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BIOTIC AND ABIOTIC STRESS

Identification and characterization of differentially expressed genes from *Fagus sylvatica* roots after infection with *Phytophthora citricola*

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Abstract *Phytophthora* species are major plant pathogens infecting herbaceous and woody plants including European beech, the dominant or co-dominant tree in temperate Europe and an economically important species. For the analysis of the interaction of *Phytophthora citricola* with *Fagus sylvatica* suppression subtractive hybridization was used to isolate transcripts induced during infection and 1,149 sequences were generated. Hybridizations with driver and tester populations demonstrated differential expression in infected roots as compared to controls and verify efficient enrichment of these cDNAs during subtraction. Up regulation of selected genes during pathogenesis demonstrated using RT-PCR is consistent with these results. Pathogenesis-related proteins formed the largest group among functionally categorized transcripts. Cell wall proteins and protein kinases were also frequently found. Several transcription factors were isolated that are reactive to pathogens or wounding in other plants. The library contained a number of jasmonic acid, salicylic acid and ethylene responsive genes as well as genes directly involved in signaling pathways. Besides a mechanistic interconnection among signaling pathways another factor explaining the activation of different pathways could be the hemibiotrophic life style of *Phytophthora* triggering different signals in both stages.

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Abbreviations

AP2/ERF	APETALA2/Ethylene response factor
cfu	Colony forming unit
DS0	Driver before subtraction
EST	Expressed sequence tag
JA	Jasmonic acid
Myb	Myeloblastosis viral oncogene homolog
NAC	Petunia no apical meristem and *Arabidopsis* transcription activation factor 1/2, and cup-shaped cotyledon 2
Ns	Non-significant
PCR	Polymerase chain reaction
PR	Pathogenesis-related protein
RT-PCR	Reverse transcription-polymerase chain reaction
S1	First subtraction
S2	Second subtraction
SA	Salicylic acid
SSH	Suppression subtractive hybridization
SNP	Single nucleotide polymorphism
TS0	Tester before subtraction
UTR	Untranslated region
WAK	Wall associated kinase

Introduction

Phytophthora, the plant destroyer, causes serious diseases in many crops and wild plants. Each of the more than 90 species of this genus of the oomycetes attacks plants.

P. infestans, e.g., causes late blight of potato and *P. sojae* is responsible for soybean root rot. Worldwide, another species *(P. ramorum)* causes the sudden oak death. *Phytophthora citricola* is a further hemibiotrophic pathogen with worldwide distribution and a wide variety of hosts, one of them *Fagus sylvatica. F. sylvatica* is the dominant or co-dominant tree in temperate Europe and an economically important species valued for its wood and in many varieties as ornamental tree. Studies indicate that synergistic interactions between root losses by soil borne *Phytophthora* species and climatic extremes are a major cause for the general decline of beech forests across Europe (Jung, in press).

In controlled and efficient root inoculations young European beech trees (*F. sylvatica*) quickly developed disease symptoms defining the interaction with *P. citricola* as compatible. In compatible interactions the pathogen is not efficiently detected by the host and activation of defense mechanisms is weak, late or even suppressed by the pathogen granting successful infection and causing disease (for review see, e.g., Schulze-Lefert and Panstruga 2003; Euglem 2005; Tyler 2009). In compatible interactions with plant roots *Phytophthora* is able to establish a short biotrophic phase with extended haustoria formation and colonization of vascular tissue. The pathogen then enters the necrotrophic phase and large parts of the root system become necrotic. In incompatible interactions on the other hand fast development of a local hypersensitive reaction of the host almost completely prevents haustoria formation and infection of vascular tissue (Enkerli et al. 1997). While interactions of herbaceous plants with pathogens including *Phytophthora* species are often analyzed with transcriptional profiling revealing many specific facets of the different pathosystems, such global studies of interactions of trees are missing. One issue is the lack of sequence information for most trees, with the exception of poplar. In case of *F. sylvatica* there were as little as 350 expressed sequence tags (ESTs) available in the public databases, so far. Suppression subtractive hybridization (SSH) is a powerful tool to enrich differentially expressed transcripts and was thus used to construct three cDNA libraries and to investigate which defense reactions are triggered by this hemibiotrophic pathogen in *F. sylvatica*.

Materials and methods

Plant and pathogen growth and infection experiments

Fagus sylvatica seeds were germinated at 4–6°C for 55 days in the dark. Individual seedlings were transferred to tubes with autoclaved tap water and grown at 18°C under natural daylight conditions until roots reached an average of 10 cm length and two leaves were developed (~19 days). Only plants free of any visual infections were selected for the experiments. *P. citricola* was maintained on V8-Agar (100 ml vegetable juice, 3 g $CaCO_3$, 16 g Agar-agar and 650 ml distilled water). Three days before plant infection young mycelia were transferred to malt agar plates [2% (w/v) malt, 2% (w/v) agar]. Mycelia from the outer circle of these plates were used for infections. For RNA extraction *P. citricola* was grown in liquid medium (Terce-Laforgue et al. 1992).

