Eur J Plant Pathol (2012) 132:407–418
DOI 10.1007/s10658-011-9886-z

Identification of genes differentially expressed in *Pinus pinaster* and *Pinus pinea* after infection with the pine wood nematode

Carla Sofia Sancho dos Santos · Marta Wilton de Vasconcelos

Accepted: 20 October 2011 / Published online: 16 November 2011

Abstract The purpose of this study was to compare the response to infestation by the pine wood nematode (PWN) *Bursaphelenchus xylophilus* between *Pinus pinaster* and *P. pinea* at an initial stage of the disease, 3 h after inoculation. The PWN is the causal agent of pine wilt disease and is destroying pine forests all over the world. In Portugal its main host is the maritime pine, *P. pinaster*, and its vector is the longhorn beetle *Monochamus galloprovincialis*. Interestingly, this disease does not seem to affect the species *P. pinea* and several factors could be behind this difference in susceptibility. With regards to the effects of the disease at a transcriptional level, the suppression subtractive hybridization (SSH) technique was utilized to identify Expressed Sequence Tags (EST) in *P. pinaster* and *P. pinea* when inoculated with PWN. EST were isolated, cloned, sequenced and identified using BlastN and BlastX, and indicated that at an initial stage of the disease there is activation of a tree defence response at a molecular level, mainly related to oxidative stress, production of lignin and ethylene and post-transcriptional regulation of nucleic acids. 58% of the isolated sequences are not yet described, which shows the lack of genomic information currently available for pine.

Keywords Early infection · *Bursaphelenchus xylophilus* · EST · Maritime pine · SSH · Stone pine

Abbreviations

(EST)	Expressed sequenced tags
(hai)	Hours after inoculation
(HR)	Hypersensitive response
(PWN)	Pine wood nematode
(PWD)	Pine wilt disease
(SSH)	Subtractive suppression hybridization

Introduction

Pine wilt disease (PWD) typically kills pine trees within a few weeks to a few months and, despite all advances in research on this subject, the pathogenic mechanism of PWD has not been clearly defined. The disease is caused by the pine wood nematode (PWN) *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle, and its vector, *Monochamus* spp., feeds on a number of conifer species. This longhorn beetle can infest conifers such as pine trees (*Pinus* spp.), but also non-pines, such as trees of the family Pinaceae, namely, *Picea*, *Abies*, *Cedrus* and *Larix* (Kobayashi et al. 1984).

C. S. S. dos Santos · M. W. de Vasconcelos (✉)
CBQF/Escola Superior de Biotecnologia,
Universidade Católica Portuguesa,
Rua Dr. António Bernardino de Almeida,
4200-072 Porto, Portugal
e-mail: mwvasconcelos@esb.ucp.pt

This disease is a serious problem in Japan, China and Korea. In Portugal, it has also become a major threat (Mota et al. 1999) where its main host is the maritime pine, *P. pinaster*, and its vector is *M. galloprovincialis*. Interestingly, this disease does not seem to affect the species *P. pinea* and several factors could be behind this difference in susceptibility.

The resulting symptoms can be divided into early and late stages, ultimately resulting in the wilting of needles and death of the tree. The main symptoms are accompanied by cessation of oleoresin exudation from the stems, a decrease in photosynthesis, cavitation of xylem and destruction of cortex parenchyma cells, traumatic resin canal formation, cambium destruction, production of phytotoxic substances, enhanced respiration and ethylene production (Fukuda 1997). It also seems that temperature and soil moisture conditions are two additional key drivers of wilt expression, as it has been observed that in regions where the nematode occurs and where environmental conditions do not result in significant tree stress, PWN does not result in wilting and host death (Evans et al. 2008)

