold, while three close members of *AtAOCs* (*gw1.123.188.1*) displayed a 2.2 fold increase. Finally, we found that one of three close homolog of *AtOPR3* (*grail3.0145000602*) was increased by 2.5 fold (Tables I and S1), suggesting putative upregulated jasmonic acid biosynthesis in poplar in the presence of the fungus.

Ethylene and JA responsive genes

In Arabidopsis, AtMYC2 and WRKY70 as well as members of the Ethylene response factors (ERF) (AtERF1, AtORA47, AtORA37, AtORA59) are among transcription factors that act downstream of JAs during stress responses (Memelink et al., 2001; Lorenzo et al., 2004). They activate for example defensins of the AtPDF family that are involved in disease resistance (Penninckx et al., 1996; Dombrecht et al., 2007). ERFs are also downstream targets of ET signalling. ERFs are particularly interesting, as some of them, such as AtERF1 and AtORA59 are activated by both, ET and JA, and constitute thus a knot between those pathways (Lorenzo et al., 2003; Pre et al., 2008). After root/fungus contact, we detected 2.3 times accumulation of *eugene3.00131043* (closest homolog of JA activated *AtWRKY70)* (Table I) but no differential expression of *AtMYC2* homologs. One of the poplar homologs of Arabidopsis defensin PDF2.5 (Fig. S8) also was two times induced (Table I). Regarding the ERF family, 147 members have been identified in Arabidopsis (Nakano et al., 2006). In the Database of Poplar Transcription Factors (http://dptf.cbi.pku.edu.cn/), 212 members of the Ethylene Response Element Binding Protein (EREBP)/APETALA (AP2) domain transcription factors have been predicted, which contain all poplar ERFs but also other AP2 domain transcription

Table I Differentially expressed genes related to JA and ET biosynthesis and downstream targets in poplar roots during interaction with *L. bicolor*

Poplar Gene Model[a]	Annotation in *P. trichocapra* (Pti)[b]	Best Hit At[c]	Protein Identity Pti vs At[d]	Annotation in Arabidopsis[e]	expression level control roots[f]	Fold change during contact[g]	p Value[h]
estExt_fgenesh4_pm.C_LG_II0741	PtACS2	At3g61510.1	329/485 (67%)	AtACS1	12	2.84	0.0434
fgenesh4_pg.C_LG_VI000988	PtACO5	At2g19590.1	215/302 (71%)	AtACO1	2359	3.73	0.0000
eugene3.00090572	PtCYP74C8	At5g42650.1	233/472 (49%)	AtAOS	61	5.79	0.0359
eugene3.00002047	PtACO2	At1g05010.1	218/314 (69%)	AtACO4	680	3.06	0.0129
gw1.123.188.1		At3g25780.1	108/144 (75%)	AtAOC3	97	2.22	0.0123
grail3.0145000602		At2g06050.1	275/369 (74%)	AtOPR3	65	2.49	0.0096
eugene3.00131043		At3g56400.1	100/229 (43%)	AtWRKY70	20	2.38	0.0040
estExt_Genewise1_v1.C_LG_XIX0272		At5g63660.1	34/75 (45%)	AtPDF2.5	2137	2.03	0.0010
eugene3.00031462	PtCHS6	At5g13930.1	331/386 (85%)	AtCHS (TT4)	1007	2.07	0.0041
gw1.X.1797.1	PtASA1	At5g05730.1	385/534 (72%)	AtASA1	58	2.32	0.0013
fgenesh4_pg.C_LG_X000601		At1g70560.1	234/352 (66%)	AtTAA1	235	2.29	0.0058

[a]Gene Model from the *JGI Populus* genome v1.1, [b]Annotation available on JGI for the respective gene, [c]Best Hit of pBLAST with poplar proteins agains *A. thaliana* proteins, [d]Protein identity revealed by pBLAST mentioned in [c], [e]Annotation on www.arabidopsis.org for the AtBestHit ([b]), [f]Expression level in control roots after background correction, [g]Expression ratio between roots in contact with *L. bicolor* and control roots, before background correction, [h] p-Value of Student T-Test.

Abbreviations: ACC Synthase (ACS), ACC Oxidase (ACO), Allene Oxidase Synthase (AOS, in Poplar: Cytochrom P450 (CYP)), Allene Oxide Cyclase (AOC), OPDA Reductase (OPR), WRKY Transcription factor, Defensin (PDF), Chalcone Synthase (CHS), Anthranilate Synthase (ASA), Tryptophane Amino Transferase (TAA)

Table II Differentially expressed EREBP/AP2 domain transcription factors in poplar roots during interaction with *L. bicolor*

Poplar Gene Model[a]	Annotation in *P. trichocapra* (Pti)[b]	Best Hit At[c]	Protein Identity Pti vs At[d]	Annotation in Arabidopsis[e]	ERF Subfamily[f]	expression level control roots[g]	Fold change during contact[h]	p Value[i]
fgenesh4_pg.C_LG_III001166		At1g64380.1	64/94 (68%)	putative AP2 dom TF	Ib	1143.00	-2.35	0.0001
fgenesh4_pg.C_LG_III000388	PtDREB51	At1g33760.1	94/174 (54%)	putative AP2 dom TF	IIIa	nd	3.41	0.0234
gw1.XVIII.2541.1		At1g77200.1	77/94 (81%)	TINY	IIIe	99.00	2.39	0.0033
eugene3.00161194	PtDREB4	At2g38340.1	55/124 (44%)	putative AP2 dom TF	Iva	1851.00	-3.26	0.0004
fgenesh4_pg.C_scaffold_44000051	PtDREB29	At1g15360.1	86/158 (54%)	AtSHN1	Va	nd	7.43	0.0003
gw1.XVIII.1599.1		At5g11190.1	110/188 (58%)	AtSHN3	Va	nd	3.06	0.0219
eugene3.00061922	PtDREB26	At5g25190.1	97/195 (49%)	putatave EREBP	Va	nd	2.10	0.0385
fgenesh4_pg.C_LG_III001839	PtERF59	At5g19790.1	114/264 (43%)	related to AtRAP2.11	Vb	77.00	8.64	0.0003
gw1.I.6432.1		At5g19790.1	113/253 (44%)	related to AtRAP2.11	Vb	487.00	6.44	0.0012
gw1.II.2522.1		At4g23750.1	87/172 (50%)	AtCRF2	VI	2680.00	-2.14	0.0037
fgenesh4_pm.C_LG_XIII000479		At4g23750.1	47/68 (69%)	AtCRF2	VI	12089.00	-2.28	0.0002
gw1.XIV.2445.1		At2g47520.1	51/85 (60%)	putative AP2 dom TF	VIIa	796.00	2.10	0.0010
gw1.132.12.1		At5g18560.1	55/56 (98%)	AtPUCHI	VIIIb	167.00	5.33	0.0017
gw1.263.10.1		At5g07580.1	96/198 (48%)	DNA binding TF	IXb	764.00	-2.37	0.0072
gw1.I.8242.1		At5g07580.1	91/190 (47%	DNA binding TF	IXb	1071.00	-2.59	0.0170
gw1.X.5466.1		At3g23230.1	41/58 (70%)	putative ERF	IXc	76.00	2.48	0.0002
gw1.V.1205.1		At3g23240.1	82/137 (59%)	AtERF1	IXc	nd	2.90	0.0135
gw1.I.7266.1	PtRAP21	At1g51190.1	314/484 (64%)	AtPLT2	(ANT-LIKE)	212.00	2.57	0.0030
fgenesh4_pg.C_LG_III001621	PtRAP23	At1g51190.1	271/423 (64%)	AtPLT2	(ANT-LIKE)	477.00	2.37	0.0021
gw1.VIII.931.1		At5g17430.1	163/192 (84%)	AtBBM	(ANT)	1419.00	2.31	0.0490
eugene3.00101225	PtRAV1	At1g25560.1	246/384 (64%)	AtTEM1	(RAV)	1848.00	-2.96	0.0037
fgenesh4_pg.C_LG_I001053	PtRAP15	At1g72570.1	177/254 (69%)	DNA binding TF		nd	3.32	0.0002

[a]Gene Model from the *JGI Populus* genome v1.1, [b]Annotation available on JGI for the respective gene, [c]Best Hit of pBLAST with poplar proteins agains *A. thaliana* proteins, [d]Protein identity revealed by pBLAST mentioned in [c], [e]Annotation on www.arabidopsis.org for the AtBestHit ([b]), [f]ERF subfamily according to (Nakano et al. 2006), [g]Expression level in control roots after background correction, [h]Expression ratio between roots in contact with *L. bicolor* and control roots, before background correction, [i]p-Value of Student T-Test.Abbreviations: Domain (dom), Transcription factor (TF), SHINE (SHN), CYTOKININ RESPONSE FACTOR (CRF), PLETHORA (PLT), BABY BOOM (BBM), TEMPRANILLO (TEM), DROUGHT RESISTANCE ETHYLENE BINDING (DREB)

factors. In our microarray data, 22 out of the 212 EREPB/AP2 domain transcription factors were differentially expressed, including 17 ERFs (Table II). Eleven ERFs were upregulated and six repressed. Nakano and co-workers (2006) have divided Arabidopsis ERFs into eleven subgroups according to their sequence-motifs and suggested functions. The affiliation of the closest Arabidopsis protein to each poplar protein whose gene was differentially expressed is indicated in Table II. Transcript levels of members of subgroup I, III, IV, V, VI, VII, VIII and IX were altered during root/fungus interaction. Subgroup V was the most represented, with five differentially expressed genes among the 17. These genes showed in average the highest induction of all regulated ERFs, with expression ratios reaching 8.6 times overexpression (*PtxaERF59*) compared to controls. A general function for the members of this subgroup has not yet been predicted (Nakano et al., 2006). The second most abundant subgroup is the group IX which counted four differentially expressed genes, from which two were induced (*gw1.X.5466.1, gw1.V.1205.1* (both in subgroup IXc)) and two others were repressed (*gw1.263.10.1, gw1.I.8242.1* (subgroup IXb)). ERFs of subgroup IX have often been mentioned in relation with defensive gene expression and are differentially regulated by ET, JA and salicylic acid (Gu et al., 2000; Onate-Sanchez and Singh, 2002; Lorenzo et al., 2003). Interestingly, we also found a poplar homolog of JA- and ET-responsive *AtERF1 (gw1.V.1205.1)* upregulated 2.9 fold within this group. It is worth noting, that expression of two members of subgroup III (fgenesh4_pg.C_LG_III000388, gw1.XVIII.2541.1) and one of subgroup VIII (gw.1.132.12.1) also was induced. These subgroups are related to growth regulation and organ development and Arabidopsis homologs of the regulated poplar genes influence LR development or root growth (Wilson et al., 1996; Hirota et al., 2007). In this context, we also detected activation of poplar homologs of Aintegumenta-like EREPB/AP2 transcription factors, whose Arabidopsis relatives (PLETHORA and BABY BOOM) are associated to meristematic and embryonic cell development (Boutilier et al., 2002; Aida et al., 2004; Passarinho et al., 2008). No differential expression was detected for homologs of *AtORAs*. Nonetheless, for the closest poplar homolog of *AtORA59* (*gw1.V.1199.1*) no microarray expression data was obtained, because oligos on the array failed our specificity threshold and data was discarded (Felten et al., in press).

Considering that JAs positively regulate biosynthesis of flavonoids through chalcone synthase (Dombrecht et al., 2007), which interfere with auxin transport (Peer et al., 2004; Besseau et al., 2007), we checked whether poplar chalcone synthases were differentially expressed. There are seven *CHS* genes in poplar and several *CHS-like* and pseudogenes (Fig. S9), referring to the one *AtCHS* and three *AtCHS-like* genes. Poplar *CHS6 (eugene3.00031462)* was induced by a factor of two during root/fungus contact, but none of the other *PtxaCHS* were differentially expressed (Table I and S1).

