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Molecular investigations in ectomycorrhizae establishment in the *Quercus robur* - *Piloderma croceum* model: influence of indole-3-acetic acid on transcripts regulation

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Abstract

We used a culture system and 55 cDNA fragments regulated in the total root system of oak microcuttings premycorrhizal with *Piloderma croceum* to compare gene regulation in lateral roots four weeks after fungal infection or after the application of exogenous indole-3-acetic acid (IAA). 67% of these genes responded to the treatments. The fungus regulated 58% and IAA 31% of the genes. All genes which responded to *Piloderma croceum* were upregulated, while up- and down-regulated genes were observed after IAA treatment. Although only one developmental stage and a sole IAA dose were analysed, 20% of the genes responded to both treatments even if not in the same way, suggesting that the complex premycorrhizal effects of *Piloderma croceum* and auxin partially activate the same genes. Regulation of genes encoding calmodulin and calcium ATPase in both treatments indicate that calcium flow could also play a role for EM formation in our system.

Introduction

Ectomycorrhizas (EM) are plant-microbe symbiosis involving soil fungi and roots of boreal, temperate but also some tropical forest trees (Smith and Read 1997). In this association the mycobiont promotes acquisition of water and nutrients from soils (Read and Perez-Moreno 2003). Symbiosis establishment involves signal exchanges and interactions between both partners with strong morphological modifications including growth promotion of mycelium (Lagrange et al. 2001) and increase of growth and health of trees (Smith and Read 1997).

Ectomycorrhizal fungi have the capacity to produce plant hormones (Ulrich 1960) or molecules able to alter hormone activity (Béguiristain et al. 1995). The hypothesis of Slankis (1973) emphasizes that indole-3-acetic acid (IAA) could be one major compound in ectomycorrhiza formation. More recently, Charvet-Candela et al. (2002) showed that fungal IAA alters expression of auxin-related genes in plant. But very little is known about the effect of auxin on symbiotic regulated genes.

In a preliminary work, we developed a system to synthesize EM on oak micro-cuttings and characterised a long premycorrhizal stage with plant growth stimulation as response to inoculation with the mycobiont *Piloderma croceum* (Herrmann et al. 1998).

With this system, 55 premycorrhiza-regulated plant genes were identified in a full root system by a subtractive suppressive hybridization (SSH) experiment (Krüger et al. 2004). In addition, we could manipulate the onset of EM formation by IAA (Herrmann et al. 2004). Although exogenous auxin did not affect significantly root growth of main or lateral roots, addition of IAA allowed an earlier mycorrhization and an extension of the spatial distribution of ectomycorrhizas (Herrmann et al. 2004). The plant growth enhancement after the pre-mycorrhizal stage as well as the accelerated EM formation after the application of IAA suggest a possible perception of exogenous IAA by oak and an implication of the hormone in symbiosis establishment.

In the present study, we compared the regulation of the 55 plant genes previously identified by SSH analysis on complete premycorrhizal root system, in lateral oak roots either premycorrhizal with *Piloderma croceum* or non-inoculated but treated with exogenous auxin. Based on the results, the hormonal role for the fungus is discussed.

Materials and methods

Biological material

Premycorrhizal interaction between micropropagated *Quercus robur* and *Piloderma croceum* was realized in a 90 mm Petri dish system according to Herrmann et al. (1998). In this system, root develops aseptically and two dimensionally within Petri dishes, while the shoot grows outside of the dish. For plants treated by auxin, indole-3-acetic acid previously dissolved in ethanol was added after sterile filtration at a final concentration of 5 µM. Each plant was placed in a 140 mm Petri dish in which humidity was regulated by moistened paper in order to prevent shoot desiccation. Control, inoculated, and IAA-treated plants were cultivated in growth chambers at 25 ±1°C, and illuminated 16h d^{-1} (70 µmol m^{-2} s^{-1}; Philips TLM 115W/33RS).

Root harvesting was performed after about 4 weeks of culture, during the first root flush, that corresponds to the beginning of the outbursting of the apical bud (Herrmann et al. 1998, 2003). Newly lateral roots formed at the end of their exponential growth flush were frozen in liquid nitrogen before RNA extraction. About 20 plants were used for each treatment in order to gather enough transcripts.

Extraction and analysis of RNA

RNA extraction was performed according to Wang et al. (2000).