When the invasion by nematodes starts, it is thought that an innate hypersensitive defence mechanism is triggered. This hypersensitive reaction (HR) results in the release of phenolics, synthesis of toxins and phytoalexins and the compartmentalization of xylem and other tissues, followed by flooding of tracheids with oleoresin and toxic substances (Myers 1988). Recent studies point out that these basal defence mechanisms against pathogens occur within the first few hours after infestation (Baldo et al. 2010) and that an HR is activated by a genetic programme, where resistance genes recognize certain effectors thus initiating a resistance response that is frequently linked to rapid cell death (Schiffer et al. 1997). As studies with cordycepin treatment (an inhibitor of mRNA synthesis) suggest that HR prevention is more successful if performed within the first 4 h, one can infer that mRNAs that are required for the HR are synthesized in the host during the first 4 h after inoculation (Hein et al. 2004).

In this paper, we describe the application of the suppression subtractive hybridization (SSH) technique (Diatchenko et al. 1996) to identify and isolate genes differentially expressed by *P. pinaster* and *P. pinea* after being infested by the PWN, at an early stage, 3 h post inoculation by the nematode.

Materials and methods

Plant material and nematode culture

We studied potted 2-year-old *P. pinaster* and *P. pinea* trees, kept in an environmental growth chamber (Aralab Fitoclima 10000EHF), with relative humidity of 80% and with a photoperiod of 16 h day (at about 490 μmol/m^{2}s and 24–26°C) and 8 h night (with temperatures of 19–20°C). Plants were watered every 2 days.

Small, square pieces of Potato Dextrose Agar with *Botrytis cinerea*, grown at 26°C for 7 days, were transferred to test tubes with barley grains previously autoclaved. *B. xylophilus* geographical isolate HF (from Setubal Region, Portugal) was cultured and placed inside the referred test tubes, after which they were incubated under the same conditions as before. The multiplied nematodes were extracted using the Baermann funnel technique (Baermann 1917) prior to inoculation. Only nematodes that had been extracted for less than 2 h were used in the subsequent experiments.

PWN inoculation and sampling time

Four plants of *P. pinea* and *P. pinaster* were inoculated following the method of Futai and Furuno (1979). In brief, a suspension with 1,000 nematodes was pipetted into a small longitudinal wound made with a scalpel on the main stem with 3–5 cm, about 40 cm above soil level. The inoculated wounds were covered with parafilm to prevent drying of the inoculum. Three hours after inoculation (hai), for each of the four experimental samples, the entire pine tree stem was cut into small pieces and stored at −80°C until further analysis.

RNA extraction and cDNA synthesis

Tester (*P. pinaster* inoculated with strain HF) and driver (*P. pinea* inoculated with strain HF) total RNA was extracted separately and the extraction was performed according to an optimized method from Provost et al. (2007). In short, approximately 200 mg of grounded pine stem was transferred to extraction buffer with 2% of β-mercaptoethanol. After vortexing, the mixture was incubated for 10 min at 65°C; 1 ml of Chloroform Isoamyl Alcohol (CIA) was added and the solution was mixed and centrifuged. Eight hundred μl

of CIA were added to the supernatant. One hundred and twenty five μl of LiCl (10 M) were added, the solution was mixed and incubated overnight at 4°C. The mixture was centrifuged and after the pellet was dried it was re-suspended with 500 μl of STE, and 450 μl of CIA were added. After centrifugation, 150 μl of Sodium Chloride-Tris-EDTA was added to the supernatant. One hundred μl of NaCl (5 M) and 1,5 ml of cold absolute ethanol were added and the solution was incubated 30 min at −80°C. The mixture was centrifuged and the pellet was re-suspended in 400 μl of cold 70% ethanol and then centrifuged. The supernatant was discarded and the pellet was dried out.

The pellet was resuspended in 30 μl of DEPC-Water (Sigma) and stored at −80°C. RNA integrity and purity was checked by UV-spectrophotometry using a nanophotometer (Implen, Isaza, Portugal). Tester (*P. pinaster*) and driver (*P. pinea*) cDNAs were synthesized using SMARTer PCR cDNA Synthesis Kit (Clontech Laboratories, USA, Cat. no. 634925), according to the manufacturer's instructions.