As JA and ET have been reported to act on auxin biosynthesis (Sun et al., 2009) through *AtTAA1* and *AtASA1*, we verified the expression of these genes in roots. We identified a 2.3 times induction of each of two genes: one close homologous (Fig. S10 and S11) of Arabidopsis tryptophan amino transferases

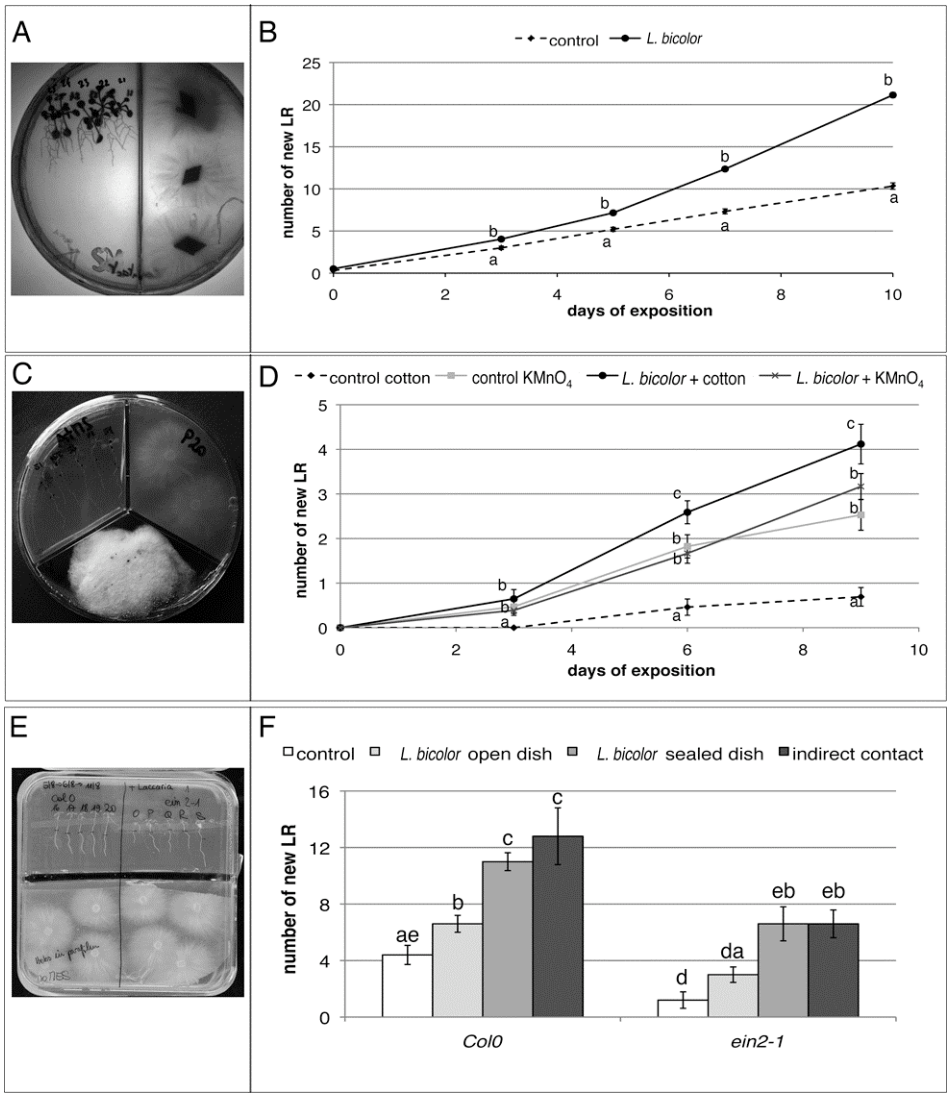

Fig. 4 LR stimulation in *A. thaliana* by volatile molecules released by *L. bicolor* mycelium. **A** Two-compartmented plate with Arabidopsis seedlings (left) and *L. bicolor* mycelium (right). **B** LR development in the presence of volatiles released by *L. bicolor*. Compared to controls, LR development was stimulated from three days of co-culturing with mycelium. Per condition 50 seedlings were analyzed. **C** Three-compartmented plate with Arabidopsis seedlings (left), *L. bicolor* mycelium (right) and sterile cotton (bottom). **D** The presence of $KMnO_4$ as an oxidizing agent stimulated LR development, but a higher stimulation was obtained in the presence of *L. bicolor*. Applying $KMnO_4$ and *L. bicolor* together reduced LR stimulation to levels observed with $KMnO_4$ alone. Per condition 13 to 20 seedlings were observed. **E** Squared plate harbouring Arabidopsis seedlings on the upper half and *L. bicolor* mycelium on lower half. **F** The tighter the plate was closed the more LRs developed in *A. thaliana* wt and *ein2-1* plants. Per condition and line 5 seedlings were analyzed. Different letters indicate significant difference (Student T-Test, $p<0.05$) between the respective conditions at each time-point (B, D, F).

AtTAA1 and *AtTAR1* (fgenesh4_pg.C_LG_X000601) the other homolog of Arabidopsis anthranilate synthase *AtASA1* (gw1.X.1797.1). fgenesh4_pg.C_LG_X000601 and its orthologous gw1.VIII.421.1 seem to be the result of a gene duplication event, but interestingly only *fgenesh4_pg.C_LG_X000601* is expressed in roots and induced upon contact.

Volatile molecules emitted by *L. bicolor* stimulate LR formation

We next asked whether *L. bicolor* could release volatile compound that could induce stress responses as well as LR induction in the plant. *L. bicolor* is known to produce ethylene (Rupp et al., 1989) and other fungi release as well jasmonates derivatives (Miersch et al., 1999a), which are stress hormones *per se* but which also interfere with LR development (Ivanchenko et al., 2008; Sun et al., 2009). We used a compartmented Petri dish system to investigate the effect of fungal volatiles on LR development in Arabidopsis (Fig. 4A). Exposing 5 dag Arabidopsis seedlings in a bi-compartmented plate to *L. bicolor* resulted in a significant LR increase compared to plants in control plates without fungus, three days after the beginning of the experiment. After 10 days the LR number of plants sensing *L. bicolor* volatiles had doubled compared to control plants (21.4 compared to 10.3 in controls) (Fig. 4B).

As both ET and MeJA can be inactivated by oxidization, we investigated whether an oxidizing agent (potassium permanganate ($KMnO_4$)) inside the Petri dish could prohibit LR stimulation by fungal volatiles. We used plates with three different compartments, one containing up to five seedlings, the second harbouring fungal mycelium on a cellophane membrane or a fungus-free cellophane membrane (controls) and a third one, in which sterilized cotton was introduced as a vehicle for potassium permanganate ($KMnO_4$) powder (Figure 4C). Note that plants were in a different environment because of the reduced space in this experiment and absolute LR numbers developing under these conditions should not be compared to results obtained before (Fig. 4B). In addition, in this assembly, fungal volatiles stimulated the development of lateral roots (Fig. 4D) and this induction followed a time course similar to what we described for the two-compartmented plate experiment (Fig. 4B), with a first significant difference from controls at three days of exposition. In control samples without fungus, the presence of potassium permanganate increased the number of LRs, maybe due to the oxidization of inhibitory molecules released either by respiration of the plant or from the medium. Thus at nine days, plants exposed to $KMnO_4$ had a mean of 2.5 LRs while from control roots had only had developed 0.5 LRs in average. The presence of $KMnO_4$ decreased the number of LRs initiated on plants exposed to the fungus to similar values observed with $KMnO_4$ alone. Together this showed that volatiles, which can be inactivated by oxidization, are released by *L. bicolor* and stimulate LR development in the plant.

Whether ethylene was indeed part of the LR inducing fungal molecules was assessed by counting the number of LRs developed by the ethylene insensitive Arabidopsis mutant *ein2-1* when exposed to *L. bicolor* volatiles. In this experiment, plants were arranged on one half of a squared plate (Fig. 4E), and the fungal mycelium was applied on a cellophane membrane on the other half of the plate. In order to inhibit the exchange of soluble molecules between plants and fungus, a narrow band of agar between Arabidopsis seedlings and *L. bicolor* mycelium was removed (Ditengou and Lapeyrie, 2000). To better compare results between wildtype (wt) and *ein2-1* seedlings, 5 seedlings of each line were arranged together in the same plate (Fig. 4E). To test effects of volatiles, plates were either entirely closed with Nescofilm™ or holes were made into the film to reduce volatile accumulation. LRs were quantified after six days of co-culturing. As expected, tighter was the plate closed, stronger appeared the LR stimulation was (Fig. 4F). Thus, whereas control plants without fungus had developed about 4.5 LRs at the end of the experiment, plants exposed to the fungus in the plate with holes in the film had formed six and seedlings in sealed plates up to 11 LRs. A comparison to indirect contact, where the roots were overlaid with the membrane containing the fungus and as well soluble as also volatile molecules could be exchanged did not show any further relevant LR stimulation. Regarding the ethylene insensitive mutant *ein2-1,* plants under control conditions without fungus formed less LRs than wt (1.5 compared to 4.5). In the sealed plate in the presence of the fungus, LR development in *ein2-1* was stimulated (up to 6 LRs). Again, indirect contacts did not further increase the LR stimulation. Thus, just like for wt, *ein2-1* responded to volatile molecules released by *L. bicolor* with stimulated LR formation by a factor of two to three. Taken together, these data suggest that ethylene released by the fungus is not the sole molecule stimulating LR development in plants during the interaction.

Discussion

In a preceding study we had observed that during contact of poplar or Arabidopsis with *L. bicolor*, the auxin gradient in roots is altered and suggested that this leads to LR stimulation (Felten et al., in press). Here we aimed on identifying the mechanisms leading to auxin alteration. A first approach focussed on auxin as a potential fungal LR inducer before we addressed the possible impact of fungal volatile molecules and the induction of a defence/stress-response in the plant during fungal LR stimulation.

Fungal auxin is not crucial for LR induction in plants

Comparison of LR induction in Arabidopsis by applied exogenous auxin or by contact with a fungus showed that LR stimulation followed different pattern. Exogenous auxin caused a rapid transient LR induction, whereas a delayed, persisting LR induction pattern was visible upon root/fungus contact. Such a difference between fungus-induced LR development and LR induction upon exogenous auxin

was already observed for the Norway spruce/*L. bicolor* interaction by (Karabaghli-Degron et al., 1998). According to their results, *L. bicolor* that secreted about 10nM IAA into the medium when grown in liquid cultures. However, to mimic the extent of LR induction observed with the fungus on agar-plates 100 µM IAA was required. In addition, the time course of LR induction by IAA and *L. bicolor* were different, which is in accordance to our data obtained with Arabidopsis roots. Given the low amount of auxin secreted by the *L. bicolor* into the medium, one could expect that adding 10µM of exogenous IAA to released fungal auxins would entirely mask the effect of the fungus, assuming that applied IAA does not interfere with IAA production by the fungus itself. However when exogenous IAA and the fungus were applied together, we observed a two-step LR induction profile, with a first phase following exogenous auxin activity and a second phase paralleling the fungal LR stimulation. This suggests that exogenous auxin and the fungus act in different ways on LR stimulation. Pathways involved do not seem to be interconnected, as a combined application of both did not interfere with the respective, characteristic phase of each process alone. We therefore exclude the possibility that fungal IAA (alone) is responsible for LR induction in the plant and searched for alternative mechanisms.

A stress reaction occurs in roots during interaction with *L. bicolor*

It has been suggested that during plant/fungus interaction upon ECM formation, a stress response is induced (Le Quere et al., 2005; Baptista et al., 2007; Sebastiana et al., 2009). Baptista and co-workers (2007) have shown the occurrence of three peaks of early ROS bursts during colonization of *Castanea sativa* roots by the ECM fungus *Pisolithus tinctorius*. As GSH:GSSG ratio reflects the presence of ROS stress (Schafer and Buettner, 2001), we quantified GSH and GSSG content during poplar/*L. bicolor* interaction. Indeed the redox balance in roots was changed favouring GSSG levels. This decreased the overall redox balance to more oxidized conditions, indicating the presence of ROS. Brassinosteroids have been suggested as stimulators of ROS production, ET and JA biosynthesis in plants (Yi et al., 1999; Müssig et al., 2000; Goda et al., 2004; Xia et al., 2009) and accordingly, are involved in stress responses (Nakashita et al., 2003). Our data show that BR biosynthesis is crucial for fungus-induced LR stimulation and auxin gradient modification. Whether this occurs through adjusting ROS levels in roots needs to be verified. In order to assess whether the presence of the fungus stimulates in addition to BR-dependent also additional phytohormone-related stress-pathways in the roots, we screened poplar roots micro-array data during the early signal exchange (soluble and volatile molecules) in contact with *L. bicolor* for differentially regulated stress-signalling related genes. We gave special consideration to JA and ET, as they are key players associated with stress-responses to biotic stimuli (Fujita et al., 2006; Wasternack, 2007). Our data revealed accumulation of transcripts related to ET biosynthesis (ACS, ACO) and JA biosynthesis (AOC, AOS, OPR). Overexpression of ET biosynthesis genes is thought to cause and thus correlate with higher ET levels in tissues (Argueso et al., 2007). On the contrary, gene expression data of JA biosynthetic genes has to

be interpreted carefully. A critical factor for JA biosynthesis is its substrate availability. In different studies, it had been shown that simple overexpression of JA biosynthetic enzymes was not sufficient to increase JA levels (Laudert et al., 2000; Stenzel et al., 2003). Nonetheless, we observed transcriptional activation of different downstream target genes of JA signalling (WRKY, ETR, ASA, PDF, CHS, see also further below), strengthening the possibility that in our experimental conditions, high levels of JA were present in roots.