For construction of the subtractive cDNA library two groups of ten *F. sylvatica* seedlings each were placed into 2-l containers with autoclaved distilled water. For the infection group ten agar plugs of 7 mm diameter with *P. citricola* mycelia were added. Further ten plants were control plants mock infected using malt agar plugs without *P. citricola*. The roots of the two groups were harvested after 6 days and each group's roots were pooled. At harvest a small piece of root from each plant was plated on selective medium (Jung et al. 1999) for re-isolation of the pathogen. Pathogen identity was verified in a PCR reaction using specific primers (Schubert et al. 1999). For independent tests of gene expression by reverse transcription-polymerase chain reaction (RT-PCR) the experiment was repeated in a consecutive year with two biological replications of 15 plants each for controls and infections. A second infection in a more natural soil-infection experiment was also analyzed to test expression of genes isolated by SSH. This experiment consisted of two biological replications of 35 mock-inoculated plants and four biological replications of 35 *P. citricola*-infected plants per replicate following the protocol of Jung et al. (1999). Sampling was performed when first symptoms appeared.

Subtractive cDNA library construction

For construction of the library RNA extraction was performed according to Chang et al. (1993). From the extracted RNA three forward libraries were construction using two separate subtraction reactions with different ratios of *F. sylvatica*:*P. citricola* RNA in the driver population. The subtractive cDNA libraries were established by vertis Biotechnologie AG (Freising, Germany).

For the first library 10 µg RNA from infected roots were used as tester and 10 µg RNA from control roots plus 0.14 µg RNA from *P. citricola* grown in liquid culture as driver. The cDNAs of driver and tester were amplified using long and accurate PCR (LA-PCR; Baskaran et al. 1996). Because of an uneven size distribution with the cDNA populations both were size-fractionated to 500–700 bp. The cDNAs of the second subtraction (S2) were cloned into pBluescript II SK+ using the following adapters: 5′-end: CCC GGG GTG AGC CAG AGG ACG

AGA CAA; 3′-end: GAA TTC TGG TGC TGG CTG TCG CTC ATG CGG CCG C(dT25).

For the second and third library 4.5 μg RNA from infected roots were used as tester and 4.5 μg RNA from control roots plus 0.14 μg RNA from *P. citricola* as driver. The cDNAs of driver and tester were amplified using LA-PCR. Before ligation into pBluescript II SK + using the following adaptors: 5′-end: GAA TTC CAT CAG GCA GAG GAC GAG ACA and 3′-end: GCG GCC GC(dT25), the S2 cDNAs were divided into two size fractions of 0.5–1 kb and > 1 kb.

Hybridization of tester and driver and colony-PCR

To confirm subtraction success 100 ng S0-cDNAs of tester and driver as well as S1 an S2-cDNAs were hybridized with P^{32}-labeled first-strand cDNA of tester and driver respectively by vertis Biotechnology. To assess insert sizes of the libraries colony-PCRs using T3- and T7-primers were performed by vertis Biotechnology. In case of the first library 48 colonies were randomly picked and single-colony PCRs were conducted. For the other two libraries 1,000 colonies each were rinsed off a plate and used for PCRs.

Sequencing and data analysis

The cDNAs were sequenced from the 5′ end. Sequencing was carried out by GATC Biotech AG (Konstanz, Germany). For DNA data handling and analyses the SEQtools software package was used. Microsatellites were searched using MSATCOMMANDER (Faircloth 2008) with minimum number of repeats set to five for di-nucleotide repeats and four for all others. Wu-Blast 2.0 searches were conducted at TAIR against the all Viridiplantae protein database from GenBank and the TAIR7 database at protein level. Additionally, BLAST searches against the GenBank non-redundant database on nucleotide and protein level were conducted. Sequence alignments were generated using Clustal-W (Thompson et al. 1994) and muscle (Edgar 2004) optimized with t-coffee (Notredame et al. 2000). Protein locations were predicted using WoLF PSORT (Horton et al. 2007) and SignalP (Emanuelsson et al. 2007). Pattern and profile searches were performed using the InterProScan Sequence Search (Zdobnov and Apweiler 2001).