The 55 cDNAs used for differential screening between the 3 treatments (control plants, inoculated plants or IAA treated plants) were selected as premycorrhiza-related plant genes in Krüger et al. (2004). Clones were amplified by PCR with M13 forward and reverse primers (94°C, 4 min; 30 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 2 min). Purity and length of all PCR products were checked by agarose gel electrophoresis.

For reverse northern blots, amplified fragments were separated on 1.5% agarose gels and blotted onto a nylon mem-

brane (Hybond N; Amersham Biosciences, Freiburg, Germany). Identical membranes were blotted for control, inoculated and IAA treated plants (Sambrook et al. 1989). Labelling of the cDNA probes was performed in the presence of 30 µCi[^{32}P]dCTP from 4 µg of total RNA with the Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). Nylon membranes were preincubated 4h at 65°C in a hybridization solution (6x standard saline citrate (SSC), 5x Denhardt, 0.5% (w:v) sodium dodecyl sulphate (SDS), 100 µg/ml salmon sperm DNA). Radioactive probes were given to a fresh hybridization mixture before membrane immersion. After 16 h of hybridization, membranes were washed 2 times for 15 min in a SSC 2x, SDS 0.1% (w:v) solution. Finally, membranes were wrapped in plastic film before exposition to a phosphorimaging screen (Molecular Dynamics). Data acquisition and transcripts quantification were performed using IMAGEQUANT Software (Amersham Bioscience). Partial pictures of each membrane are presented on Figure 1.