Together this data shows that stress-indicative compounds (ROS) or biosynthetic genes of stress-related signals accumulate (ET, JA) or are required (BR) during the early signal exchange between roots of Arabidopsis or poplar and the ECM fungus *L. bicolor*. Their quantification over time of root/fungus interaction will help to clarify their specificity to one or different phases of ECM development.

Stress-response and LR induction can be linked

ROS, BR and JA can in addition to stress responses be related to auxin pathways and thereby with LR development. BRs interact with auxin and influence root PAT (Nakamura et al., 2003; Goda et al., 2004; Nakamura et al., 2004; Li et al., 2005). Interestingly, BRs have been shown to stimulate *AtPIN2* transcription. During fungus-induced LR stimulation in poplar the transcription of *PtaPIN9* – the closest *AtPIN2* poplar PIN – has been shown to be induced and to be crucial for LR stimulation (Felten et al., in press). Moreover, LR stimulation during *L. bicolor/A. thaliana* interaction caused auxin accumulation at the basal meristem, which was inhibited in the Arabidopsis *pin2* mutants as well as by BRZ application (this study and Felten et al., in press). Altogether these data suggest a putative link between BR and the modification of *AtPIN2/PtaPIN9* expression during fungal LR stimulation.

The microarray data had shown that downstream-targets of ET and JA pathways are impacted by the fungus/root interaction. These putative targets can be divided into two groups. First, targets related to stress-pathways and second, those who intervene in root development. As stress-related downstream targets, data suggest poplar homologs of AtWRKY70, subgroup VI and IX ERFs and the defensin AtPDF2.5 (Penninckx et al., 1996; Lorenzo et al., 2003; Lorenzo and Solano, 2005; Nakano et al., 2006). Especially high expression of a poplar homolog of AtERF1 is interesting, as this gene assiociated to stress reactions, is activated by JA and ET (Lorenzo et al., 2003) and has also been mentioned in connection with root nodule symbiosis in *Lotus japonicus* (Asamizu et al., 2008). It is thus possible that ERF1 may have a broad spectrum of putative downstream targets and can connect different pathways. Root development related downstream targets are subgroup III and VIII ERFs as well as EREBP/AP2 domain transcription factors of the Aintegumenta family (Aida et al., 2004; Nakano et al., 2006; Galinha et al., 2007; Hirota et al., 2007). The fact that ET and JA downstream

targets of both, the stress- and developmental groups are affected, may and explain how these phytohormones link stress and developmental processes. Investigation of the role of ERFs subgroup V, which were most prominent within the differentially expressed ERFs and had the highest induction rate will be interesting to extent the knowledge about ERFs and their functions. ET and JA transcription factors mentioned above act downstream of JA and ET. They may also interact and modify other pathways. An important result is for example the induction of a possible JA activated chalcone synthase (*PtaCHS6*) and genes involved in JA and ET-directed auxin biosynthesis (*TAA1, ASA1*). Herein may lay an explanation for the auxin response increase and altered auxin distribution in roots upon contact with the fungus, and suggest auxin signalling to be controlled by stress-hormones ET and JA.

Recently, Sun and co-workers (2009) have elegantly shown how jasmonates affect lateral root development through activation of auxin biosynthesis and PAT (Sun et al., 2009). Hence, application of MeJA can activate in a jasmonates signalling dependent way the expression of several auxin biosynthesis genes, including ASA1 and that this induction is crucial for LR induction by MeJA (Sun et al., 2009). Moreover MeJA stimulated expression of PAT facilitors *PIN1* and *PIN2*, but however resulted in decreased amounts of these proteins (Sun et al., 2009). The authors proposed a model in which stimulation of auxin biosynthesis through ASA1 and inhibition of PAT through an ASA1-independent but MeJA-dependent (and thereby possibly flavonoids dependent) mechanism leads to auxin accumulation within the basal meristem where a higher number of cells are primed for LR initiation. Interestingly, parallels can be drawn between the effect of MeJA and the effect of the fungus on roots. In addition to LR stimulation, four similar observations can be made in both MeJA treatment and contact with the fungus: First, during both exogenous MeJA treatments and fungus/root interaction, transcription of *AtASA1* or *PtaASA1* was activated. Second, MeJA treatments activated *AtPIN2* expression, and similarly contact with the fungus activated the homologous of *AtPIN2*, the poplar *PtPIN9*. Third, both treatments caused an increased auxin response at the root apex, most probably due to auxin accumulation. Finally, another striking parallel is the activation of an auxin response in the basal meristem as monitored with *pDR5:GUS* expression pattern in Arabidopsis plants, either treated with MeJA or in contact with the fungus (Sun et al., 2009).

Volatile molecules released by the fungus may cause stress-responses and LR induction in the plant

We have seen that (i) not fungal auxin is responsible for the LR induction in the plant but that (ii) many stress responses are activated upon plant/fungus contact, which have connections to PAT and LR development. We therefore hypothesized that the fungus might release compounds that trigger auxin redistribution and LR induction through stress-responses. Candidates for those compounds are ethylene and jasmonates. It has already been demonstrated that *L. bicolor* is able to release ethylene

(Rupp et al., 1989). Concerning jasmonates, it has been reported that fungi are able to produce those derivatives but confirmation is needed in the case of *L. bicolor* (Miersch et al., 1999a; Miersch et al., 1999b). As JAs and ET can both occur in form of volatiles (e.g. jasmonic acid methyl esters (JAME) or methyl-jasmonates (MeJA) are volatile JAs), we tested whether an exposition of A. thaliana seedlings to volatiles released by *L. bicolor* may alter LR development. Indeed, our data showed that fungal volatiles induced LRs similarly to what is observed during physical interaction between both partners. These data reinforced again our assumption that fungal auxin cannot be the major player in early LR stimulation, because our experimental conditions did not allow any exchange of soluble molecules. We next monitored LR development during volatile communication in the presence of an oxidizing reagent. This experimental condition nullified fungal LR induction. This seemed in agreement with our expectations, as ethylene could, as part of the LR stimulating fungal molecules, be oxidized and degraded it into CO_2 and water, loosing thus its activity. Unexpectedly, we found that *L. bicolor*'s volatiles were still able to induce LRs in the ethylene insensitive Arabidopsis mutant *ein2-1*. A similar observation has recently been reported by Splivallo and co-workers (2009), when they monitored LR stimulation in Arabidopsis ethylene insensitive plants in response to ethylene releasing truffle fungi. Taken together, these data rule out the possibility for ethylene to act as a fungal key molecule for LR stimulation during the interaction with its plant partner. As mentioned earlier, we had detected activation of JA biosynthetic enzymes in the plant. Interestingly it has been demonstrated that only exogenous JAs stimulate endogenous JA production (Miersch and Wasternack, 2000). Thus, an external source of JA must have been present in our experimental setup. The fungus itself could represent this source of JAs, which could have been then perceived by the plant. Monitoring LR stimulation in a JA insensitive plant and quantifying JAs released by the fungus may enable to confirm this assumption.

Conclusion

Taken together we have identified four different stress-related messengers, ROS, ET, JAs and BRs, whose activation can be related to fungus-induced LR development. As all four messengers are involved as well in stress-reactions and also impact root development via auxin signalling pathways, they may constitute an important knot for stress-induced LR stimulation. This study opens new perspectives for further investigation. As it appears clearly that fungal IAA and ET are unlikely to be the fungal stimulators that induce LRs in the plant, we suggest further investigations towards understanding the role of JAs and BR during fungus/plant interaction.

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Supplemental Data

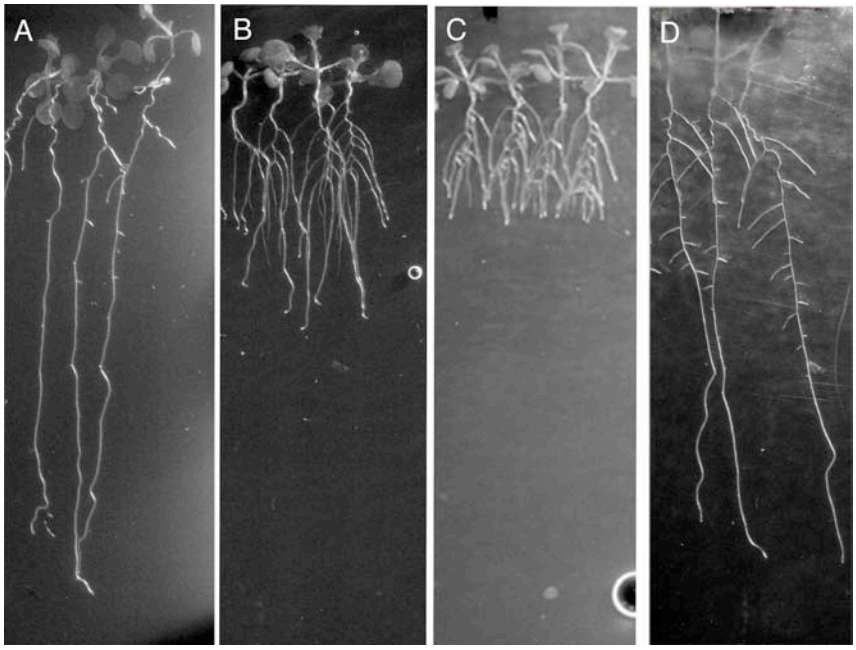

Fig. S1 Primary root length of *A. thaliana* seedlings after eight days under control conditions (A), 1µM IAA (B) and 10µM IAA (C) treatment or contact with *L. bicolor* (D). Note root elongation inhibition with increasing exogenous IAA concentration but not after contact with the fungus.

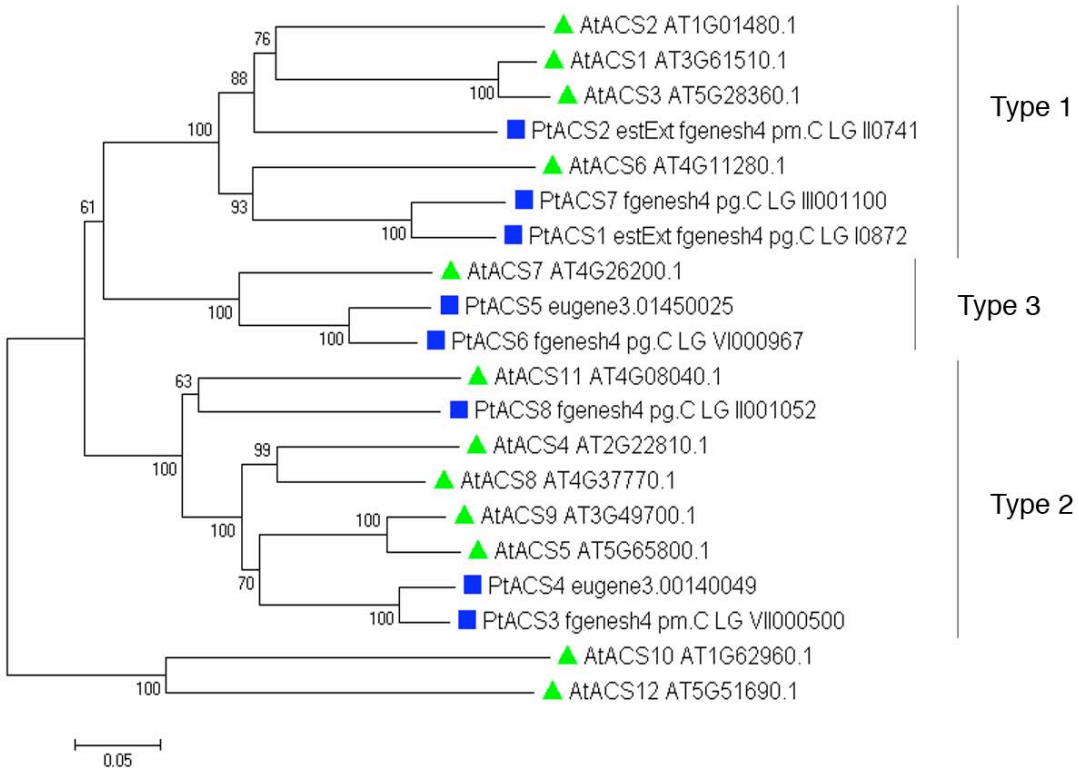

Fig. S2 Neighbor-joining cladogram of Arabidopsis and Poplar **ACC synthase (ACS**) protein sequences. Annotations referring to www.arabidopsis.org and http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