Construction of SSH libraries

Suppression subtractive hybridization was carried out as described by Diatchenko et al. (1996) using PCR-Select cDNA Subtraction Kit (Clontech, USA, Cat. no. 637401), according to the manufacturer's instructions. Tester (*P. pinaster* inoculated with *B. xylophilus*) and driver (*P. pinea* inoculated with *B. xylophilus*) cDNAs were digested with RsaI and ligated to adaptors. Two hybridizations and PCR amplifications were performed to enrich the differentially expressed sequences. The resulting PCR product was purified and cloned into the pCR2.1-TOPO Vector (TOPO TA Cloning Kit, Invitrogen, USA). *Escherichia coli* TOP10 Blue competent cells were transformed with the ligated products. Individual bacterial colonies were selected, grown and their DNA was extracted with GenElute Plasmid Miniprep Kit (Sigma, Cat. Nr. PLN70). Samples were sequenced in Macrogen, Korea.

Bioinformatic analysis for gene identification

All 71 genes were functionally annotated using BlastN and BlastX at NCBI, and categorizated based on Gene Ontology (GO) annotation. The GO provides an ontology of defined terms representing gene product properties, in particular with regards to cellular components, molecular functions and biological activity. Only sequences that had BlastN E-values of e^{-05} or lower were included in the analysis.

Confirmation of expressed genes

Quantitative Real-Time PCR (qRT-PCR) assays were taken to confirm the differential expression results obtained with SSH technique, using four of the genes isolated from the libraries related with defence mechanisms.

About 1 μg of tester and driver total RNA were used to synthesize cDNA, using First Strand cDNA Synthesis Kit (Fermentas), following the manufacturer's instructions. Genes codifying for histone H4 (AT1G07660), Phenylalanine ammonia-lyase (AY641535.1), *P. pinaster* mRNA up-regulated during drought stress (AJ309123.1) and an unknown gene isolated from *P. pinaster* subtracted library (JG391969) were studied by qRT-PCR. Abundance of transcripts was normalized using the housekeeping gene 18S-rRNA, which primers were designed using Pine Gene Index entry TC110284 and Primer3. Primers were designed in the conserved regions and synthesized by Macrogen (Korea) (Table 1).

Real-time PCR amplifications were performed in a 25 μl reaction volume containing 8.5 μl of cDNA, 1.25 μl of each primer, 1.5 μl of Milli-Q water and 12.5 μl of iQ SYBR Green Supermix (Biorad), using a Chromo4 thermocycler (Biorad).

All reactions were performed in duplicate and standard curves were established for each gene. The specificity of the amplified fragments was verified by melting curve analysis and relative quantification of gene amplification was performed using the cycle threshold (ct) values, obtained with Opticon Monitor 3 software (Biorad). Gene expression was presented using 2^(ΔΔCT) method (Livak and Schmittgen 2001) in order to compare the expression levels between tester and driver samples.

ESTs sequence accession numbers

The Expressed Sequence Tags (EST) reported in this study have been deposited in GenBank database under the accession numbers from JG391956 to JG392009.

Table 1 Primers used for quantitative RT-PCR

Gene	Sequence
18S	F 5′—TTAGGCCATGGAGGTTTGAG—3′
	R 5′—GAGTTGATGACACGCGCTTA—3′
Histone H4 from driver subtracted library (D14)	F 5′—AGGGAGGACCTGCTATTCGT—3′
	R 5′—ATCCCCCAAATCGGATTAAC—3′
Unknown from tester subtracted library (T8)	F 5′—TACCTCATAAGGGTGAATGG—3′
	R 5′—CTCGATATGTTCTGGCTCAT—3′
Phenylalanine ammonia-lyase (Ut-9)	F 5′—GGCACGAGGCTCTGCTCTGC—3′
	R 5′—GCCAGACGAGCCGAAGTCCG—3′
mRNA up-regulated during drought stress (Ud-5)	F 5′—ACCAGCAGCGCCATCCATGG—3′
	R 5′—CCCGGGACAAGAAAGGCCCG—3′

Results

Extracted RNA and subtraction

The extracted total RNA obtained was quantified by spectrophotometry and purity evaluated by A_{260}/A_{280} ratio (1.8>2.0). The results suggested that the extracted RNA was of suitable quality to proceed with the experiments.

