Identification of premycorrhiza-related plant genes in the association between *Quercus robur* and *Piloderma croceum*

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Summary

• An *in vitro* system with micropropagated oaks (*Quercus robur*) and the ectomycorrhizal fungus *Piloderma croceum*, which is characterized by a delayed mycorrhiza formation, was used to identify plant transcripts upregulated in the premycorrhizal phase.

• Complementary DNA (cDNA) populations of uninoculated roots and fungal mycelium were subtracted from a cDNA population of inoculated roots. Differential expression was confirmed by reverse Northern and 50 clones for different polypeptides were found to be up-regulated. Twenty-nine clones were investigated in more detail.

• For approximately half of the cDNA fragments no homologies could be identified in databases. The residual fragments code for polypeptides with homologies to known proteins involved in signal perception and transmission, stress responses, metabolism and growth.

• Since many of the identified genes have not yet been described in the context of symbiotic events, their potential roles during early phases of the recognition process are discussed.

Key words: ectomycorrhiza, *Quercus robur* (common oak), *Piloderma croceum*, premycorrhiza related plant genes, suppression subtractive hybridization.

 163: 149–157

Introduction

Ectomycorrhizas (EM) are mutualistic symbioses between fine roots of higher plants and fungi that involve trees of boreal, temperate and sometimes also tropical forests and a wide range of Ascomycetes and Basidiomycetes (Smith & Read, 1997). Tree survival in ecosystems concerned substantially depends on the symbiosis, as the fungal partners promote acquisition of water and nutrients from the soils (Read & Perez-Moreno, 2003). To investigate the EM functions, *in vitro* culture systems between various fungi and poplar, oak, eucalyptus or pine have been established (Kottke *et al.*, 1987; Hilbert *et al.*, 1991; Hampp *et al.*, 1996; Herrmann *et al.*, 1998). Such models have been used to elucidate the molecular mechanisms underlying the nutrient exchanges (Nehls *et al.*, 1998, 2000, 2001; Javell *et al.*, 2001) or an increase in heavy metal tolerance in EM (Jacob *et al.*, 2001). Corresponding gene regulation is characteristic of functional EM having a developed mantle and penetrating hyphae between the rhizodermic root cells that form the Hartig net. However, physiological changes and related modifications of gene expression have been detected at the presymbiotic stage, before the establishment of any interface (Balasubramanian *et al.*, 2002). Studies, some of which are based on screens of large, expressed sequence tag (EST) collections are beginning to consider molecular events at different stages of the EM formation (Martin *et al.*, 2001). In the present work, we investigated regulation of plant genes in the premycorrhizal stage with the suppression subtractive hybridization

(SSH) technique. We used a gnotobiotic culture system with oak (*Quercus robur*) microcuttings and the EM fungus *Piloderma croceum*, which is characterized by a delayed EM formation. The extended premycorrhizal phase is associated with morphological and physiological effects on the host plant. The oak plantlets display enhanced lateral root elongation followed by an increase in leaf area although the Hartig net is still not formed (Herrmann *et al.*, 1998). At this stage, photosynthetic activity is also markedly stimulated by the presence of the fungus (Herrmann *et al.*, 2004). This indicates that intensive physiological interactions occur before establishment of the symbiosis. Different morphological markers were described, which allow one to define individual steps before ectomycorrhizal establishment (Herrmann *et al.*, 1998, 2004). We used this well-defined model system to identify genes and proteins involved in the premycorrhizal stage.

Materials and Methods

Mycorrhizal colonization

Quercus robur L. microcuttings were inoculated with *Piloderma croceum* J. Erikss. & Hjortst. in a Petri dish system, as described by Herrmann *et al.* (1998). Fungal plugs were precultured on MMN culture medium (Marx, 1969) with additional carbohydrate source and characterized by production of the yellow pigment corticrocin (Schreiner *et al.*, 1998). With an optimal plug size of *c.* 7 mm diameter obtained after 8–11 d of preculture (23°C in the dark) the fungus was used for root inoculation. The mycorrhization system consisted of a sealed 90 mm Petri dish in which the roots grow two-dimensionally on a MMN medium with 1 : 10 nitrogen and phosphorus and without any carbohydrate source, while the shoot part grows outside of the dish. To reduce the loss of plant material during acclimatization, each of these systems was placed in a 140 mm Petri dish in which humidity was regulated with moistened paper. After 2–3 wk of acclimatization, during which the shoot flush initiated before the inoculation developed, a new root flush started after a variable rest period.