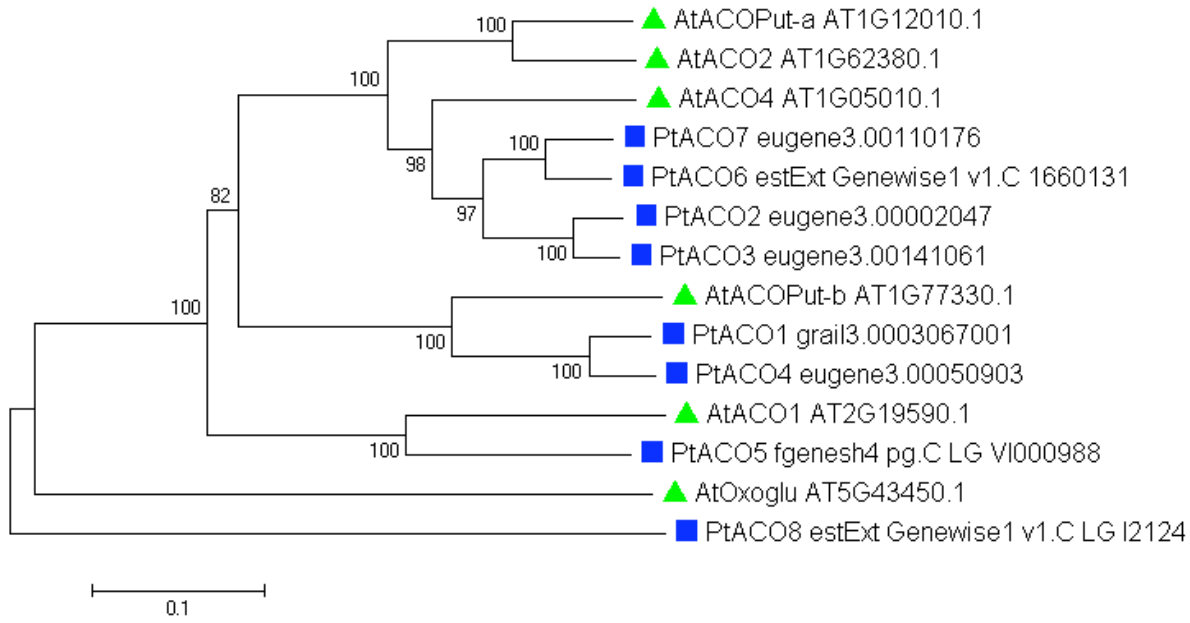

Fig. S3 Neighbor-joining cladogram of Arabidopsis (At) and Poplar (Pt) **ACC oxidase (ACO)** protein sequences. Annotations referring to www.arabidopsis.org and http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

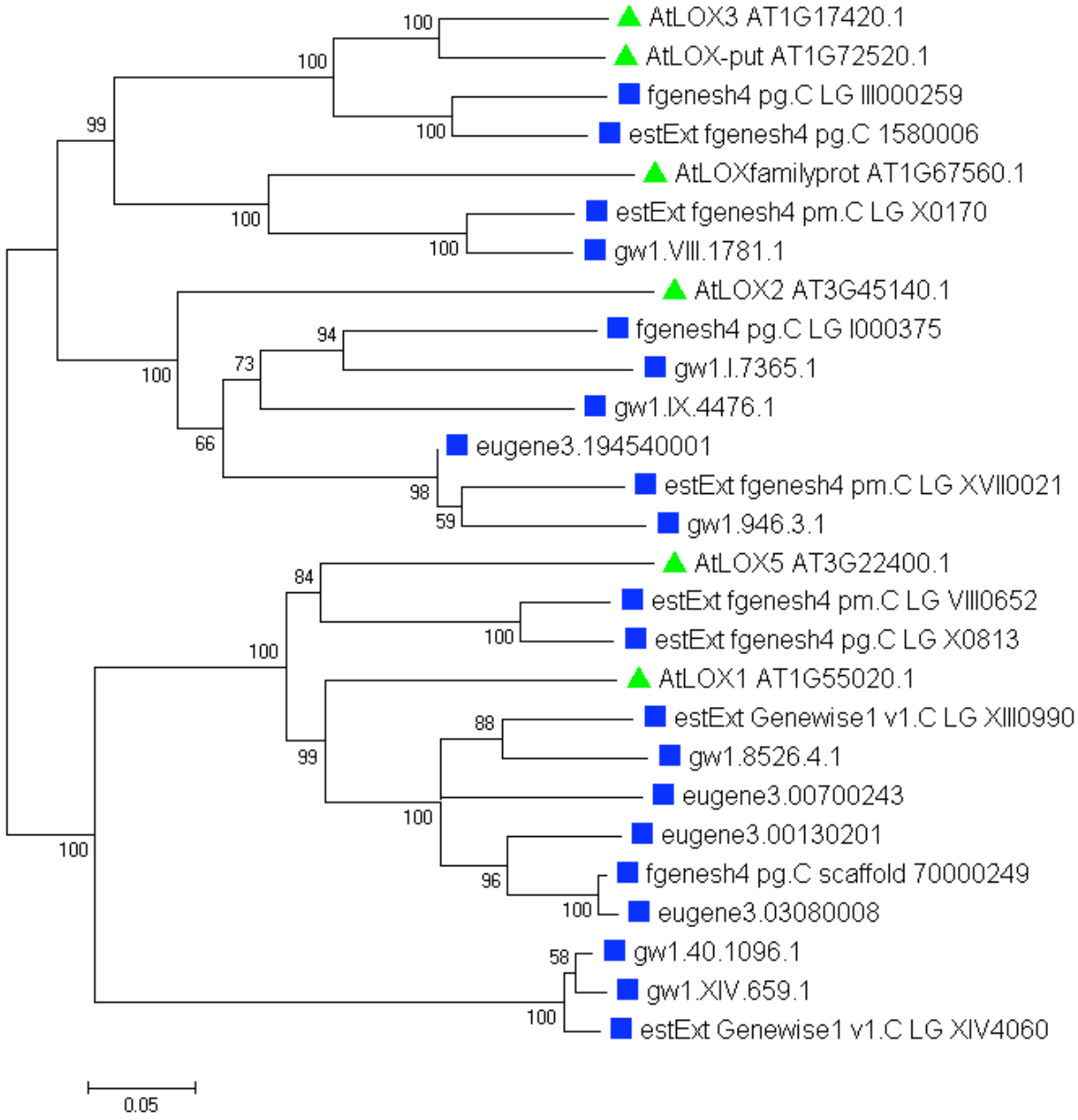

Fig. S4 Neighbor-joining cladogram of Arabidopsis (At) and Poplar **lipoxygenases (LOX)** protein sequences**.** Annotations referring to www.arabidopsis.org and gene names to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

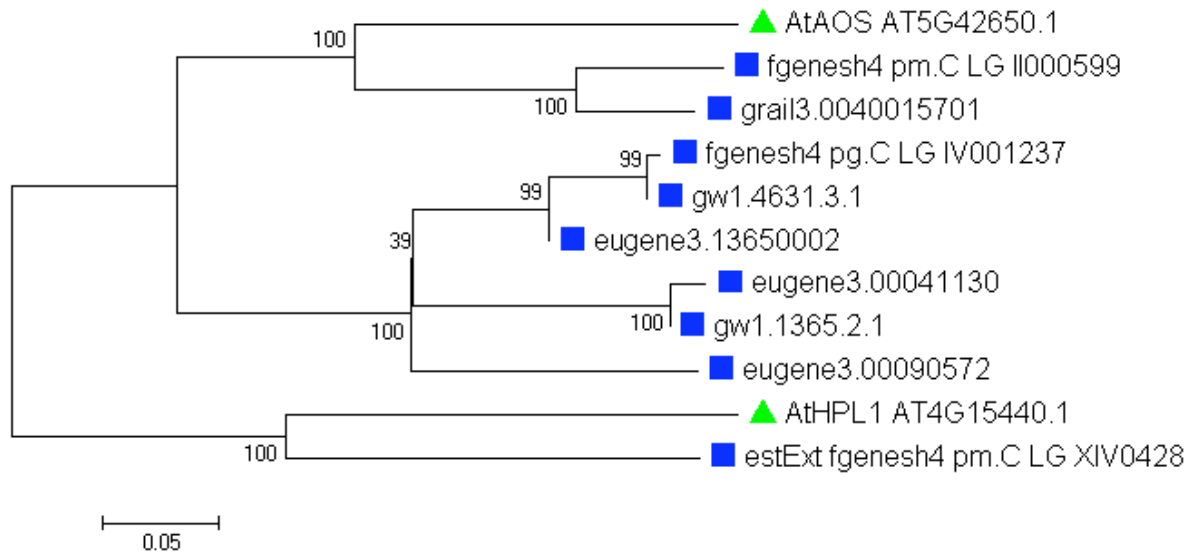

Fig. S5 Neighbor-joining cladogram of Arabidopsis (At) and Poplar **allene oxide syntases** (AOS) protein sequences. Annotations referring to www.arabidopsis.org and gene names to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

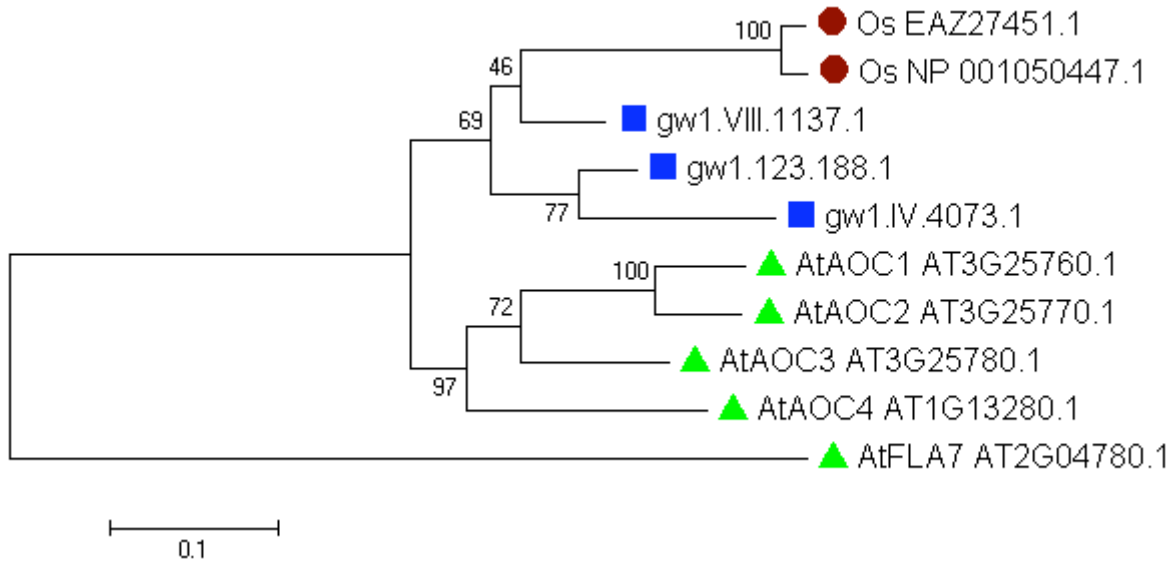

Fig. S6 Neighbor-joining cladogram of Arabidopsis (At), Oryza sativa (Os) and Poplar **allene oxide cyclase (AOC)** protein sequences as well as Arabidopsis Fasciclin-like Arabinogalactan (FLA7) to root the tree. Annotations referring to www.arabidopsis.org and gene names to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html for poplar and NCBI for rice.

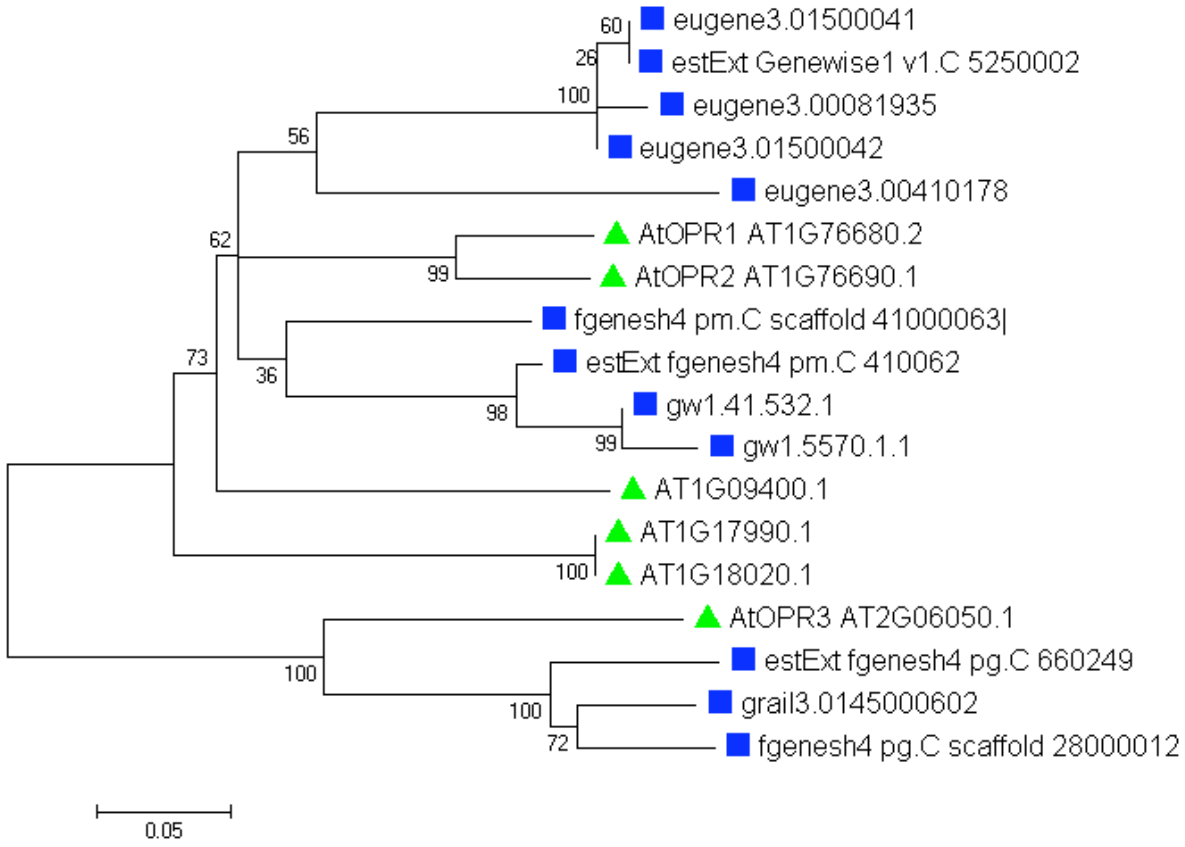

Fig. S7 Neighbor-joining cladogram of Arabidopsis (At) and Poplar **OPDA reductase (OPDR)** protein sequences. Annotations referring to www.arabidopsis.org and gene names to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