Semi-quantitative RT-PCR

Two independent biological replicates were established in the in vitro experiment. Two biological replicates for the control and four for infection were analyzed in the soil infection. For each of those two technical replicates were performed for root samples in in vitro and leaf samples in soil infections, respectively. RNA for RT-PCRs was isolated using the following protocol. Tissue was ground in liquid nitrogen and 500 mg were added to 8 ml pre-warmed extraction buffer [100 mM Tris–HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, 2% (w/v) PVP 360000, 0.5% (w/v) spermidin], 240 μl β-mercaptoethanol and 3 μl RNase inhibitor (Roche, Mannheim, Germany) were added. Lysis was performed at 55°C for 15 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added. After mixing 350 μl of Nucleon Phytopure Resin (GE Healthcare, Freiburg, Germany) was added and the mixture was shaken for 3 min. Samples were centrifuged at 11,000 g for 15 min at 4°C. The chloroform extraction of the supernatant was repeated as before. The RNA was precipitated with an equal volume of isopropanol for 15 min at room temperature and centrifuged at 12,000 *g* for 30 min at 4°C. The pellet was washed with 70% ethanol and dried for 5–10 min before it was resuspended in 180 μl water. Ambion DNA free Kit (Applied Biosystems, Darmstadt, Germany) was used for DNA removal following the manufacturer's protocol supplemented with 1 μl of RNase inhibitor. First strand cDNA was synthesized from 1 μg of total RNA for test genes and 1 ng for 5.8S RNA, used as control. First strand synthesis was performed with M-MLV reverse transcriptase RNase minus point mutant (Promega, Mannheim, Germany) following the manufacturers protocol at 51°C with gene specific primers (−2 primers listed hereafter) or Oligo (dT) 15 primer (Promega, Mannheim, Germany). PCR amplification was conducted using GoTaq polymerase (Promega, Mannheim, Germany) from 5 μl cDNA for test genes and 1 μl for 5.8S with these primer sets: Fs_Pc_002_D5_1: AAT TAA GGC TGG CAA AGA CAA GG and Fs_Pc_002_D5_2: TGA ACT TCT CTG GCA ACA AAT CAA C (annealing at 55.8°C); Fs_Pc_002_G7-1: AGA TGA AGA AGC AGG GCA GAT TCC and Fs_Pc_002_G7-2: CCA CTC TCC TAT TCG TTG GGT ATA TTG (annealing at 54°C); Fs_Pc_007_C12_1: CGG GAG GGC TCA TGG TAT TTA TGT G and Fs_Pc_007_C12_2: AAC CAC GAG CCA ACC TGA ATA TCC (annealing at 56.2°C); Fs_Pc_008_A12-1: CGC ATA CTG GGT GTG GGC GAC CCG A and Fs_Pc_008_A12-2: CCG TTA TTG GCA CCG TTA TCC TCC TC (annealing at 60.3°C); Fs_Pc_009_F7-1: ACG CAC TGC GAG GTG ACA AAG and Fs_Pc_009_F7-2: CCG CGA GGG TGA TAA TAA AGT TTC C (annealing at 54°C); Fs_Pc_012_H10_1: TGA GAA GTC AGG AAG GGT GTT GGA and Fs_Pc_012_H10_2: CAG GAT TCA AAA TTG TGG AAG GGA A (annealing at 55.7°C); Fs_Pc_013_G4_1: GGA TTC ACT CCT CCC TCA ACT GGT A and Fs_Pc_013_G4_2: CAT GAA AGA GAT GAG TGT GGT GGT G (annealing at 54.3°C); 5.8S-1: ACG ACT CTC

GGC AAC GGA T and 5.8S-2: GTG ACA CCC AGG CAG ACG T (annealing at 57°C). Following an initial denaturation for 1 min at 95°C all steps of the PCR lasted 30 s. PCR cycle numbers were empirically optimized to avoid over-cycling. For Fs_Pc_002_D5, Fs_Pc_002_G7, Fs_Pc_009_F7, Fs_Pc_012_H10 and Fs_Pc_013_G04 30 cycles, for Fs_Pc_007_C12 and Fs_Pc_008_A12 25 cycles of amplification and for 5.8S RNA 25 cycles in roots and 20 cycles in leaf samples were carried out. RT-PCRs were run on agarose gels, stained with ethidium bromide and photographed with a digital camera (Kodak Electrophoresis Documentation and Analysis System 120). Bands were quantified using the ImageQuant software (Nonlinear Dynamics, Newcastle upon Tyne, UK) using local average background subtraction and normalized to 5.8S RNA amplicon.

Results

Disease symptoms and confirmation of infection

In the in vitro infection slight browning of some parts of the root system was visible 3–4 days post infection. After 6 days the fine roots showed extended necrosis and also the root collar showed lesions. In the repeated experiment used for RT-PCR the time-scale was the same but necrosis were less intense and the root collar did not show any symptoms when samples were taken. In the soil experiment first leaf symptoms were evident 7 days post infection at the time of sampling.