Selection of premycorrhiza-related genes

Root harvest for RNA extraction was performed at the end of the root flush (i.e. after 4 wk of co-cultivation) which correlates with outbursting of the apical shoot bud. Newly developed parts of the main and lateral roots of control and inoculated plants (*c.* 10 plants each per extraction) were frozen in liquid nitrogen before RNA extraction according to Wang *et al.* (2000). The RNA was extracted from at least three independent biological replicates. One RNA preparation was used for SSH, another for Northern or reverse transcriptase polymerase chain reaction (RT-PCR) analyses as outlined below. The RNA from the fungal mycelium was isolated by using an RNA isolation kit (Rneasy; Qiagen, Hilden, Germany). SSH was performed by using the SMART polymerase chain reaction (PCR) cDNA Synthesis Kit and the Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences, Palo Alto, CA, USA) according to the manufacturer's instructions. For this purpose, total RNA from control oak roots (treated in the same way as roots grown in the presence of the fungal mycelium) and mycelium of *P. croceum* (driver) was subtracted from the RNA obtained from premycorrhizal, inoculated oak roots (tester). The subtractive cDNA library obtained by SSH contained 250 clones, which were further used for differential screening. The inserts were amplified by PCR with M13 forward and M13 reverse primers under standard conditions (94°C, 4 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1.5 min). Amplified fragments were separated in duplicates on 1% agarose gels, blotted onto a nylon membrane (Hybond N; Amersham Biosciences, Freiburg, Germany) and hybridized with the radioactively labelled cDNA probes for 18 h at 64°C, under high stringency conditions (Sambrook *et al.*, 1989). The ^{32}P-cDNA probes (1.4×10^6 c.p.m. ml^{-1}) were prepared from 4 μg of total RNA from the control or inoculated roots with Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). After hybridization, membranes were washed at 64°C (2 × 5 min, 2× standard saline citrate (SSC), 0.1% (w : v) sodium dodecyl sulphate (SDS); 15 min, 1 × SSC, 0.1% (w : v) SDS), exposed to Hyperfilm ECL (Amersham Biosciences) and quantified using IMAGEQUANT Software (Amersham Biosciences). The intensity of the hybridization signal from control roots was set as 1.0 and the signal from the inoculated material expressed relative to it. A total of 65 clones were found to be upregulated and sequenced on an automatic sequencer (Li-Cor Biosciences, Lincoln, NE, USA) with the dideoxy chain termination method (Thermosequenase Kit; Amersham Biosciences, Freiburg, Germany). The fluorescently labelled (IRD700 and IRD800) M13 forward/reverse primers were used. Homology searches in data bases were performed by using the BLAST algorithm (Gish & States, 1993) at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) (BLASTX and BLASTN).

Construction of a cDNA library and isolation of the full-length genes

A cDNA library from inoculated, premycorrhizal roots was constructed by using the SMART™ cDNA Library Construction Kit (BD Biosciences). Three genes were amplified from the library by using the Expand High Fidelity PCR System (Roche, Mannheim, Germany) with library-specific (5′ λ TriplEx2) and gene-specific primers as follows: metallothionein 5′-GCGGATATGCAAAACCCAGGGC-3′, formate dehydrogenase 5′-CATCACACAAGAAGCACAAGC-3′ and expansin-like 5′-TGGCAAGTCTAAGCCCTTCCG-3′. The PCR conditions were: 94°C, for 5 min, followed by 30 cycles of 94°C for 45 s, 55°C for 45 s and 72°C for 2.5 min. The sequence reactions were performed as described earlier.

Northern analysis and RT-PCR

Total RNA (1 μg) was separated on 1.2% (w : v) agarose gel containing formaldehyde and transferred to a positively charged nylon membrane (Roche) (Sambrook *et al.*, 1989). Blots were probed with *in vitro* transcribed antisense RNAs of the full-length genes, labelled by DIG-11-UTP and detected with CDP-Star (Roche).