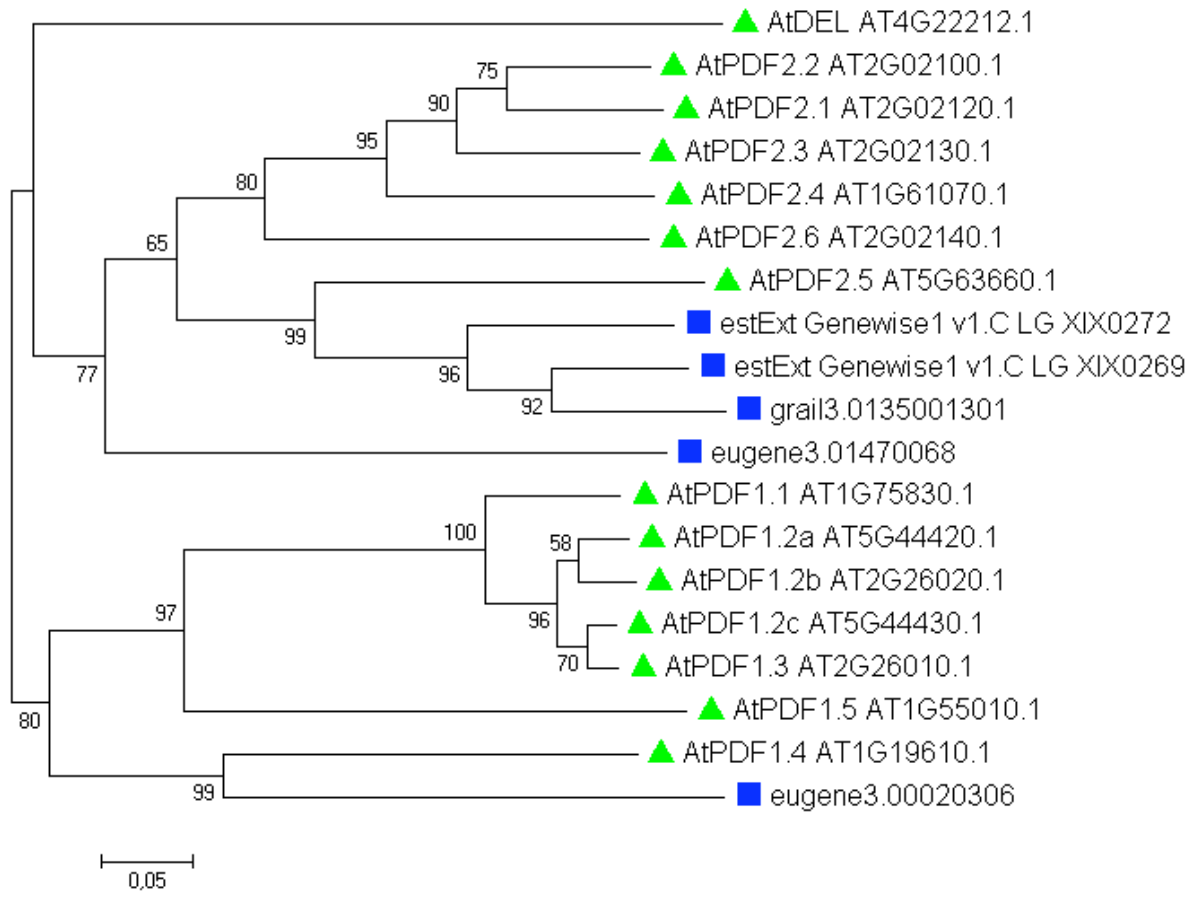

Fig. S8 Neighbor-joining cladogram of Arabidopsis (At) and Poplar **Defensin (PDF)** and one Arabidopsis Defensin-like (DEL) protein sequences. Annotations referring to www.arabidopsis.org and gene names to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

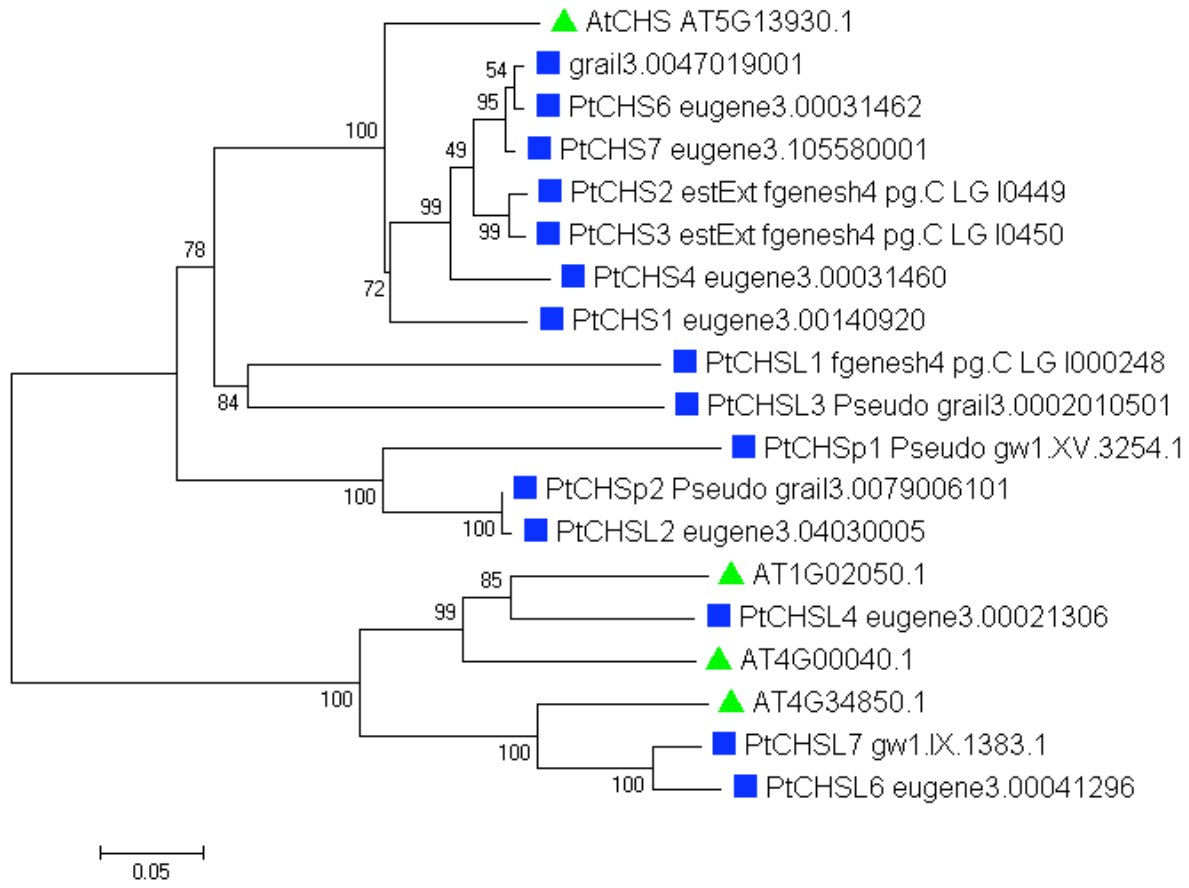

Fig. S9 Neighbor-joining cladogram of Arabidopsis (At) and Poplar **Chalcone Synthase (CHS)** and Chalcone Syntase Like (CHSL) protein sequences. Annotations referring to www.arabidopsis.org and to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

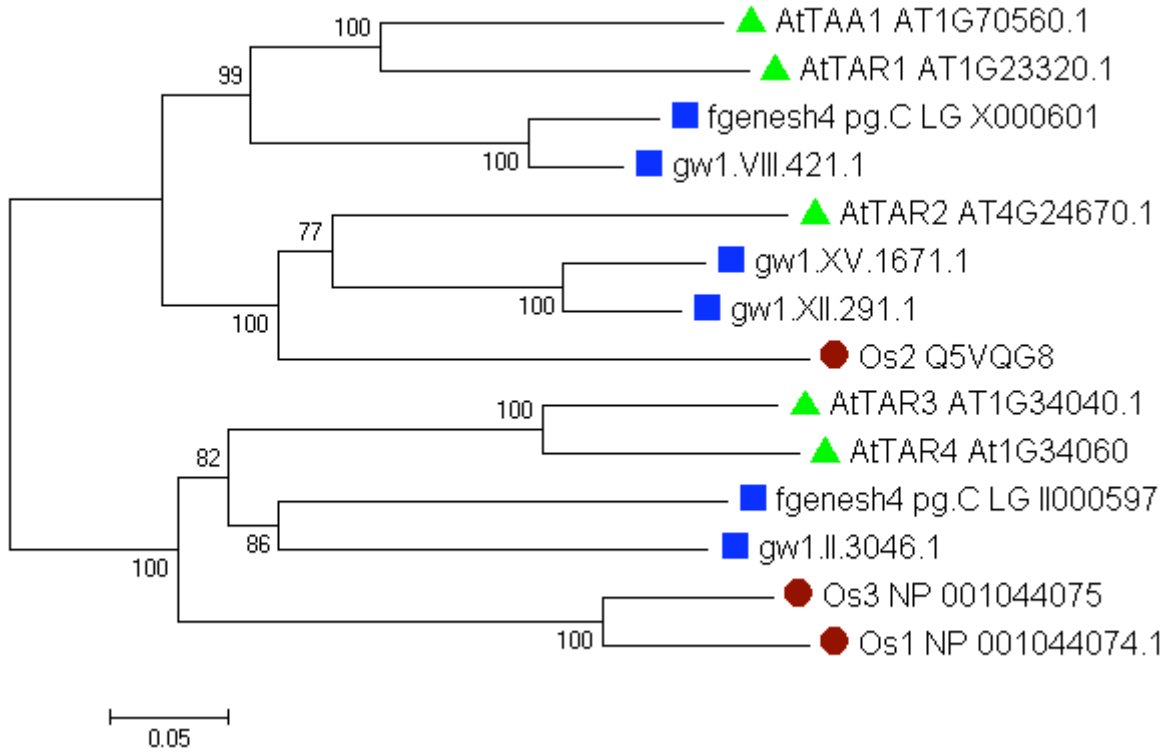

Fig. S10 Neighbor-joining cladogram of Arabidopsis (At), Rice (Os) and Poplar **tryptophan amino transferase (TAA/TAR)** protein sequences**.** Annotations referring to www.arabidopsis.org or Stepanova et al., 2008 and gene names to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

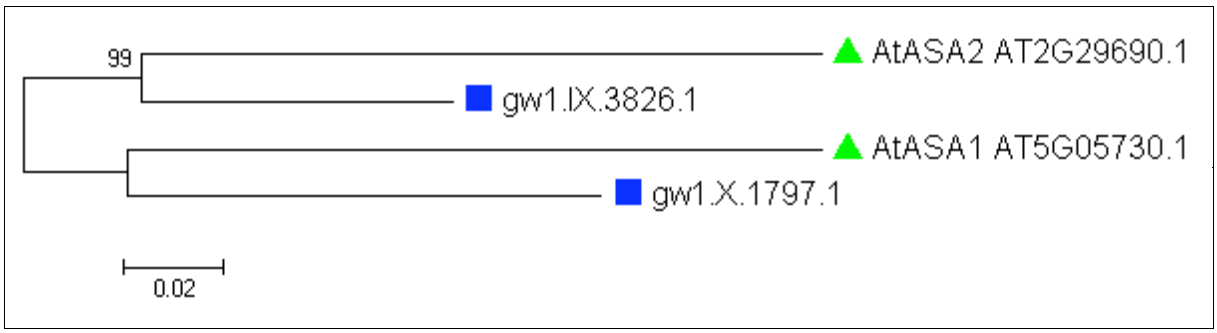

Fig. S11 Neighbor-joining cladogram of Arabidopsis and Poplar **anthranilate synthase (ASA)** protein sequences**.** Annotations referring to www.arabidopsis.org and gene names to http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html