Phytophthora citricola re-isolation and PCR identification from infected roots was successful and all control roots were clean. Microscopic inspection of the agar plugs used for in vitro infection revealed zoosporangia with zoospores and also empty zoosporangia.

Properties of the cDNA libraries

After cDNA synthesis for the first library it became evident that the length of the driver population was shorter than of the tester (Supplementary Figure 1a). The cDNAs of both tester and driver were therefore size fractionated to 500–700 bp before subtraction (Supplementary Figure 1b). Aliquots of first and second subtraction (Supplementary Figure 1c, d) were analyzed on agarose gels before the S2 population was cloned. The first library had a yield of 500,000 colony forming units (cfu) and 87.5% insert size above 500 bp, 10.5% below 500 bp and only 2% had no insert.

The cDNA synthesis was repeated in order to increase the yield of longer fragments. Additionally, a change in ratio of RNA from control roots to RNA from *P. citricola* within the driver population was made. For the construction of the second and third library the new tester and driver cDNAs were used. This time both cDNA populations had the same size range (Fig. 1). To test the enrichment of tester transcripts during subtraction cross hybridizations of tester and driver were performed. Differences in gene expression are already evident in the cDNAs before subtraction (S0) with obviously higher expression of a fair amount of transcripts within the tester compared to the driver. In S2 cDNAs with stronger expression in the tester are clearly enriched (Fig. 1). The S2 cDNAs were divided into two size fractions of 500 bp–1 kb and > 1 kb for more efficient cloning, especially of larger cDNAs. These are referred to as second and third cDNA library within the paper. The second library had a yield of 100,000 cfu and the third of 33,000 cfu. The insert sizes of both libraries were analyzed by mass-PCR of 1,000 colonies per library and confirmed presence of cDNAs up to 2 kb within the fraction of above 1 kb (Supplementary Figure 2).

Sequence analysis

After removal of empty vectors 1,149 EST sequences were further analyzed (success rate 92%). Sequence data were deposited in GenBank under the accession numbers

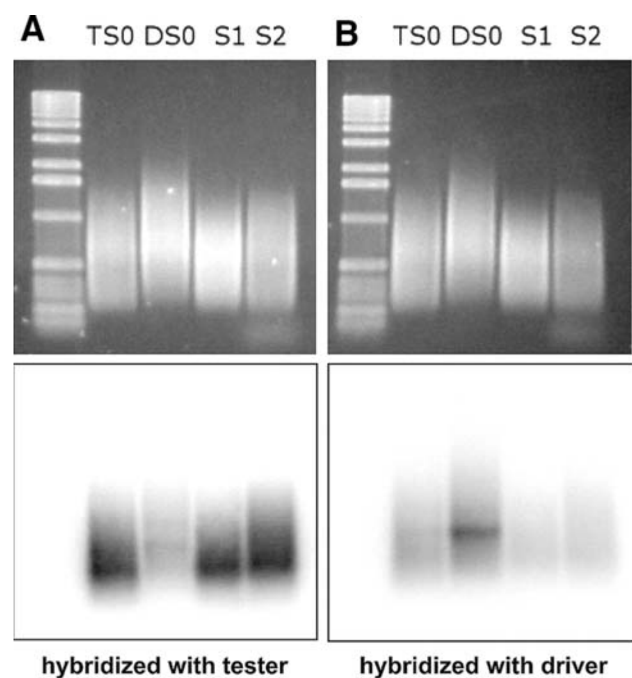

Fig. 1 Cross hybridization of tester and driver cDNAs. **a** upper part gel image, lower part hybridization with tester. **b** upper part gel image, lower part hybridization with driver. *TS0* tester before subtraction, *DS0* driver before subtraction, *S1* first subtraction, *S2* second subtraction

FL482109–FL483257. The sequences have been trimmed to remove vector, adapter and low quality sequences and were organized into functional categories based on the MIPS plant functional catalogue (Ruepp et al. 2004). Scanning for microsatellites within the ESTs revealed 47 di-,85 tri-, 11 tetra-, and two penta- and hexa-nucleotide repeats respectively (Supplementary Table 2). Clustering of the ESTs revealed a high proportion of singletons (72.8%). The cDNA sequences had an average content of 45.9% GC after vector and adaptor clipping. The average length sequenced per clone was 515 bp. From the first library 535 sequences were retrieved, from the second 273 and from the third 341. The complete collection enfolded 46.6% ESTs from the first subtraction and 53.4% from the second. There was a slightly higher percentage of ESTs without database hit within the second subtraction (46.1% compared to 42.1% in the first). In general, small sequence differences like single nucleotide polymorphisms (SNPs) are more likely to reflect the different individuals pooled for library construction and demonstrate genetic diversity of these genes. Differences resulting in more distinct amino acid sequences as for example in the four groups of protease inhibitors found in the EST collection (Supplementary Figures 3, 4) are likely to be assigned to different isoforms of genes which are often found among PR proteins.