For quantitative RT-PCR, cDNA was generated by reverse transcription of 1 μg total RNA from control or inoculated roots (Superscript II; Invitrogen) and amplified with DyNAzyme II EXT DNA polymerase (Finnzymes OY, Espoo, Finland) under the following conditions: 94°C for 4 min, followed by 20 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min for the 26SrRNA and 25 cycles for the pheromon receptor (PheR)-like gene. The following primers were used: PheR-like forward, 5′-CAAAGGCACCAATTTCTGCTG-3′; PheR-like reverse, 5′-ACGTGCAAGTGTTCCAAAGCC-3′; 26SrRNA-oak forward, 5′-GACTCTCTTAAGGTAGCCAAATGCC-3′; 26SrRNA-oak reverse, 5′-AACGCTTGGCTGCCACAAGCC-3′.

Results

We compared control roots with inoculated roots during the extended premycorrhizal phase at the level of gene expression. Much attention was focused on the homogeneous physiological stage of the root material, in order to restrict the genes to those specific for the recognition stage. The oak microcuttings are characterized by rhythmic growth with successive root and shoot growth flushes which are out of phase. In a previous study (Herrmann *et al.*, 1998) we showed that during the first root flush after inoculation, the maximal elongation rate of lateral roots strictly correlates to a 'swelling' of the apical shoot bud, announcing the onset of a shoot flush. The following 'outbursting' of the shoot buds is a brief, light observable and very precise developmental stage offering a high physiological homogeneity. The RNA was isolated from roots at this step. Even if analysis of fungal genes was not our intention, *P. croceum* was also handled carefully and inoculated under defined, homogenous conditions. Preculture of the fungus on carbohydrate culture media stimulates formation of the yellow pigment corticrocin. After plant inoculation on the carbon-depleted and nitrogen- and phosphorus-reduced medium, white effuse hyphae grew from the plugs towards the roots and, after 2–3 wk, depigmentation of the plugs was observed. Corticrocin synthesis was observed anew in hyphae surrounding short roots announcing the beginning of the EM formation. Using this coloured indicator, root harvest was performed before corticrocin was visible, which ensured that all studies were performed exclusively during the premycorrhizal stage.

About 250 clones were obtained by the SSH technique and further analysed by differential screening in an independent experiment. Finally, 65 plasmids were sequenced; 50 of these encoded different polypeptides. From these, 29 cDNAs with more than 150 nucleotides are deposited in the NCBI (EMBL) database (Table 1). Approximately half of the sequences did not show similarity to any gene deposited in the databases; the residual cDNAs were similar to plant genes of known function, expressed sequence tags (ESTs) or putative open reading frames (Table 1). We could not identify any gene that is exclusively expressed in inoculated roots. This confirms that the cDNA fragments identified are from oak and not from the fungus and become upregulated during early phases of the interaction. Differential expression was confirmed in a third set of experiments by Northern analysis or RT-PCR. This is demonstrated for the metallothionein and expansin genes (because they were full-length) and for the relatively long cDNA encoding the pheromone receptor-like protein (Figs 1 and 2).

A total of 14 cDNAs encoded polypeptides with significant similarities to either known proteins or characterized epitopes (Fig. 3). This includes proteins involved in recognition processes (an inhibitor of apoptosis, a rev interacting protein with similarity to mis3, and the serine carboxypeptidase), in signal perception and transduction (a polypeptide with sequence similarities to a pheromone-receptor from yeast, a peptidyl-prolyl-isomerase and two kinases) and those involved in stress