From the *forward subtraction* we obtained 12 sequences that produced no significant hits in BlastN and BlastX searches of NCBI (data not shown). The forward-subtracted sample consists of sequences that are specific to *P. pinaster*. Performing subtraction in the forward direction allowed us to obtain the genes that were differentially expressed by nematode inoculated *P. pinaster*.

From the 33 sequences obtained by *reverse subtraction*, five produced hits in NCBI search databases, all of which were similar to putative histones H4 of *Picea* spp (Table 2). The reverse-subtracted sample consists of sequences that are specific to *P. pinea*. Performing subtraction in the reverse direction allowed to obtain the genes that were differentially expressed by nematode inoculated *P. pinea*.

Unsubtracted tester and driver

We also looked into the genes expressed in *P. pinaster* and *P. pinea* prior to subtraction from the tester and driver samples. Of the 11 colonies from *P. pinaster* (unsubtracted tester) only one produced no significant hit in the NCBI database. Of the other 10 classified proteins from the expressed genes in *P. pinaster* (Table 3), a putative alfa tubulin was isolated, similar to *Picea wilsonii* species. Tubulins belong to a multigene family in plants and are involved in the formation of earlywood microfibril angle (González-Martínez et al. 2007) and have a role in microtubule and spindle organization (Dryková et al. 2003).

Also, a possible cytosolic Fe-S gene was identified, and these are usually involved in numerous metabolic processes such as isomerization and dehydration reactions, and serve as electron carriers in various redox reactions (Kispal et al. 1999).

Table 2 Sequence similarities of subtracted driver, *P. pinea*, cDNA (D)

Seq.	Size (bp)	Protein/DNA similarity	Origin of similar sequence	Blast X		Blast N	
				Acc No. of similar sequence	Similarity	Acc No. of similar sequence	Similarity
D1	507	Histone H4	*Picea sitchensis*	ABK21562.1	103 aa	EF082188.1	3e–44 (79%, 615 bp)
D2	519	Histone H4	*Picea sitchensis*	P35057.2|H4_SOLLC	7e–38 (98%, 103 aa)	EF081475.1	9e–41 (82%, 654 bp)
D11	950	Histone H4	*Picea glauca*	NP_001077477.1	5e–19 (74%, 86 aa)	BT115137.1	6e–30 (74%, 612 bp)
D12	950	Histone H4	-	XP_001768205.1	4e–05 (61%, 103 aa)	–	–
D14	872	GJ17461-like (H4)	*Picea glauca*	XP_002686736.1	2e–04 (80%, 138 aa)	BT105693.1	1e–18 (72%, 914 bp)

Table 3 Sequence similarities of unsubtracted tester, *P. pinaster*, cDNA (Ut) selected following SSH