The distribution of functional categories among ESTs with classification is shown in Fig. 2. All sequences with similarity to proteins expressed in other plants but without clear functional classification were denoted by expressed proteins. Sequences with no database hit or similarities to organisms like human or mouse were categorized as unclear. The first subtraction yielded more ESTs involved in cell structure while in the second the percentage of disease/defense-related and those of energy metabolism was higher. Within the category of cell structure cell wall proteins were found to be most dominant (84%) with half of the sequences coding for proline-rich proteins alone. Half of the ESTs in the category of signal transduction were characterized as kinases. The category of disease/defense was compiled from almost 50% PR-proteins. Within the protein destination and storage category most were involved in proteolysis followed by folding. From the sequences with homology to other *Phytophthora* species elicitins and ribosomal proteins were the most frequent functional classes. In the interaction of *P. sojae* with soy bean elicitins were found to be up regulated late when the pathogen entered the necrotrophic growth stage (Moy et al. 2004). The strong presence of elicitins in the libraries is therefore in agreement with the observed necrosis at the time of sample harvest. A complete list of ESTs and BLAST results is given in the Supplementary Table 1.

Transcription factors

Among the transcription factors of the libraries several with known functions in stress response regulation were identified. Two ESTs exhibited similarity to ethylene responsive transcription factors. One of them (Fs_Pc_003_D11) contained the InterPro domain IPR001471 characterizing

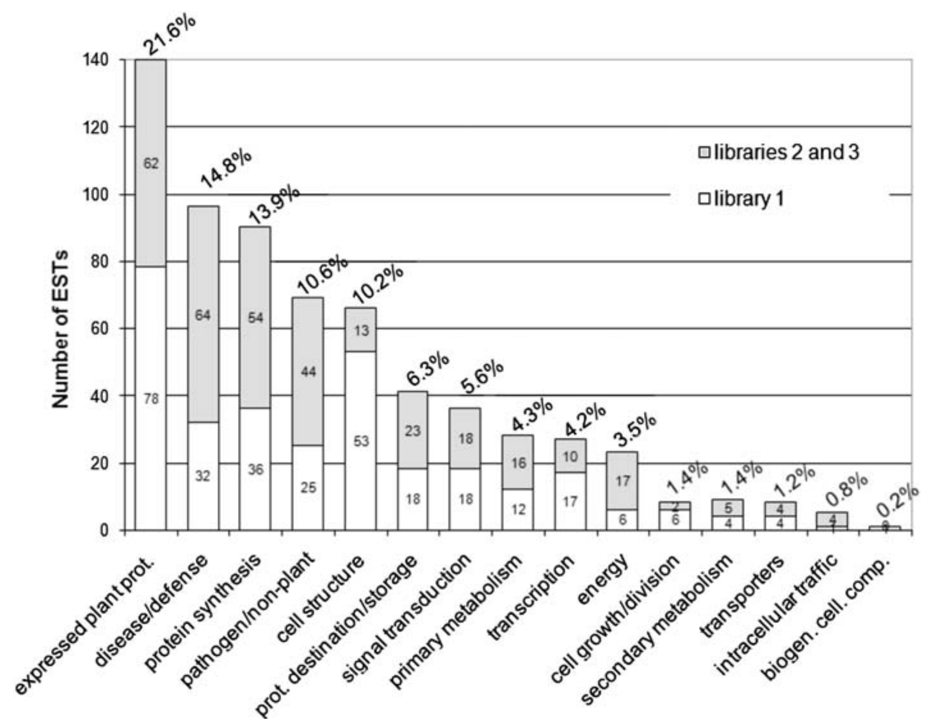

Fig. 2 Contribution of the two subtraction reactions to the functional categories. ESTs without any homology are omitted. Percentages given of total collection

pathogenesis-related transcriptional factors with AP2/ERF (APETALA2/Ethylene Response Factor) domain. The other one (Fs_Pc_001_A6) enclosed an InterPro IPR001387 Helix-turn-helix type 3 domain and similarity to a mulit-protein binding factor 1b of *Arabidopsis* that is also involved in ethylene signaling (Riechmann and Meyerowitz 1998). The library also contained three members of the WRKY transcription factor family suggested to play a key role in regulating pathogen-induced defense (Euglem et al. 2000). A further EST included a NAC domain (petunia no apical meristem and *Arabidopsis* transcription activation factor 1/2, and cup-shaped cotyledon 2) and showed high homology to ATAF1, a wound and pathogen inducible *Arabidopsis* transcription factor (Collinge and Boller 2001). The collection also enfolded two myeloblastosis viral oncogene homolog (myb)-family transcription factors and one homolog of an AG-motif binding protein-1 which mediates activation of a wound-inducible myb gene in tobacco (Sugimoto et al. 2003). This EST's up-regulation in infected roots was verified by RT-PCR (Fig. 3). Besides involvement in wound response some myb-family members are also part of pathogen reaction (Yang and Klessig 1996).