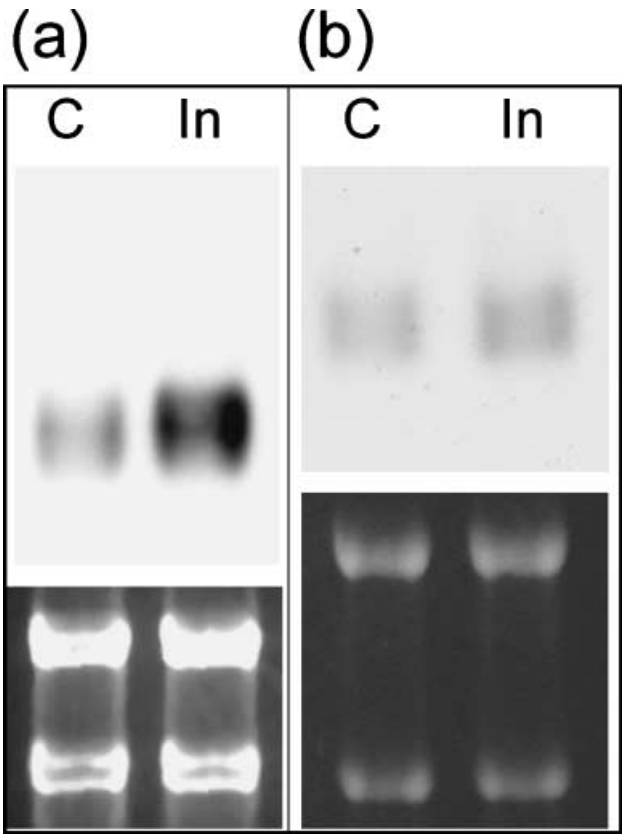

Fig. 1 Expression pattern (Northern analysis) of metallothionein (a) and expansin (b) from *Quercus robur*. Total RNA was isolated from control (C) and inoculated (In) oak roots. Ethidium bromide-stained agarose gels demonstrate equal loading (lower panels).

New Phytologist

Table 1 Symbiosis-related genes, upregulated in the premycorrhizal stage of the association between *Quercus robur* and *Piloderma croceum* (cf. also information deposited in the EMBL Database). For description of E-value, cf http://www.ncbi.nlm.gov

GenBank Accession no.	Database matches	Fragment size (bp)	E-value	Induction level
AJ580022	Peptidylprolyl *cis–trans* isomerase (*Triticum aestivum*)	433	5e-42	1.6
AJ580023	No match	367		2.0
AJ580024	Serine carboxypeptidase III (*Hordeum vulgare*)	427	2e-16	1.2
AJ580025	No match	518		1.9
AJ580026	No match	796		1.8
AJ580027	Putative protein kinase (*Arabidopsis thaliana*)	654	2e-04	2.6
AJ580028	Phosphoglyceromutase (*Ricinus communis*)	650	4e-13	2.8
AJ580029	Poly(A)-specific ribonuclease (PAN1) (*Saccharomyces cerevisiae*)	687	4e-10	2.0
AJ580030	Rev interacting protein mis3-like (*Arabidopsis thaliana*)	521	2e-43	1.7
AJ580031	Putative protein kinase (*Arabidopsis thaliana*)	380	5e-07	2.0
AJ580032	No match	654		1.6
AJ580033	No match	620		2.0
AJ580034	Hypothetical protein (*Arabidopsis thaliana*), similarity to inhibitor of apoptosis protein gblU45881 from *Drosophila melanogaster*	187	8e-08	1.3
AJ580035	Ribosomal protein L17 (*Castanea sativa*)	553	1e-56	1.6
AJ580036	No match	199		1.8
AJ580037	No match	314		2.2
AJ580038	No match	338		1.3
AJ580039	No match	562		1.2
AJ580040	No match	647		1.9
AJ580041	No match	806		3.7
AJ580042	No match	233		1.7
AJ580043	No match	567		2.2
AJ580045	No match	328		2.6
AJ580046	No match	613		2.1
AJ616018	Ca^{2+}-transporting ATPase-like protein (*Arabidopsis thaliana*)	401	8e-06	1.2
AJ577263	Metallothionein-like protein (*Quercus suber*)	454	3e-11	1.2; N 3.8
AJ577264	Expansin-like protein (*Arabidopsis thaliana*)	923	2e-87	1.2; N 1.7
AJ577265	Pheromone receptor-like protein (*Arabidopsis thaliana*)	783	5e-15	1.9; R 2.9
AJ577266	Formate dehydrogenase (*Hordeum vulgare*)	1258	e-177	1.7

Induction levels are derived from differential screening, except for N (from Northern) and R (from reverse transcriptase polymerase chain reaction).