Conclusion & Perspectives

Conclusion

The aim of this work was to decipher how the ECM fungus *L. bicolor* activates LR development in a mycorrhizal or non-mycorrhizal host (poplar or Arabidopsis) through plant endogenous auxin pathways. My work has enlightened fungus-induced LR development through the following major findings:

(i) LR induction is a process initially activated during the early phase of interaction and continues during colonization, but comes to an end before colonization is accomplished

(ii) LR stimulation is caused by diffusible fungal signalling molecules

(iii) LR induction overlaps with a stress response and with an increased auxin response at the root apex and in the basal meristem

(iv) Polar auxin transport (probably basipetal from the root apex) and auxin signalling are required for fungal LR induction

(v) The termination of LR induction coincides with the induction of a target involved in auxin-conjugation

Auxin fluxes at a root level

Based on the present results, a scenario can be proposed of how LR induction occurs (Fig. 19) that enlarges upon the model proposed in chapter II. The early phase of interaction, when LR stimulation starts, is suggested to overlap with a stress response. The fact that this response might be the signal to start LR induction, which can occur as a broad unspecific response in diverse plant partners, would explain why LR stimulation is not limited to mycorrhizal host-plants and why it does not depend on recognition of the partners. We propose that volatile and diffusible signalling molecules released by the fungus (ethylene, jasmonates, ROS etc.) trigger the stress response in the plant. The plant itself reacts with increased ethylene, jasmonates and ROS production until recognition of the partners occurs and the defence-response gets repressed, in the case of symbiosis (Duplessis et al., 2005). Ethylene and jasmonates may then trigger auxin biosynthesis is roots while brassinosteroids, involved in ROS homeostasis, act on polar auxin transport. In addition, other signalling molecules, which have yet to be identified, may also have a direct impact on auxin transporters. These different processes, both those identified here as well as those hypothesised to exist, can enhance basipetal polar auxin transport from the root apex (potentially through PtaPIN9 action). Currently, we do not know whether the auxin accumulation at the root apex is a consequence of, or caused by, the altered auxin transport. Basipetal auxin fluxes from the root apex feed the auxin reflux loop, involved in priming of pericycle founder cells (Laskowski et al., 2008) and enhance auxin transport towards the

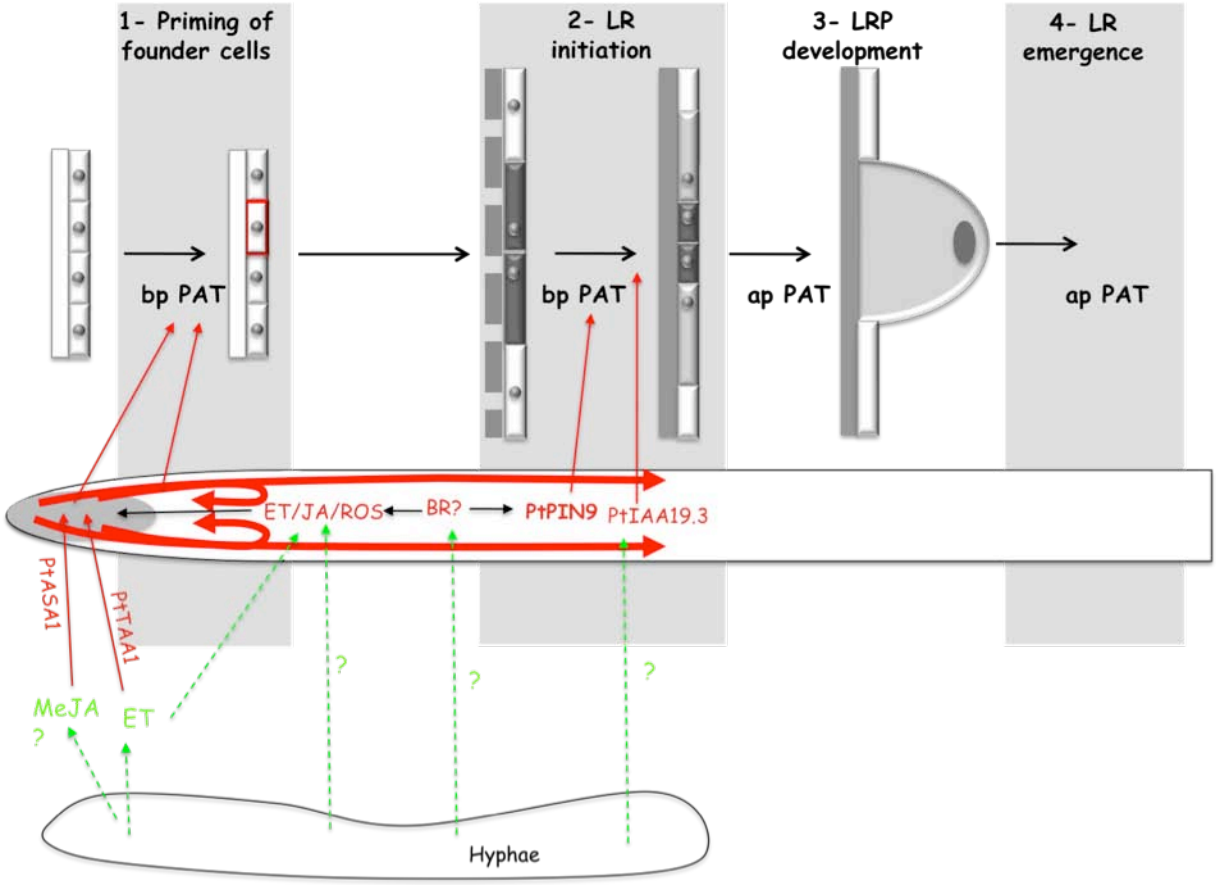

Figure 19: Hypothetical model of auxin homeostasis and transport changes in the <u>early phase</u> of ECM development on a whole-root level.

Fungi produce ethylene and methyljasmonate that are perceived by the plant and lead to increased root endogenous auxin biosynthesis via PtaTAR1 and PtaASA1. Auxin increase as well as action of BRs enhance basipetal auxin transport and favour priming of pericycle founder cells in the basal meristem. Epidermis (E), Cortex (C), Pericycle (P), Ethylene (ET), Methyl-jasmonate (MeJA), Indole-3-Acetic Acid (IAA), Brassinosteroids (BRs), Reactive Oxygen Species (ROS), fungus derived molecules are coloured in green, plant derived molecules and auxin fluxes in red.

proximal elongation zone, where LRI occurs (Fig. 13, Introduction). Stimulation of these auxin fluxes from the root apex, where the increased auxin response and an increased auxin accumulation likely takes place, may thus enhance founder cell specification prior to LRI. Intriguingly, we found that the decline of LR stimulation was mirrored by the induction of genes coding for auxin-conjugating enzymes, and that this correlated to a decrease in *PtaPIN9* levels. By these means the auxin content and basipetal auxin transport inside the root would be decreased and LR stimulation curtailed during the intermediate phase. This stresses further that the auxin accumulation and redistribution within the root are critical for LR stimulation.

The proposed scenario does not contradict previously published studies, which suggest that fungal auxin is responsible for LR stimulation. For instance, a *Hebeloma cylindrosporum* mutant that over-produes auxin enhances LR formation in the plant host more strongly than the wildtype strain (Tranvan et al., 2000). This could also be explained by an increased stress in the plant because auxin is also known to stimulate ROS accumulation (Jiang and Feldman, 2005).

Taken together, we propose that the early stress response (through ET, JA, BR, ROS) triggers auxin accumulation at the root tip. This auxin is redistributed by basipetal auxin transport towards the basal meristem, where it stimulates founder cell priming, as well as to the proximal elongation zone where LRI occurs based on auxin signalling (Fig. 19). When the stress-response is repressed upon recognition of the symbiotic partners during the intermediate phase, auxin conjugation increases, basipetal transport decreases and LR development occurs as in control plants at the rhythm driven by root internal programs.

Auxin fluxes at a cellular level

Our results have allowed us to explain LR induction as a result of auxin fluxes and accumulation at a root-tissue level during plant/fungus contact. Our results can also be used to propose a hypothetical model regarding auxin fluxes on a cellular level that can explain root colonization and Hartig net formation. The phase during which the rate of LR development is increased paralleled the colonization phase of *L. bicolor*, indicating that there may be a mechanistic connection between the two processes (Fig. 20). This hypothesis is supported by the literature which shows that disturbing auxin balances in the plant by application of auxin transport inhibitors NPA or TIBA decreased or even completely prohibited LR induction as well as root colonization by symbiotic fungi (Rincon et al., 2001; Rincon et al., 2003). We can assume that the auxin related processes during indirect interactions between poplar roots and *L. bicolor* as discussed earlier occur in the same way when the poplar roots are in physical contact with *L. bicolor*. At least at 3 days of direct and indirect interaction real-time PCR based gene expression analysis revealed similar results (data not shown). As shown in figure 18 (Introduction) a role for auxin during Hartig net formation can be proposed based on the “Acid growth theory” (Rayle and Cleland, 1992) and/or induction of cell wall

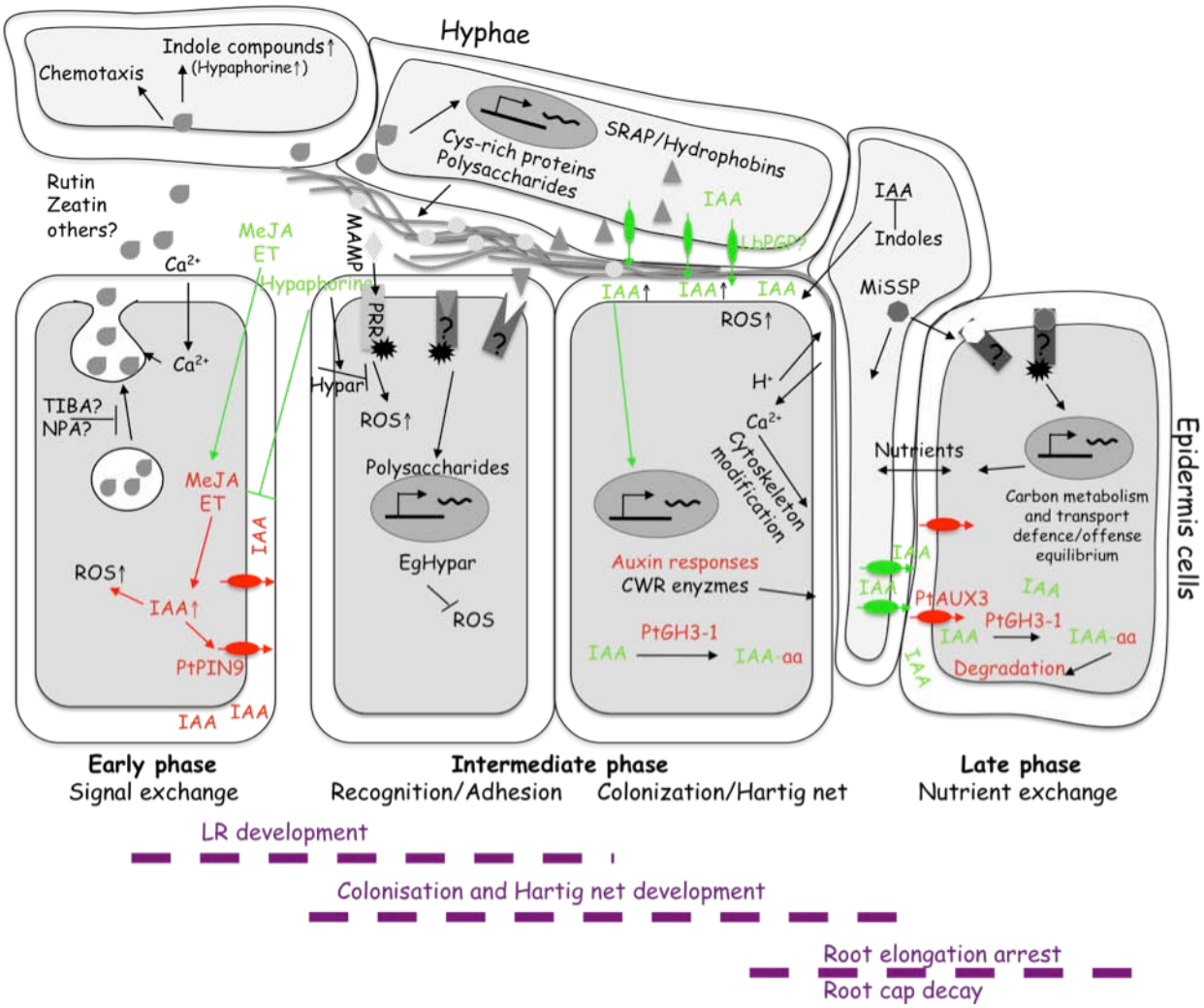

Figure 20: Hypothetical model of auxin homeostasis and transport in distinct phases of ECM development at a cellular level.