PR-1

The libraries contained three sequence coding for *pathogenesis-related* protein 1 (PR-1) homologs, all of them with amino acid differences in their deduced protein sequence. One of them was a full-length clone (Fs_Pc_009_H09) coding for a basic isoform (calculated pI 8.97) and probably located in the vacuole. Basic PR-1 proteins can be induced by ethylene (Agrawal et al. 2001). In general, PR-1 proteins were shown to be up regulated after pathogen infection, salicylic acid (SA) treatment (van Loon et al. 2006), herbivore feeding (Moran and Thompson 2001), wounding (Agrawal et al. 2000) as well as in rice after jasmonic acid (JA) treatment (Agrawal et al. 2000; Mitsuhara et al. 2008). Inhibition of pathogen growth by PR-1 was also demonstrated (Niderman et al. 1995). Overexpression of a pepper PR-1 in tobacco enhanced resistance to *P. nicotianae*, *Ralstonia solanacearum* and *Pseudomonas syringae pv. tabaci* (Sarowar et al. 2005).

β-1,3-glucanases—PR-2

Plant β-glucanases are capable of releasing oligosaccharides from cell walls of pathogens eliciting plant defense (Keen and Yoshikawa 1983) and have been shown to be induced by pathogen (Castresana et al. 1990) or ethylene treatment (Boller 1988). The six isolated EST sequences with homology to β-1,3-glucanases could be divided into two groups. The first group contained four sequences with some amino acid polymorphisms belong to PR-2 class I. PR-2 class I proteins are basic proteins localized in the cell vacuole (Van den Bulcke et al. 1989) and possess antifungal activity (Sela-Buurlage et al. 1993). The second group consisted of two sequences with only a SNP in the coding region and a length difference in the 3′ untranslated region (UTR) but with identical amino acid sequence. These sequences aligned to the C-terminus of a new class of

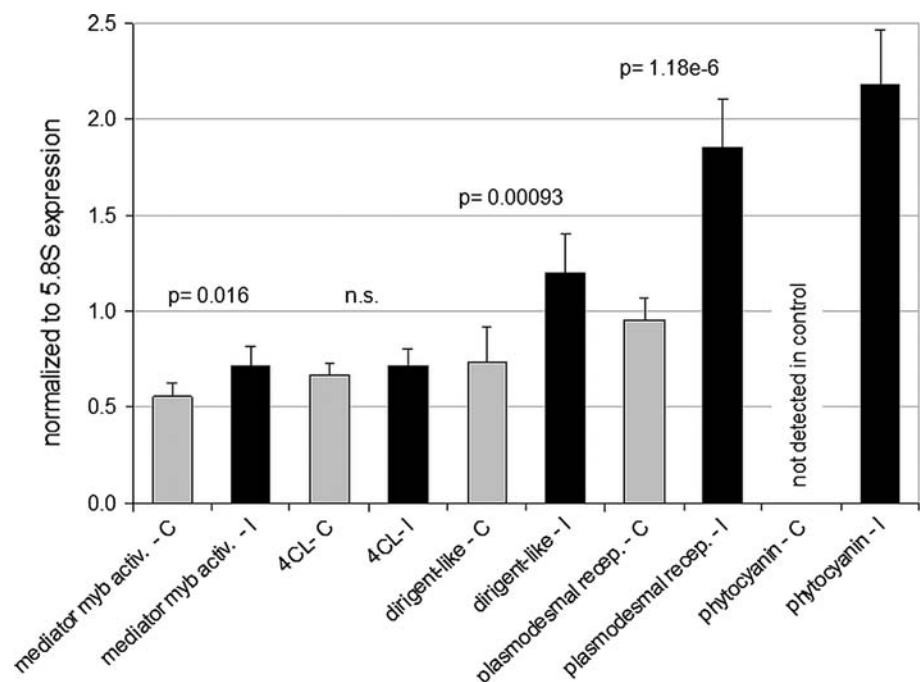

Fig. 3 Reverse transcription-polymerase chain reactions of sample genes. Measurements are normalized to 5.8S expression. For significant changes *P*-values are given. *Grey* control, *black* infected. *4CL* 4-coumarate-CoA ligase

glucanases and contained a carbohydrate-binding domain (X8/CBM43) that is absent in the other PR-2 sequences.