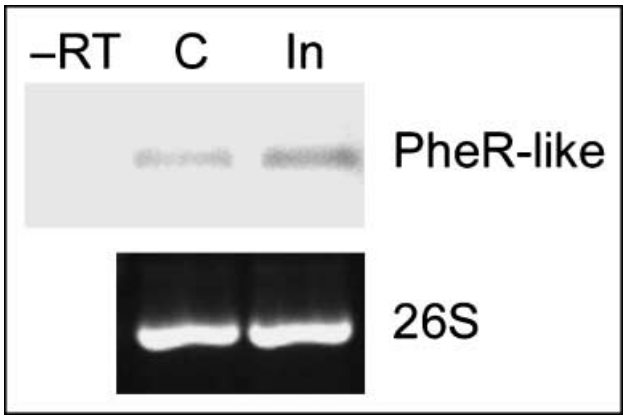

Fig. 2 Expression pattern of the pheromone receptor-like gene (PheR-like) from *Quercus robur*. Total RNA was isolated from control (C) and inoculated roots (In) and used for reverse transcriptase polymerase chain reaction analysis with PheR-like specific (upper panel) or 26S rRNA specific (lower panel) primers. The inoculated sample was subjected to polymerase chain reaction amplification without transcription (–RT) in order to exclude possible contamination with genomic DNA.

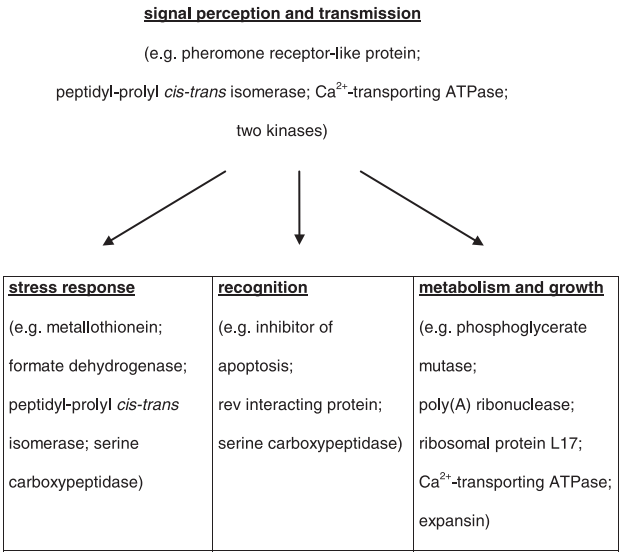

Fig. 3 Summary of cellular functions involved in various processes in the premycorrhizal stage of the association between *Quercus robur* and *Piloderma croceum*. The results are based on DNA sequences obtained after suppression subtractive hybridization (cf. Table 1; for details, see text).

responses (formate dehydrogenase and metallothionein). We also identified genes involved in metabolism and growth, such as phosphoglycerate mutase, poly(A)-specific ribonuclease, ribosomal protein L17, Ca^{2+}-transporting ATPase-like protein and expansin.

Discussion

Suitability of the model to study gene regulation in the premycorrhizal stage

The aim of the present study was to identify genes that are especially involved in the early recognition phase between *Q. robur* and *P. croceum*, before mycorrhiza formation. We have shown previously (Herrmann *et al.*, 1998) that in its long premycorrhizal stage, *P. croceum* influences root development during the limited time window of a growth flush. An important prerequisite to characterize gene regulation at this given premycorrhizal stage was to isolate RNA from material at homogeneous physiological stage. We took advantage of the endogen rhythmic growth of the oaks and have chosen the precise and brief morphological marker 'bud bursting' which corresponds to the end of a root flush to extract RNA from root material. To ensure that only premycorrhizal roots were analysed, we used the easy to handle marker 'nonproduction of the yellow pigment corticrocin' by the fungal partner. With this set of morphological criteria, we could finally analyse root material as homogeneous as possible and at a stage at which premycorrhizal recognition events between the partners have already proceeded, but not the numerous regulation processes that are related to the nutrient transfer in fully differentiated EM. We gathered from these precautions in selecting the analysed roots that a successful screen should fulfil three criteria. (1) The number of known genes, which are characteristic for later phases of mycorrhizal symbioses, as well as the number of genes for enzymes of the primary and secondary metabolism should be low. Consistent with this concept, we did not isolate genes involved in mycorrhiza-initiated changes. In addition we identified a gene for one ribosomal protein while others remain unaltered. Also, none of the genes identified is directly involved in defence, such as genes for the phenylpropanoid metabolism (He *et al.*, 1998), or is a component of the ubiquitin/proteosome pathway (Hilbert *et al.*, 1991; Burgess *et al.*, 1995). (2) The collection of positive clones should include candidates already known to be involved in early phases of symbiotic interactions: expansin is such a clone (Cosgrove *et al.*, 2002). (3) The screen should identify signal perception and transmission components which are highly specific and differ from those involved in general signalling processes of eukaryotic cells. We identified a new receptor-like protein (Fig. 4).