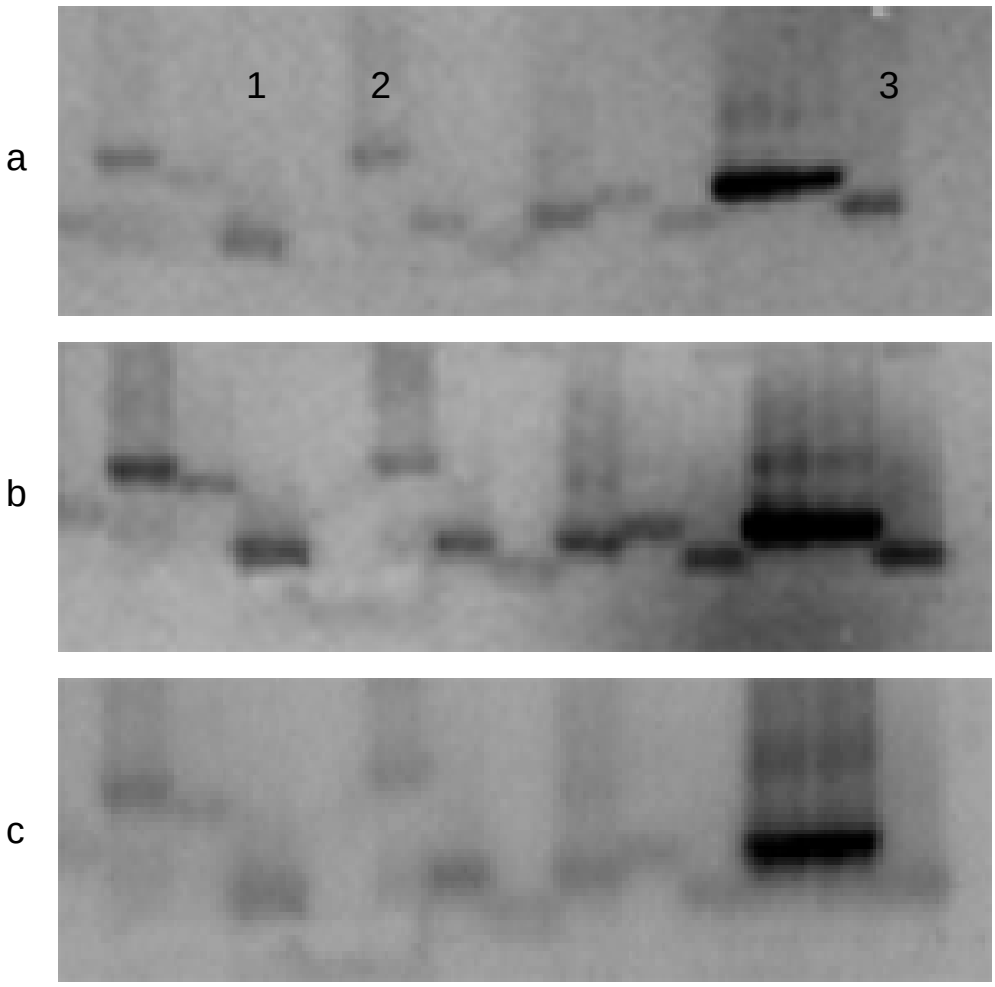

Figure 1: Effects in lateral roots of *Piloderma croceum* or of exogenous IAA on premycorrhiza related genes. Reverse northern blots for (a) control-plants, (b) inoculated-plants, (c) IAA treated-plants. Three example clones are selected: (1) no homology_AJ580040; (2) expansin_AJ577264; (3) no homology_AJ873912. These genes are marked * in Table 1. (1) is only regulated by *Piloderma croceum;* (2) is not regulated for both treatments; (3) is differentially regulated during fungal- and IAA-treatments.

Results-Discussion

Ectomycorrhizal symbiosis formation involves a molecular cross-talking between both partners. It provokes modifications of the root structure that allow establishment of a mixed plant-fungus interface where direct transfer of nutrients takes place. These modifications cause drastic alterations at the transcript level (Duplessis et al. 2005; Johansson et al. 2004). In the present study, we have followed the regulation of 55 pre-symbiotic related genes in the *Quercus robur/Piloderma croceum* model and assessed to which extent these genes were also regulated by exogenous IAA.

From the 55 considered genes, 37 were regulated in at least one of the treatments with either *Piloderma croceum* or IAA (Table 1; see also examples of northern blot experiments in Figure 1). This high proportion (67%) indicates that the SSH bank used was adequate for studying premycorrhizal events. About one third of the genes were not regulated in any of the treatments, which may correspond to the fact that their expression could be specific of main roots, as the SSH bank was obtained from whole root systems (Krüger et al. 2004), and only lateral roots were studied here. It is also possible that some genes of the SSH were not regulated in lateral roots during the narrow development window in which the material was collected for this study. In the following paragraphs we discuss some traits of the regulation pattern found in each treatment. Afterward we compare the patterns of both treatments to enlighten to which extent auxin could be part of the *Piloderma* effect.

Regulation pattern in the Piloderma treatment

In presence of *Piloderma croceum*, 58% of the plant genes regulated in the total roots system (Krüger et al. 2004) were also regulated in the lateral roots (Table 1). Some of these genes, which are upregulated by the fungus, are implicated in signal perception and transmission. Peptidyl-propyl cis-trans isomerase (AJ580022) acts during protein-protein interactions with receptor proteins (Tai et al. 1992; Yem et al. 1992). Calmodulin, (AJ873911) and the identified kinase (AJ580031) play a role as signal transducer in plants (Snedden and Fromm 2001) and as receptor in bacterial or fungal symbiosis (Stracke et al. 2002), respectively. For this functional category of genes, we can therefore hypothesise an implication in the perception pathway, prior to morphological and cellular reorganisation during the EM formation. Further genes of the SSH bank might be implicated in growth and meristemic cell division, i.e. two phosphoglycerate mutases (AJ580028, AJ873927) (Mazarei et al. 2003) and expansin (AJ577264, see Figure 1) (Cosgrove et al., 2002). However, these genes were not regulated in the inoculated lateral roots although in accordance with our previous finding (Herrmann et al. 1998), their growth was strongly stimulated in comparison to the one of non infected plants. This apparent contradiction does not indicate that these genes are not involved in the growth response to the inoculation. Since the roots were harvested at the end of their exponential growth stage toward the end of a root flushing, expression of these genes might have already been down-regulated again.

Table 1: Patterns of expression of premycorrhiza-related genes in lateral roots during the plant-fungus interaction or after IAA treatment compared to control-plants. Levels of expression are indicated by + or –: +, 2-3 fold, ++, 3-5 fold, +++ more than 5 fold upregulated; -, 2-3 fold, --, more than 3 fold repressed; 0, not regulated clone. * refers to reverse northern blot experiments shown in Figure 1.