Chitinases—PR-3

Chitinases were found in low amounts in healthy plants, but induction has been observed after inoculation with fungi (Kombrink et al. 1988), bacteria (Lummerzheim et al. 1993) or viruses (Margis-Pinheiro et al. 1993), wounding (Hamel and Bellemare 1995), ethylene (Boller et al. 1983) or SA (Kang et al. 1998) and JA treatments (Davis et al. 2002). Even though treatment with β-1,3-glucanases or chitinases alone can inhibit fungal growth in vitro, more commonly combinations of the two enzymes are required for antifungal activity (reviewed in Boller 1993) explaining the co-expression even in response to a pathogen that lacks chitin. Four ESTs with homology to PR-3 were isolated. Two of them were basic proteins of class I with differences at four amino acids and a probable vacular localization (Neuhaus et al. 1991). The third only showed weak similarity to a basic class I protein. Lummerzheim et al. (1993) showed that transcripts hybridizing with a bean basic class I chitinase accumulated in *Arabidopsis thaliana* during both compatible and incompatible interactions with *Xanthomonas campestris*. The forth EST belonged to PR-3 class IV and is predicted to be secreted. In *Arabidopsis* induction of class IV was measured shortly after infection with *X. campestris* (Gerhardt et al. 1997).

Osmotin—PR-5

The group of PR-5 proteins can be divided into several subgroups. Members of the subgroup of osmotins have been isolated from a variety of plant species and are presumed to accumulate in the vacuole (for review see Anžlovar and Dermastia 2003). The mode of osmotin activity is unclear. However, inhibitory activity against *Phytophthora* was demonstrated for osmotins in vitro (Woloshuk et al. 1991) and overexpression of PR-5 in orange enhanced tolerance against *Phytophthora* (Fagoaga et al. 2001). Two sequences of osmotins were found in the libraries with only minor nucleotide and amino acid differences.

Protease inhibitors—PR-6

Some insects and many pathogenic microorganisms secrete proteolytic enzymes to increase the availability of amino acids for their growth. As defense mechanism plants posses various inhibitors of these proteases (van Loon et al. 2006). Homology searches revealed 15 ESTs coding for members of the potato type I serine protease inhibitor family. These sequences were divided into two groups (Supplementary Figure 3). The first one comprised ten sequences that encode five different amino acid sequences. The second group included five sequences coding for three different amino acid sequences. A large group of 17 ESTs exhibited similarities to miraculin/Lemir. These sequences were organized into two groups based on their nucleotide sequences. In both categories at least one full-length sequence was present. The first category consisted of four ESTs with some amino acid polymorphisms. The second contains 13 sequences coding for five slightly different proteins. Both groups share the soybean trypsin inhibitor (Kunitz) family signature (Supplementary Figure 4). Antifungal activity of a Kunitz-type trypsin inhibitor has been reported from the roots of *Pseudostellaria heterophylla* (Wang and Ng 2006).

PR-10

Even though PR-10 proteins are known to be induced by pathogens (El-kereamy et al. 2009) or abiotic stresses (van Loon et al. 2006), their particular biological functions are still not clear. They show sequence similarities to ribonucleases and ribonucleolytic activity was demonstrated (e.g., Bantignies et al. 2000; Liu et al. 2006). Other proposed functions include cytokinin binding (Fujimoto et al. 1998), metabolic activity in secondary metabolism (Samanani et al. 2004) or steroid carrier function (Markovic-Housley et al. 2003). PR-10 proteins from *Oxalis tuberosa* and *Solanum surattense* exhibited antibacterial and antifungal activities (Flores et al. 2002; Liu et al. 2006). The library contained seven sequences of PR-10 with some differences in amino acids and also some differences in the 3′ UTR.

Genes associated with chemical defense

Germin-like proteins generate hydrogen peroxide that can be toxic to attackers and also stimulates plant-defense (e.g., Hu et al. 2003). There were four ESTs present with homology to germin in the library encoding protein sequences with differences at single amino acid positions. Another sequence harbored the typical domain of berberine bridge enzymes involved in alkaloid biosynthesis (IPR012951). Additionally, two transcripts for superoxide-dismutases were found.