The hypothetical events in the three phases of root colonization are related to root developmental changes observed during poplar root colonization with L. bicolor (violet lines at bottom of scheme). During the **early phase** the presence of the fungus stimulates auxin biosynthesis and efflux in plant epidermal cells. Thus auxin accumulating in the apoplast is of plant origin. This process coincides with LR induction. During the **intermediate phase**, when colonization takes place, the fungus releases actively IAA through PGP-like carriers into the apoplast of root epidermis cell wall. Together with indole compounds released by the fungus and increased proton effluxes through plasma membrane H+-ATPases this acidifies the cell wall, which facilitates cell-spacing and penetration by the fungus. Cells start to decrease excess IAA through conjugation. In the **late phase**, the fungus still releases IAA into the epidermis cell wall, but plants import this auxin actively and degrade it to prevent further apoplast acification and ongoing penetration of the fungus. Increased auxin degradation may decrease the total auxin content in the cells and also influence root growth arrest and root cap decay that were specifically observed in the late phase. Green metabolites and proteins are of fungal origin and red ones of plant origin.

remodelling enzymes by auxin (Swarup et al., 2008). I used the gene expression data obtained in my work to add to this model the auxin fluxes that may occur between the plant and fungus (Fig. 20).

In the early phase of interaction auxin biosynthesis and efflux from the cells are stimulated, most likely through the aforementioned stress response. As soon as plant and fungus are in physical contact, during the intermediate phase, both plant auxin and fungal auxin may accumulate to a significant extent in the cell wall of plant epidermis cells. In this proposed scenario, the question arises concerning how auxin can exit the hyphae. The genome of *L. bicolor* does not code for any PIN auxin efflux carriers, but three homologs of the Arabidopsis auxin carrier ABCB/PGP family are present (see Annex A2). Expression data published in (Martin et al., 2008) for *L. bicolor* in contact with poplar or Douglas fir indicated up-regulation of one of these genes in greenhouse-grown ECM by 5.8 fold. It still needs to be addressed whether those transporters can actually carry auxin out of the fungus, but the possibility to have a mediated auxin efflux, which is enhanced in ECM, may stress the importance of fungal IAA during colonization. As the induction of plant auxin efflux carriers declines during colonization it is most probably that auxin accumulating in plant cell walls is of fungal origin. This auxin may trigger cell wall acidification together with indole compounds released by the fungus (e.g. hypaphorine). Furthermore, auxin accumulation may give rise to a short ROS accumulation (observed by Duplessis et al., 2005) during Hartig net formation which can, together with cell wall peroxidases (Mensen et al., 1998), low pH and the activity of certain cell wall remodelling enzymes stimulate cell wall loosening during Hartig net formation. Induction of IAA-amido synthetases during the intermediate phase is likely to re-adjust the auxin content in the epidermis cells that is likely to increase due to the release of auxin by the fungus into plant cells. Within the late phase of interaction we have observed induction of a plant auxin influx carrier in the roots. These may actively imports fungal auxin that accumulates in the cell wall, in order to degrade it in the cytoplasm and to re-stabilize the cell wall to inhibit further colonization. Indeed, Wallender et al. (1992) observed a decrease in auxin levels in ECM compared to non-colonized plants. This may be a result of the balance of auxin production by the fungus and auxin degradation by the plant in mature ECM where auxin levels may be drastically reduced to prohibit further penetration deeper into plant tissues. Together, this model (Fig. 20) proposes a gradual change from plant to fungal auxin accumulation in plant cells at the plant/fungus interface that modulates cell wall characteristics in the specific phases of plant-fungus interaction and facilitates colonization and Hartig net formation, downstream of recognition and adhesion.

In conclusion we propose that auxin homeostasis adjustment through polar auxin transport and auxin-conjugation are important mechanisms to regulate LR stimulation as well as root colonization during ECM development. It had long been known that PAT inhibitors influence ECM symbiosis establishment and LR stimulation (Karabaghli-Degron et al., 1998; Rincon et al., 2001; Niemi et al., 2002; Rincon et al., 2003). With the discovery of the importance of

PtaPIN9 during fungus induced LR development, we have now proposed for the first time a target through which these alterations may occur. Its further investigation on a cellular and protein-level will permit to decrypt in detail how it impacts auxin fluxes during LRI at the plant/fungus interface and to affine and confirm the hypothetical models proposed here.

Perspectives

Additional experiments for completion of manuscripts

Additional experiments will be added to the manuscripts in preparation (Chapter I and II) to confirm and extent the findings described.

Concerning **chapter I:** For detailed analysis of root development (root cap and meristem organization) during ECM formation as well as the exact starting point of colonization on LRs, additional longitudinal root sections are required. In the present sections the number and organization of meristematic cells at the root apex could not be exactly visualized. Possible alterations in the root meristem may be at the source of arrested root elongation growth.

Concerning **chapter III:** Root development and ROS quantification experiments have either been conducted on poplar or on Arabidopsis. Those data should be homogenized by extending each experiment to both plants.

We have based the quantity of IAA released by *L. bicolor* on results published by Karabaghli-Degron et al. (1998). IAA quantification in this literature has been realized from four weeks old liquid *L. bicolor* cultures using an ELISA based technique with fluorescence labelled anti-IAA antibodies. We would like to quantify IAA in the culture conditions in which LR monitoring experiments were realized, thus in the cellophane-overlaid agar plate. Preliminary HPLC analysis realized in collaboration with R. Splivallo, University of Göttingen have yielded results between 5 and 10nM IAA in the medium directly under the fungal colonies (Procedure published in (Splivallo et al., 2009)). GC-MS analysis realized in collaboration with M. Chalot, Nancy University for IAA quantification did not yield satisfying results, probably due to sample preparation. The protocol needs to be optimized. Once we will know the exact IAA concentrations secreted by L. bicolor similar concentrations need to be applied exogenously and the effect on *pDR5:GUS* or *pDR5:GFP* as well as LR development have to be assessed in Arabidopsis and poplar. Similar experiments (quantification and mimicking) are needed for ethylene and jasmonates.

An interesting experiment to verify whether ROS production during plant/fungus contact indeed arises through a mechanism involving BRs would be ROS quantification during Arabidopsis or poplar contact with *L. bicolor* in the presence of BR biosynthesis inhibitor BRZ.

Different mutant lines are available mostly in Arabidopsis and poplar to test the requirement of ROS or ET, BR and MeJA perception and response during plant fungus contact

(i) Arabidopsis *rbohD/F* mutant line (Torres et al., 2002) (available in K. Palme's group) produce lower amounts of ROS

(ii) Arabidopsis *bri1* line (available in K. Palme's group) is insensitive to brassinosteroids due to a mutation in the BR receptor.

(iii) Arabidopsis and poplar *etr1-1* lines (Vandenbussche et al., 2007; Love et al., 2009) (Arabidopsis line available in K. Palme's group, poplar line by collaboration with B. Sundberg, UPSC, Sweden) are stronger ethylene-insensitive lines than *ein2-1* and can be used to re-test the role of ethylene signalling during fungal LR induction

(iv) Arabidopsis *asa1-1* line (Sun et al., 2009) (available in K. Palme's group) that is insensitive to LR stimulation by exogenous (applied or fungal) MeJA.

Further projects

The role of plant auxin during ECM development

It will be interesting to assess the role of plant auxin in ECM formation further. In order to confirm the above shown models protein abundance and localization of PtaPIN9 during poplar/*L. bicolor* interaction needs to be investigated. Antibodies for these targets are already available in our group and await testing in Western Blot and immunolocalization experiments. Through collaboration with the group of V. Busov at Michigan Technological University we have obtained transgenic *PtaPIN9* overexpressor and antisense lines. Preliminary tests on antisense lines, which show 17 times reduced *PtaPIN9* mRNA levels compared to wildtype, indicated insensitivity to LR stimulation by *L. bicolor*. However these tests need to be repeated and there is a strong requirement to assess protein levels in these transgenic plants in order to be able to draw conclusion from these experiments. If the insensitivity to LR stimulation is confirmed with these lines, their mycorrhizal capacity will be addressed. This may finally permit to bridge the gap between the need of LR stimulation for ECM formation.

Furthermore the localization and abundance of PtaGH3-1 protein in poplar during ECM formation with *L. bicolor* will be assessed based on immunolocalization techniques. This target may have an important function in establishing an auxin balance in epidermis or cortex cells involved in or in vicinity to the Hartig net. Together with observations on poplar p*DR5:GFP* or p*DR5:GUS* lines the PtaGH3-1 localization will help to understand auxin responses in ECM roots. Quantification of IAA in ectomycorrhizal roots is needed to confirm whether the auxin response visible in p*DR5:GFP* or p*DR5:GUS* can be related to auxin level alterations.

The role of fungal auxin during ECM development

As already pointed out in the introduction, the role of fungal auxin in ECM is not clear but it might be an important factor during colonization and Hartig net development downstream of recognition of both partners. As a molecule, which impacts diverse developmental processes at a low concentration, auxin levels need to be fine-tuned also at the plant-fungus interface. We have revealed here induction of auxin efflux and influx carriers at specific stages during ECM formation and have furthermore *in silico* identified PGP-like proteins in *L. bicolor* that have homology to the auxin transporting PGP carrier in Arabidopsis. This can be the starting point for a new project aiming on **deciphering auxin fluxes at the poplar/*L. bicolor* interface during different stages of ECM development**.

In a first attempt it is required to assess whether fungal auxin is taken up into plant cells in any phase of ECM development. It has been shown that ECM fungi produce increased amounts of IAA when grown on Trp. Feeding *L. bicolor* with labelled Trp prior to co-culturing can be used to trace the destination of fungal auxin in medium and plant. The investigation of the nature of fungal IAA in plant cells (free, amino-acid or sugar linked) can reveal whether storage, degradation or signalling and further transport may occur. If it can be confirmed that fungal auxin is taken up into the plant, it would be highly interesting to follow its flow in the plant.

A possibility to do this would be the analysis of radioactive labelled auxin in different cell types (epidermis, cortex, endodermis, pericycle). Two possibilities to separate cells of different types are given by laser microdissection or by the use of lines that express tissue specific fluorescence markers and whose cells can be sorted through Fluorescence assisted cell sorting (FACS). The latter possibility has been used with success to profile auxin levels in roots (Petersson et al., 2009). Even if constructing such lines in poplar would be time-consuming IAA quantification may be more successful than using laser microdissection, which requires to immobilize IAA in plant cells and extract it after a long series of sample preparation, probably causing degradation (IAA is not a stable compound). To my knowledge, auxin quantification has not been realized on laser microdissected samples. Thus establishing a protocol may be as time-consuming as construction poplar cell-specific marker lines.

Once the direction of auxin fluxes in the different phases of ECM development will be analyzed one can imagine focusing on the auxin transporters that mediate this transport. As mentioned above, targets in poplar and *L. bicolor* have already been revealed. It needs to be confirmed, however, whether those are able to transport auxin. Such experiments can be realized by heterologous expression (Petrasek et al., 2006; Mravec et al., 2009) of the proteins and analyzing auxin fluxes between the cells and the extracellular medium. These results together with immunolocalization of *L. bicolor* and poplar auxin carriers at the plant/fungus interface can help to understand the auxin fluxes between both partners in the respective phases of ECM development.

After having studied whether and where auxin fluxes occur from the fungus to the plant, it will be ultimately necessary to analyze how ECM formation is altered when those auxin fluxes coming from

the fungus are inhibited. Inhibiting those fluxes can either be realized by generating *L. bicolor* mutants for the respective auxin carriers (a technique for genetic transformation of this fungus is already established (Kemppainen et al., 2005)) or if this is not possible one could imagine to screen for drugs that inhibit the carriers. Genetic transformation will be difficult if auxin carriers in *L. bicolor* are redundant and double or higher order mutants are needed to inhibit auxin efflux. As well, the use of inhibitors has disadvantages because PGP auxin carriers in the plant may also be affected. This part may be the most challenging of the proposed project due to technical difficulties. If it is possible to obtain a means to inhibit auxin efflux from *L. bicolor*, we hypothesize that alterations in ECM formation will be found. We would suggest, based on our data that difficulties in Hartig net formation and penetration of the fungus would occur. A highly interesting question would then be if normal (paraepidermal) Hartig net formation can be restored by expression of auxin biosynthesis genes under a fungal-inducible epidermis-specific promotor (e.g. from the endodermis cell file marker line) upon contact with the fungus. This may mimic auxin accumulation in epidermis cells during contact with the fungus. The fungal-inducible plant promotor should be selected at the basis of data from gene expression of plant genes upon contact with the fungus. It would be best to use the promotor of a gene which responds at the same time during colonization as fungal auxin accumulation occurs in plant cells in order to mimic auxin accumulation within the right interaction-phase. These experiments could dissect the role of fungal auxin during ECM development.