GenBank Accession no.	Database matches	E value	Inoculated/Control	IAA/Control
cDNA upregulated during the premycorrhizal stage and under IAA treatment				
AJ580039	No match		++	+
AJ873910	No match		+	+
cDNA upregulated during the premycorrhizal stage and repressed under IAA treatment				
AJ580035	60S Ribosomal protein L17	1e-56	+	-
AJ873911	Calmodulin	3e-13	+++	-
AJ616018	Calcium ATPase	8e-06	++	-
AJ577263	Metallothionein	3e-11	+	-
AJ580025	No match		+	-
AJ580037	No match		+	-
AJ873913	No match		++	-
AJ873912*	No match		+	--
AJ873914	No match		++	--
AJ873915	No match		+	-
cDNA upregulated during the premycorrhizal stage, not regulated under IAA treatment				
AJ580022	Peptidylpropyl cis-trans isomerase	5e-42	+	0
AJ580027	Protein kinase	2e-04	+	0
AJ873919	mRNA capping enzyme	1.0	+	0
AJ580042	Probabl sensory box	1.8	+	0
AJ580043	Maltose transporter	3.9	++	0
AJ580044	60S ribosomal protein L23	9e-17	+	0
AJ580045	Chemokine receptor like	1.8	++	0
AJ873917	No match		+	0
AJ873918	No match		+	0
AJ873920	No match		+	0
AJ873921	No match		+	0
AJ580036	No match		+	0
AJ873916	No match		+	0
AJ580040*	No match		++	0
AJ873922	No match		+	0
AJ873923	No match		+++	0
AJ873924	No match		+	0
AJ873925	No match		++	0
AJ873926	No match		+	0
AJ580041	No match		+++	0
cDNA not regulated during the premycorrhizal stage, upregulated under IAA treatment				
AJ580026	Ribosome biogenesis regulatory protein	3e-06	0	+
AJ580031	Putative protein kinase	5e-07	0	+
AJ580028	Phosphoglycerate mutase	4e-13	0	+
AJ873927	Phosphoglycerate mutase	7e-11	0	+
AJ580023	No match		0	+
cDNA not regulated during the premycorrhizal stage and under IAA treatment				
AJ580034	Hypothetical protein, similarity to apoptose inhibitor	8e-08	0	0
AJ577264*	Expansin	2e-87	0	0
AJ580032	Phospholipid-transporting ATPase	0.39	0	0
AJ577266	Formate deshydrogenase	e-177	0	0
AJ580024	Serine carboxypeptidase III	2e-16	0	0
AJ580030	Rev interacting protein mis3-like	2e-43	0	0
AJ580047	Activation associated secreted protein like	1.8	0	0
AJ580046	No match		0	0
AJ580029	No match		0	0
AJ873929	No match		0	0
AJ873930	No match		0	0
AJ873931	No match		0	0
AJ873932	No match		0	0
AJ873928	No match		0	0
AJ873933	No match		0	0
AJ873934	No match		0	0
AJ873935	No match		0	0
AJ580038	No match		0	0

Regulation pattern in the IAA treatment

Morphological effects of auxin on oak were followed in a previous work (Herrmann et al. 2004). We found no growth difference and in particular no significant effect on root growth compared to control plants. Here, we confirmed the finding as the strong increase of lateral root length induced by *Piloderma croceum* could not be obtained by exogenous IAA application. At the molecular level, only 17 pre-mycorrhizal plant genes (i.e. 31 %) were regulated by the IAA treatment and we did not observe any clear relation between the regulation pattern and the functional category to which these genes belong (Table 1). For example, the genes for the identified kinase (AJ580031) and calcium ATPase (AJ616018) showed differential regulations, and were respectively upregulated and repressed in IAA-treated material. Similarly, the growth related genes encoding phosphoglycerate mutases (AJ580028, AJ873927) were upregulated, while expansin (AJ577264) was not regulated (Figure 1). The absence of regulation of 38 genes by exogenous IAA could have two reasons, (1) these genes are not under auxin control, or (2) these genes or some of them are under auxin control but the used IAA concentration and the application mode were not able to trigger their expression.