Features of the identified polypeptides

Polypeptides involved in early signal perception and transmission events should perceive fungal signals, or transmit them to downstream events or regulate signal transmission processes. A number of genes and proteins have been described to be involved in plant–microbe interactions (Baker *et al.*, 1997; Bladergroen & Spaink, 1998; Hirsch & Kapulnik, 1998; Endre *et al.*, 2002; Stracke *et al.*, 2002) and several authors suggest a pivotal role of heterotrimeric G proteins in the initial defence response (Beffa *et al.*, 1995) or in root development (Ullah *et al.*, 2003). At least one gene of our screen codes for a soluble protein able to interact with heterotrimeric G-proteins at their receptor-binding sites (Table 1); this protein shows the strongest homology to a pheromone receptor from yeast. The 191 amino acid long open reading frame of the oak protein is also homologous to two different genes from *Arabidopsis* (Accession nos. AY050988 and NP365789). The alignment of the oak protein with the putative pheromone receptor AR781 from *A. thaliana* (Accession no. AY050988) and pheromone receptor Map3 from *Schizosaccharomyces pombe* is shown in Fig. 4a. The homologies between the oak and the *Arabidopsis* protein are particularly striking for their *C*-terminal parts, which share three highly conserved domains. This suggests that both proteins belong to the same family and possibly have related functions. Since the hydrophilic protein from *Arabidopsis* possesses nuclear targeting signals, it might be involved in transferring signals from the cytoplasm to the nucleus. An alignment with the membrane-bound pheromone receptor from *S. pombe* Map3 uncovered that the conserved regions are located within the *C*-terminal inner domains of the receptor (Fig. 4) (i.e. the region which is involved in the interaction of the receptor with the G protein) (Celic *et al.*, 2003). Suharsono *et al.* (2002) have shown that the rice heterotrimeric G-protein functions upstream of small GTPases in early steps of the defence response in disease resistance. Other genes identified in our screen code for putative kinases and for a peptidyl-prolyl *cis–trans* isomerase (Table 1). Although the function of the latter enzyme in plants is little investigated it appears to play a major role in prokaryotic and eukaryotic organisms by catalysing *cis–trans* isomerization of Xaa–Pro peptidyl bonds. Some members of this gene family are induced by stress and involved in physiological adaptation processes. The enzyme participates in protein folding (Matouschek *et al.*, 1995; Rassow *et al.*, 1995; Rospert *et al.*, 1996), protein–protein interactions, in particular interactions with receptor proteins (Tai *et al.*, 1992; Yem *et al.*, 1992; cf. Chen *et al.*, 1997), interaction with Ca^{2+} channels and signal transduction processes related to Ca^{2+} (Brillantes *et al.*, 1994; Cameron *et al.*, 1997), cell growth (cf. Steiner *et al.*, 1997) and mitosis (Lu *et al.*, 1996).