Annex

A1. Optimization of an *in vitro* culture system for poplar/*L. bicolor* ECM formation

I adapted a plate culture system, called sandwich culture system, which was reported by Chilvers et al. (1986) and Burgess et al. (1996) for *Eucalyptus* colonization by *Pisolithus microcarpus* or *Paxillus involutus*.

Since this system had never been used before in our laboratory, my first task had been to set up a protocol enabling the establishment of functional ectomycorrhizae between poplar and *L. bicolor*. I performed a series of tests considering the parameters, which influence the ECM establishment including the composition of the medium, the pH, the gas exchanges, the conditions of temperature and of light. Two important factors that I addressed in detail were medium composition and medium pH. Their optimization is reported in the following.

The co-culture system developed here uses carbon-reduced (1g/L glucose) Pachlewski P20 medium, which is a standard medium for *L. bicolor* cultures and which fulfils furthermore the requirements of the media composition for successful ECM formation with other species, such as the correct P:N ratio (Brun et al., 1995). Reducing both the phosphorous and nitrogen concentration of the medium to 1/10 as reported by Brun et al. (1995) for MMN medium to favour ECM formation or omitting any carbohydrate source from the medium did negatively interfere with mycelium and plant growth and was thus not suitable for co-culturing. The incorporation of the pH-indicator Bromocresolgreen into the medium showed that the presence of the fungus rapidly decreased the pH during co-culturing (Fig. A1), from pH 5.8 in the beginning of the experiment to 3 at 10 days of co-culturing. We therefore included 1g/L MES buffers into the medium to stabilize the pH in order to limit stress-related artefacts in plant development. MES buffer is known for its minimal biological activity and has been tested in fungal cultures (Good and Izawa, 1972; Wong and Fortin, 1989). This was sufficient to stabilized the pH during the first three days of interaction and to slow down the pH decrease over the entire co-culturing period. The so-optimized medium was used for all further experiments. The detailed protocol of the optimized procedures, used for all experiments during my thesis, has been included briefly in the following article (in preparation) and in more detail in the article presented in chapiter II.

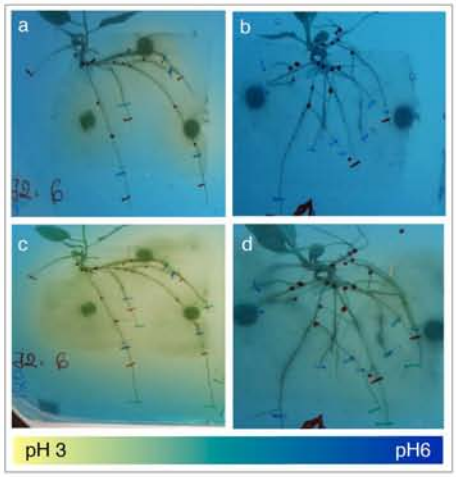

Figure A1: pH decrease during co-culturing visualized by Bromocresolgreen

To detect an eventual pH changed in the P20 medium during co-culturing, 0.06% (w/v) Bromocresolgreen was included into the medium before autocaving. A six-step pH scale from pH 6 to 3 was realized with HCl in liquid P20 medium and is schematized at the bottom of the picture. **a** Roots and fungus at 3d on unbuffered medium and **b** on buffered medium containing 1 g/L MES sodium salt. For both conditions the medium was adjusted to pH 5.8 at the beginning of the experiment. **c** and **d** The same samples at 10d. A strong pH decrease was observed in the unbuffered medium (**c**), while in the MES-buffered medium the pH only decreased slightly (**d**).

A2. Possible auxin carries in *L. bicolor*

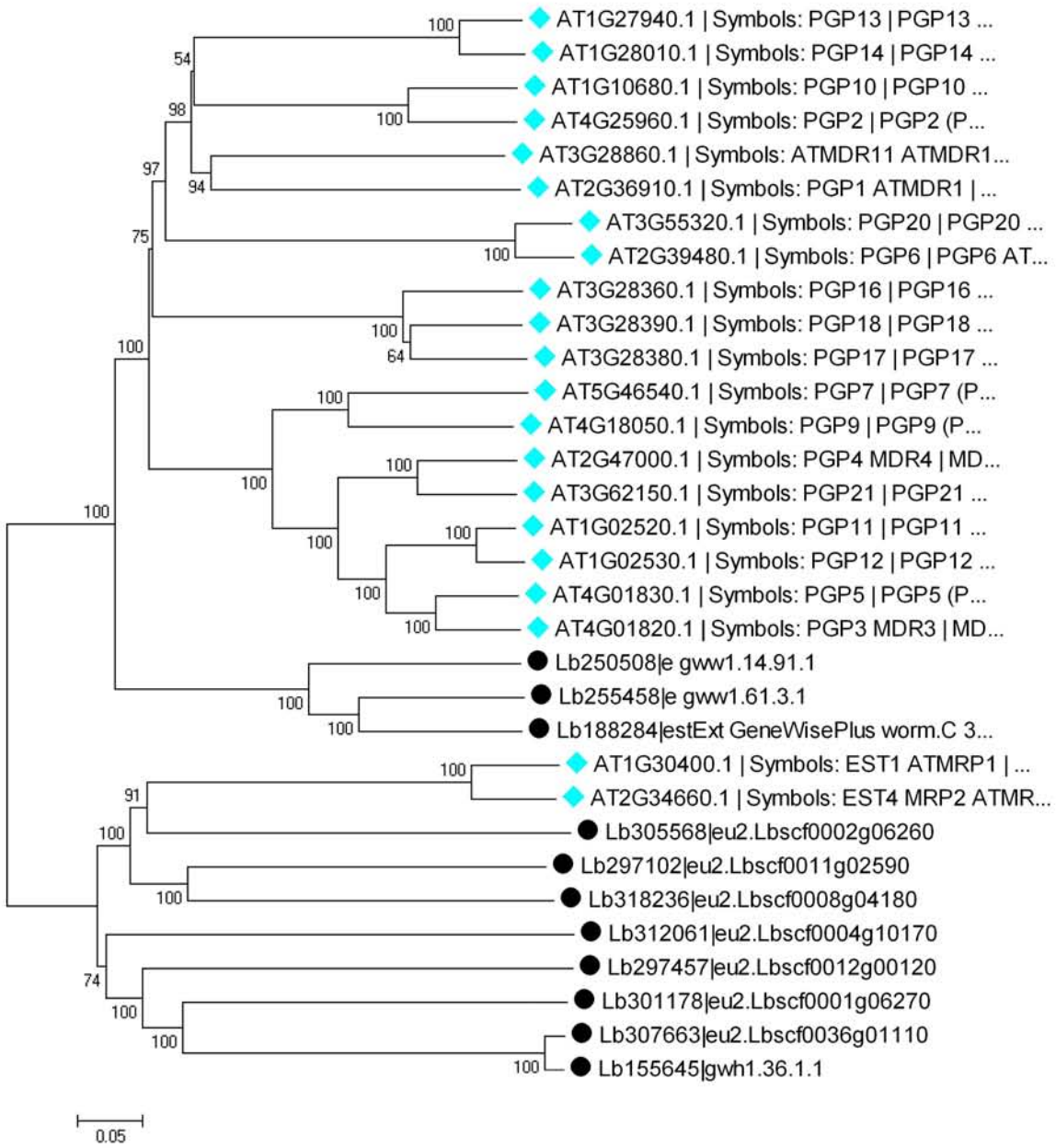

Fig. A2: Phylogenetic tree of *Laccaria bicolor* and *Arabidopsis thaliana* ABCB/PGP multridrug transporters. (Neighborjoining, pairwise deletion, 5000 bootstrap repetitions) as well as two distinct multidrug transporters (AtMRP1 and 2). Three homologs of Arabidopsis PGPs were identified in Laccaria: Lb250508, Lb255458 and Lb188284. In data from Martin et al. (2008) Lb255458 was upregulated in Douglas fir and poplar ECM by 5.8 times.

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Poplar root development in response to fungal signals during onset of ectomycorrhiza development

The early phase of the interaction between tree roots and ectomycorrhizal (ECM) fungi, prior to symbiosis establishment, is accompanied by a stimulation of lateral root (LR) development. This thesis aims on understanding by which molecular mechanisms the interaction of plant and fungus induces LR stimulation. Therefore the ECM fungus *L. bicolor* in interaction with one of its mycorrhizal hosts, *Populus tremula x Populus alba* or with the non-mycorrhizal herbaceous model plant *Arabidopsis thaliana* was studied. Both plant partners react with LR stimulation to the presence of the fungus. First we assessed the time course of poplar/*L. bicolor* ECM development in a newly for these species optimized plate-sandwich culture system. Next we identified gene networks that regulate LR development during the early signal exchanges between *Populus tremula x Populus alba* and the ECM fungus *Laccaria bicolor*. We focussed on auxin transport and signalling pathways, as those are key actors regulating LR development. Experiments with poplar and Arabidopsis transgenic auxin response marker lines revealed that the presence of fungal signalling molecules modified auxin gradients in roots. Using microarray- and quantitative Real-time PCR based transcript profiling of poplar roots we uncovered the accumulation of transcripts of the polar auxin efflux carrier *PtaPIN9* as well as of auxin responsive transcription factors. *A. thaliana* transgenics defective in these targets showed that they are crucial for fungus induced LR stimulation. Finally we identified an involvement of ethylene, jasmonates, brassinosteroids and ROS (Reactive Oxygen Species) signalling during fungal LR induction. These pathways are known to be activated upon stress responses in the plant and to interact with auxin pathways. Together these data show how ECM fungi stimulate LR development in plants by interfering with endogenous auxin-levels, -distribution and -signalling most probably through stress signalling pathways.

Keywords: ectomycorrhiza, auxin, lateral root, polar auxin transport, phytohormones

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Développement racinaire du peuplier en réponse aux signaux fongiques lors de la mise en place de l'ectomycorhize

L'interaction précoce entre les racines des arbres et les champignons ectomycorhiziens (CEM), est accompagnée d'une forte stimulation du développement de racines latérales (RLs) de la plante hôte et ceci avant l'établissement de la mycorhize. L'objectif de cette thèse est de décrypter les mécanismes moléculaires impliqués dans le développement des RLs du peuplier en réponse au champignon ectomycorhizien *Laccaria bicolor*. Pour atteindre cet objectif, nous avons utilisé une des plantes hôtes de *Laccaria bicolor*, le peuplier *P. tremula* x *P. alba* ainsi qu'une plante non-mycorhizienne, *Arabidopsis thaliana*. *Laccaria bicolor* stimule la formation des racines latérales dans ces deux espèces. Après avoir mis au point un système de mycorhization *in vitro*, nous avons analysé et décrit la cinétique de la formation des mycorhizes peuplier/*L. bicolor*. Nous avons ensuite identifié des réseaux de gènes qui régulent le développement des RLs pendant l'interaction précoce à partir des micro-arrays NimbleGen. L'auxine étant considérée comme un élément clef de la régulation du développement des RLs, nous avons ensuite focalisé notre étude sur les protéines impliquées dans le transport et la signalisation de cette hormone. L'analyse de la cinétique d'expression de ces gènes candidats a permis de montrer une régulation précoce de *PtaPIN9* (homologue de *AtPIN2*) impliquée dans le transport polarisé de l'auxine ainsi que des facteurs de transcription répondant à l'auxine. Parallèlement, un changement du gradient auxinique dans l'apex des racines de peuplier et *A. thaliana* a été mis en évidence en réponse à *Laccaria bicolor*. L'utilisation des mutants d' *A. thaliana* confirme la fonction importante de PtaPIN9 lors de la stimulation des RLs en réponse au champignon. De plus, nos résultats suggèrent l'implication des voies de l'éthylène, des jasmonates, des brassinostéroides et des ROS (Reactive Oxygen Species) pendant l'interaction plante/champignon, suggérant une implication des voies de signalisation dépendantes des stress dans les étapes précoces de l'interaction. L'ensemble de ces résultats m'a conduit à proposer un modèle des voies de signalisation impliquées dans les étapes précoces de l'interaction ectomycorhizienne : *Laccaria bicolor* stimulerait le développement des RLs en modifiant la répartition et la signalisation de l'auxine endogène de la racine via les voies de signalisation du stress.

Mots clés : ectomycorhize, auxine, racine latérale, transport polaire de l'auxine, phytohormone