Comparison of the expression profiles after Piloderma and IAA treatments

The expression patterns found for each treatment clearly indicate that the premycorrhizal interaction between *Piloderma* and oak microcuttings cannot be restricted to a single exogenous IAA effect. First, while 32 genes were upregulated by *Piloderma,* only 7 reacted so in the IAA experiment, and only two of them were identical (Table 1). Second, no gene was down-regulated in the *Piloderma* treatment, while 10 genes were down-regulated in the IAA treatment. Third, the 10 genes which were down-regulated by IAA were upregulated by *Piloderma*. These differential patterns do not mean that IAA is not at all involved in the pre-mycorrhizal *Piloderma* effect. However, the auxin release known from EM fungi (Gay et al. 1992; Rudawska et al. 1997) will probably be balanced by other effects after the inoculation of the root systems. In addition, the effects of an initial and unique application of IAA to the medium on regulation of plant genes are not comparable to the continuous release of fungal auxin during an inoculation experiment over several weeks. In this context, it is remarkable that from 55 studied genes, 12 (i.e. more that 20%) were regulated by both treatments even if not in the same direction. This suggests that an auxin effect is part of the interaction with *Piloderma croceum*.

Only two genes were upregulated in lateral roots of inoculated and auxin treated plants. These genes do not exhibit any homology to known proteins, and their role is still not clear. The fact that auxin and mycorrhizal fungus can equally stimulate identical genes was also observed by Nehls et al. (1998). They found a glutatione-S-transferase (GST) upregulated in *Eucalyptus* ectomycorrhizae and after an auxin treatment, whereas the precise function of this GST is still unclear. We suppose that the control of such genes involves identical per-

ception systems for recognition of the mycobiont and of exogenous IAA.

Five genes upregulated after the IAA-treatment were not regulated by the fungus. Two of them (AJ580028 and AJ873927) code for phosphoglycerate mutases. They are controlled by hormones and are upregulated by IAA in *Arabidopsis* (Mazarei et al. 2003). Mazarei et al. (2003) also showed an induction of this group of enzymes during a plant-parasitic interaction with nematodes. In the *Quercus robur-Piloderma croceum* model, phosphoglycerate mutases are upregulated in the total root system during the premycorrhizal stage (Krüger et al. 2004), but are apparently not regulated in the lateral roots in presence of *Piloderma croceum* or at least not at the development stage in which these roots were harvested.

Some genes are differentially regulated by *Piloderma croceum* and exogenous IAA. Ten genes upregulated in inoculated plants were down-regulated by IAA at the tested concentration. We hypothesise that these genes are under hormonal control, but that their regulation pattern during the premycorrhiza stage was not mimetically reproduced by the IAA application. Calmodulin (AJ873911) and the calcium ATPase (AJ616018) illustrate this differential regulation by IAA and *Piloderma*. The genes encoding both enzymes were upregulated in presence of the fungus but repressed in the IAA treatment. Calmodulin and Ca-ATPase are in direct relation with Ca concentrations in the cell (Snedden and Fromm 2001), and De Ruijter et al. (1998) demonstrated the importance of calcium flow during plant-microbe symbiosis. Yang and Poovaiah (2000) established a relation between calcium-calmodulin and auxin action on gene regulation. They showed that proteins coded by small auxin up RNAs (*SAURs*) are calmodulin binding proteins involved in calcium/calmodulin-mediated signalling during auxin-mediated signal transduction. The presence of those genes in the SSH bank used in this work and the fact that they were again regulated in the present assay strongly suggests that *Piloderma croceum* could regulate calcium metabolism in the presymbiotic stage.

Conclusion

After our demonstration that exogenous auxin triggers the onset of EM formation in the system *Piloderma croceum/Quercus robur* (Herrmann et al. 2004), the two regulation patterns of plant genes by *Piloderma croceum* and by exogenous IAA strongly suggest a fungal control on hormone balance that might facilitate EM formation. The barely detectable morphologically effect of exogenous IAA could be related to an indirect action of regulated genes in plants. According to our results, the IAA concentration tested seems to act on the calcium pathway, but not directly on growth related genes. The low number of presymbiosic related genes reacting after IAA treatment implicates that the fungal recognition by oak involves additional signals. However, a strong limitation of the present work was that only one concentration of external auxin was tested and only one premycorrhizal stage observed. The results obtained with one set of plants must be validated by independent repetitions considering more stages in the *Piloderma*-oak association and consecutive statistical analysis. A better un-

derstanding of the role of auxin in this plant/fungus interaction requires the development of a small array system on the basis of the SSH and other plant/microbe-related genes.

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