The genes for metallothionein and formate dehydrogenase are normally expressed in response to stress. Metallothioneins, cysteine-rich, metal-binding proteins, are induced by various stimuli, including heavy metals, hormones, endogenous programs or exogenously applied agents acting on signalling processes (Choi *et al.*, 1996; Chatthai *et al.*, 1997; Butt *et al.*, 1998; Andrews, 2000; Ghoshal & Jacob, 2001; Laplaze *et al.*, 2002). They are also involved in the protection against radiation,

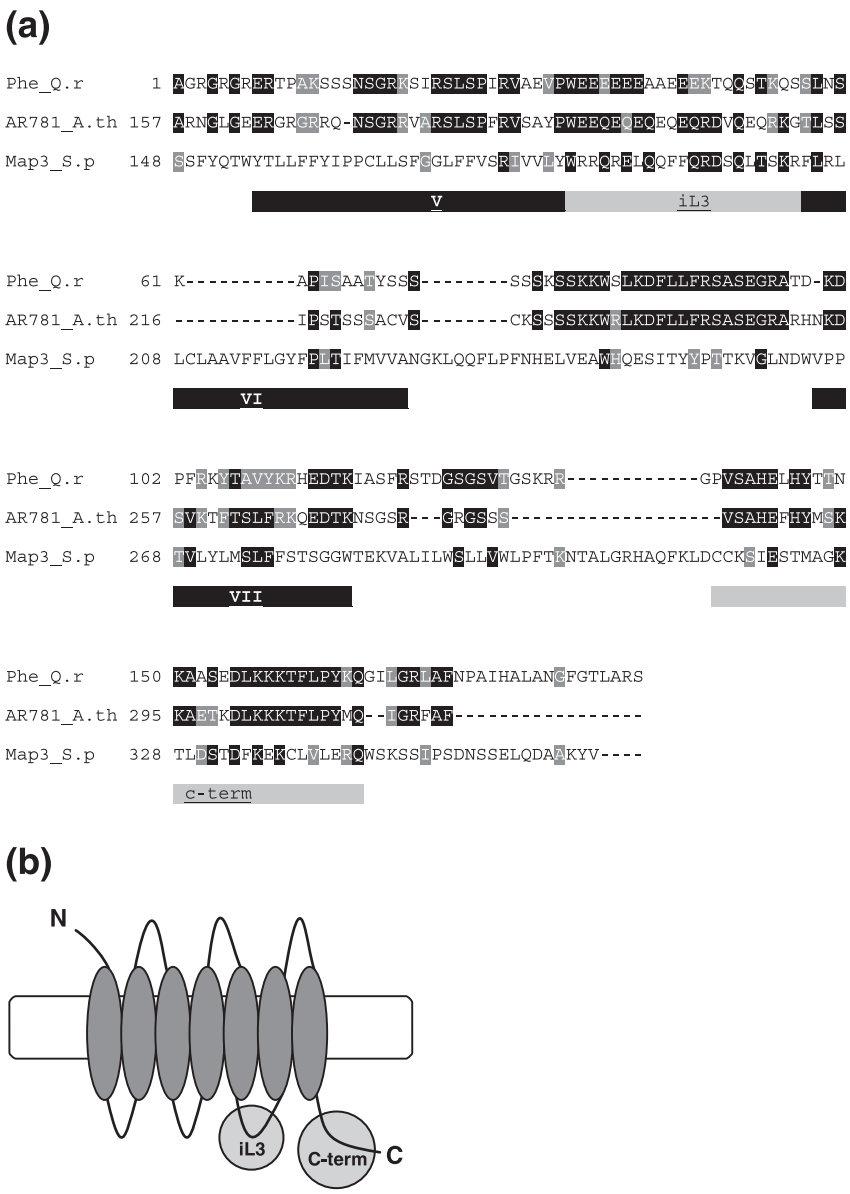

Fig. 4 (a) Alignment of the deduced *Quercus robur* polypeptide (Phe_Q.r., Accession no. AJ577265) with *Arabidopsis thaliana* putative pheromone receptor protein AR781 (AR781_A.th., Accession no. AY050988) and pheromone M-factor receptor from *Schizosaccharomyces pombe* (Map3_S.p.; Accession no. P31397). Conserved domains are marked grey/black, whereas the position of the transmembrane domains in the *S. pombe* protein is underlined by black bars (domains I–IV not shown). Grey bars show regions of homology between the plant proteins and Map3 located in the third inner loop (iL3) and the *C*-terminal cytoplasmic domain (c-term). (b) A model of heterotrimeric G-protein coupled receptors showing the positions of the homologous regions between the plant proteins and Map3 in the iL3 and the *C*-terminal (cytoplasmic domain C-term).