Seq.	Size (bp)	Protein/DNA similarity	Origin of similar sequence	Blast X		Blast N	
				Acc No. of similar sequence	Similarity	Acc No. of similar sequence	Similarity
Ut-1	864	Unknown	*Picea sitchensis*	ABK24055.1|	1e–107 (89%, 299 aa)	EF084744.1	0.0 (92%, 1,227 bp)
Ut-2	866	EST, clone 14–5 JM E7(20)	*P. pinaster*	B8LK84.1|DRE2 1_PICSI	9e–14 (100%, 285 aa)	FN257026.1	4e–132 (100%, 481 bp)
Ut-3	867	Alfa tubulin (TUA1)	*Picea wilsonii*	XP_002981542.1	1e–146 (99%, 449 aa)	EU268195.1	0.0 (95%, 1,356 bp)
Ut-4	950	–	–	–	–	–	–
Ut-5	191	Cytochrome oxidase	*Ixodes ricinus*	AAP93883.1	7e–81(100%, 158 aa)	AY327035.1	8e–05 (100%, 477 bp)
Ut-6	867	Unknown	*Picea sitchensis*	ADE77904.1	2e–93 (86%, 274 aa)	BT124669.1	0.0 (91%, 1,122 bp)
Ut-7	538	Unknown	*Pinus taeda*	ABK20926.1	1e–55 (95%, 123 aa)	FJ104302.1	3e–163 (93%, 598 bp)
Ut-8	866	Translationelongation factor-1	*Picea sitchensis*	CAC27139.1	1e–149 (95%, 444 aa)	BT071362.1	0.0 (93%, 1,872 bp)
Ut-9	609	Phenylalanineammonia-lyase	*P. pinaster*	ACS28225.2	1e–102 (97%, 718 aa)	AY641535.1	0.0 (99%, 2,708 bp)
Ut-10	866	Unknown	*Picea sitchensis*	ABK21322.1	3e–62 (100%, 120 aa)	EF676404.1	1e–48 (85%, 639 bp)
Ut-11	867	Unknown	*Picea glauca*	ABK25472.1	3e–71 (84%, 367 aa)	BT102048.1	0.0 (89%, 1,644)

As referred before, disruption of water flow is one of the symptoms of nematode infection (Tan et al. 2005). In fact, two of the genes isolated from the unsubtracted tester were related to water or oxidative stress. The first, a likely cytochrome oxidase subunit I, is reported to be abundantly expressed during drought and/or heat stress (Rizhsky et al. 2004). The second, although it was unknown in NCBI database, was found to have a conserved domain (please see Table 5, Ut-10) belonging to a putative thioredoxin.

A putative translation elongation factor-1 (EF1α), was also identified, that has the role of presenting amino acyl-tRNA to the A site of ribosomes. Under stress conditions (e.g. hypoxia), it appears to form non-functional complexes with polysome-associated mRNA and prevents peptidyl synthesis and translocation (Hochachka et al. 1996).

A gene expressing phenylalanine ammonia lyase (PAL) was also identified; it is responsible for the production of secondary metabolites in the phenylpropanoid pathway (Mauch-Mani and Slusarenko 1996). Four other sequences were isolated, but could not be classified in NCBI databases. Therefore, their conserved domains were identified in order to obtain further information (Table 5). Genes of (or related to) RNA recognition motif (RRM), also known as RNA-binding domain or ribonucleoprotein domain were expressed (Ut-1) and they usually form complexes with RNAs and/or proteins.

One other unknown sequence was identified and, based on its conserved domain analysis, it was concluded that it may be a xyloglucan endotransglycosylase (XET) and was detected in *P. pinaster* expressed genes (Ut-6). This enzyme preferentially cleaves xyloglucan polymers in plant cell walls and has the ability to re-ligate to a different acceptor chain, thereby controlling wall extensibility (Fry et al. 1993; Eckard 2004).

The conserved domains also allowed us to identify a non-specific lipid-transfer protein type 1 (Ut-7) that has the ability to catalyse the transfer of lipids between membranes (Torres-Schumann et al. 1992) and a likely FMN-dependent alpha-hydroxyacid was also isolated from *P. pinaster* (Ut-11), an oxidizing enzyme located in the peroxisomes that plays an important role in photorespiration, where it oxidizes glycolate to glyoxylate (Stenberg and Lindqvist 1997).

One of the classified proteins obtained in *P. pinea* (Table 4) was a putative clavata-like receptor, an important regulator of cellular processes and protein kinase that might be involved in tailoring the defence response against pathogens (Chisholm et al. 2006).