RT-PCR

Semi-quantitative RT-PCR was performed for seven genes in two independent infection experiments to analyze gene expression. Genes for RT-PCR were randomly chosen from different categories that could be involved in pathogen response (signal transduction, transcription, cell structure,

disease/defense, secondary metabolism). Four of the genes (Fs_Pc_009_F07: mediator of myb transcription factor activation, Fs_Pc_013_G04: phytocyanin homolog, Fs_Pc_012_H10: plasmodesmal receptor, Fs_Pc_007_C12: drigent-like) showed significant up regulation in infected samples (Fig. 3). A slight but not significant up regulation was detected for a putative 4-coumarate-CoA ligase (Fs_Pc_002_G7). For an EST with weak homology to PR-10 (Fs_Pc_002_D5) no amplification could be detected and RT-PCR with primers for a zinc finger (MYND type)/F-box protein (Fs_Pc_008_A12) resulted in unspecific reactions. The up regulation of selected genes after infection measured with RT-PCR verified that pathogen-induced genes were isolated.

Discussion

The purpose of this study was to identify and characterize genes regulated after infection of *F. sylvatica* with the hemibiotrophic pathogen *P. citricola*. SSH was chosen to establish a cDNA library enriched for genes expressed during the host–pathogen interaction.

The late time-point of infection was selected for the isolation of defense-related ESTs because in compatible interactions a delay of plant defense reaction was observed (e.g., Rinaldi et al. 2007) and this is reflected by the low number of transcripts of secondary metabolism isolated in this study. The formation of strong symptoms confirmed that *P. citricola* did not only colonize *F. sylvatica* externally but also infected and caused disease at the time the RNA was isolated. The SSH procedure efficiently selected tester over driver cDNAs (Fig. 1) and the libraries were in fact enriched with transcripts involved in pathogen response with more than 30% belonging to the categories disease/defense, cell structure and signal transduction (Fig. 2). Actually, PR proteins formed the biggest group of clearly classified ESTs. As regarding to signal transduction the libraries contained 15 different protein kinases. One of them exhibits high homology with cell wall associated WAK-like (wall associated) kinases which are required for pathogen response (He et al. 1998). WAKs also function in cell elongation. As a unifying feature both processes require synthesis and secretion of new material to either seal off pathogen-damaged cells or to maintain the structural integrity of the recently expanded walls (McQueen-Mason 1997; Scheel 1998). The presence of a WAK-like kinase is in good agreement with the numerous transcripts for cell wall proteins represented in the EST collection, most of them known to respond to stress. The ~21% transcripts with homologs in other plants but yet unclear functional characterization are an interesting pool for further analyses. Some of their database homologs were isolated from stressed tissue or show similarities to stress-response genes.

A number of cDNAs for a *P. citricola* elicitin were isolated in this study. Elicitins can induce a hypersensitive reaction and further defense mechanisms in the host (reviewed in Kamoun 2006). In congruence with elicitin sequences of the pathogen several EST involved in plant oxidative or respiratory burst like superoxide dismutases and NADPH oxidase were isolated (Supplementary Table 1). The hypersensitive reaction triggered by elicitins would promote growth of the pathogen in the necrotrophic stage while it would inhibit during biotrophic growth. The late induction of elicitins observed in hemibiotrophic pathogen interaction (Moy et al. 2004) is therefore probably a component of *Phytophthora*'s pathogenic strategy under positive selection. This explains the late but extended formation of necroses observed in this study.

Plant protease inhibitors were found to form the biggest functional subcategory besides ribosomal proteins. They also showed a high diversity in amino acid sequence which points to a fast evolution of these genes. Rapid evolution of another group of PR proteins, the chitinases, was proposed to be caused by co-evolution with pathogen effectors (Bishop et al. 2000). Usually R-genes are in the focus of co-evolution studies. Protease inhibitors might also be under selective pressure of effectors involved in the arms race between plants and pathogens as anticipated by Misas-Villamil and van der Hoorn (2008).

In accordance with the results of transcriptome changes during pathogen attack in *Arabidopsis*, which demonstrated an extensive cross-talk between signaling pathways (De Vos et al. 2005), the libraries contained a number of jasmonic acid responsive genes but also SA and ethylene responsive ones. Genes directly involved in signaling pathways were also present in the libraries. Three ESTs coding for ACC synthase (1-aminocyclopropane-1-carboxylate synthase) were identified. ACC synthase catalyzes the first committed step in ethylene biosynthesis. In addition to the PR-proteins known to be responsive to JA cullin 1 was isolated. Cullin 1 is part of the SCF complex (Skp, Cullin, F-box containing complex), a major component of the JA signaling pathway (Katsir et al. 2008). PR-1, -2 and -5 are widely used as markers for the SA signaling pathway (Thatcher et al. 2005), for all of them several ESTs were found as a good indication of induction of this pathway. Besides a mechanistic interconnection another factor causing activation of different signaling pathways could be the hemibiotrophic life style of *P. citricola*. During the biotrophic growth different signaling might be triggered than during necrotrophic growth.

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