lipid peroxidation or oxidative stress (Liu & Thiele, 1997; Sato & Kondoh, 2002). An increase in oxygenic species normally proceeds cell wall loosening and thus promotes growth. In addition, metallothioneins are stimulated by nitric oxide as a defence response (Klessig *et al.*, 2000; Wendehenne *et al.*, 2001). Thus, stimulation of metallothionein accumulation represents an initial defence response of the roots which might turn into a growth response. Metallothioneins have been reported to be involved in plant–microbe interactions, although they appear to be downregulated during later phases of mycorrhization (Voiblet *et al.*, 2001; Johansson *et al.*, 2004). In three independent experiments we confirmed that the metallothionein gene belongs to the most upregulated genes in our oak screen (Table 1; Fig. 1a). Eleven cDNAs which have been isolated in our screen code for a second stress response protein, formate dehydrogenase. This enzyme is located in mitochondria and catalyses the oxidation of formate to carbon dioxide, reducing NAD to NADH. Formate dehydrogenase plays a crucial role in the biosynthesis of compounds involved in energetic metabolism and in stress-induced signal transduction pathways. In potato leaves expression of the enzyme is stimulated by various stresses inducing hypoxia (Des Francs-Small *et al.*, 1993; Suzuki *et al.*, 1998).

The expression of genes for an inhibitor of apoptosis, a rev interacting protein and a serine carboxypeptidase suggests involvement of processes characteristic for programmed cell death and those inhibiting apoptosis. Inhibitors of apoptosis have been identified in insects and mammals and are characterized by the *N*-terminal baculovirus IAP repeats and a *C*-terminal RING-FINGER domain involved in protein–protein interactions

(Liston *et al.*, 1996). This *C*-terminal RING-FINGER domain is highly conserved in our oak protein with similarities to an inhibitor of apoptosis. These proteins can bind tumour-associated factors and thus inhibit a death signal (Uren *et al.*, 1996). The involvement of inhibitor of apoptosis proteins during the establishment of mycorrhiza is not surprising, since the prevention of cell death is a prerequisite for successful Hartig net formation. Finally, the rev interacting protein exhibits homology to mis3. Rev proteins are involved in RNA nuclear export and in targeting cytoplasmic RNAs to polyribosomes to activate their translation efficiency (Boris-Lawrie *et al.*, 2001). Mis3 is essential for ribosome RNA processing and thus ribosome biogenesis and is implicated in the start of cell growth and the S-phase checkpoint (Kondoh *et al.*, 2000).

The premycorrhizal period is characterized by a substantial promotion of root growth, thus a stimulatory effect on growth and metabolism related genes is not surprising. It has been reported that expansin gene expression clearly proceeds the growth response. During the colonization of legume roots by *Rhizobium* and the formation of nodules, the delivery of expansin to the cell wall correlates with root growth and changes in the root hair shape (Cosgrove *et al.*, 2002).

Little is known about most of the proteins identified in this screen. Epitopes of several oak symbiosis-related proteins exhibit substantial similarities to *Arabidopsis* proteins. Although *Arabidopsis* does not form mycorrhiza it was recently shown to interact with *Piriformospora indica* (Varma *et al.*, 2001; T. Peškan-Berghöfer, unpubl. data), a fungus belonging to the Sebacinaceae. The availability of knock-out lines in *Arabidopsis* provides an additional tool to analyse the role of some genes identified in oak–*P. croceum* mycorrhiza, and to address the question whether they are also involved in other plant–microbe interactions.

Our results obtained with the SSH approach allowed a first insight in the gene regulation during the premycorrhizal stage. Progress in genomic investigations offers a large number of available ESTs for poplar (Kohler *et al.*, 2003) and oak (C. Plomion, unpubl. data). Available transcripts expressed in oak will now be used for microarray analysis in our oak ectomycorrhizal system to study gene regulation at a larger scale and under the influence of different parameters involved in the onset of mycorrhization (Herrmann *et al.*, 2004).

Acknowledgements

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