A further potential PWD-related expressed sequence was a possible S-adenosyl methionine synthetase 2, as it plays a role in the biosynthesis of polyamines and ethylene (Peleman et al. 1989). A likely copper resistance protein, CopC—blue copper, was also isolated, and these proteins are predicted to be involved in antioxidant defence, by reducing reactive oxygen species produced in response to pathogens and/or other stresses (Verica et al. 2004). We also identified a putative mRNA up-regulated during drought stress, which can be considered a molecular response to water-deficit (Bray 2002).

Table 4 Sequence similarities of unsubtracted driver, *P. pinea*, cDNA (Ud) selected following SSH

Seq.	Size (bp)	Protein/DNA similarity	Origin of similar sequence	Blast X		Blast N	
				Acc No. of similar sequence	Similarity	Acc No. of similar sequence	Similarity
Ud-1	866	Unknown mRNA	*Picea sitchensis*	ABK21925.1	5e–36 (96%, 77 aa)	EF082569.1	0.0 (88%, 717 bp)
Ud-2	867	Unknown	*Picea glauca*	–	–	BT116785.1	6e–09 (84%, 1,746 bp)
Ud-3	866	Clavata-like receptor	*Picea glauca*	ABF73316.1	1e–108 (83%, 998 aa)	DQ530597.1	0.0 (84%, 2,997 bp)
Ud-4	865	Unknown	*Picea glauca*	ABK23251.1	1e–136 (90%, 299 aa)	BT103799.1	0.0 (89%, 1,272 bp)
Ud-5	537	mRNA up-regulated during drought stress	*P. pinaster*	–	–	AJ309123.1	1.0e–100 (89%, 348 bp)
Ud-6	705	Unknown	*Picea sitchensis*	ABK22226.1	5e–76 (87%, 160 aa)	BT071582.1	1.0e–168 (86%, 945 bp)
Ud-7	866	N14 matrix protein	*Pinctada maxima*	BAA90539.1	140 aa	AB032612.1	3e–05 (93%, 814 bp)
Ud-8	519	Unknown	*Picea glauca*	ABK24266.1	1e–45 (97%, 535 aa)	BT115938.1	0.0 (92%, 1,907 bp)
Ud-9	518	s-adenosyl methionine synthetase 2	*P. taeda*	ACO57105.1	3e–35 (100%, 94 aa)	AY874759.1	0.0 (98%, 541 bp)
Ud-10	511	CopC	*Pseudomonas fluorescens*	ACL13563.1	1e–35 (96%, 127 aa)	EU927287.1	1.0e–109 (86%, 4,741 bp)
Ud-11	783	Unknown	*Picea glauca*	ACN40917.1	1e–126 (91%, 437 aa)	BT107125.1	0.0 (89%, 1,540 bp)
Ud-12	412	Unknown	*Picea sitchensis*	ABK21554.1	93 aa	EF082180.1	0.0 (85%, 614 bp)
Ud-13	442	Unknown	*Picea sitchensis*	ABK20902.1	1e–29 (100%, 60 aa)	EF677978.1	2e–94 (88%, 445 bp)
Ud-14	863	Unknown	*Picea sitchensis*	ABK22836.1	248 aa	EF083489.1	5e–47 (82%, 1,215)
Ud-15	689	Anonymous locus	*P. pinaster*	–	–	FN257100.1	3e–128 (87%, 365)

Ten other *P. pinea* expressed genes were unclassified in NCBI databases, but six (Table 5) had a conserved domain. A possible gene expressing a protein belonging to the 4 F5 protein family (Ud-1) was identified. The functions of these proteins are still unknown, but it is known that they are rich in aspartate, glutamate, lysine and arginine, and are ubiquitously expressed (Scharf et al. 1998).

Similarly to one protein obtained in *P. pinaster* (Ut), a probable RNA recognition motif was identified (Ud-4) in *P. pinea* that, as already indicated, is present in post-transcriptional events. Also, a sequence expressing a putative protein belonging to Class-II DAHP synthetase family was isolated (Ud-8); these are involved in the first step of the shikimate pathway (Herrmann and Weaver 1999).

A gene sequence encoding a protein similar to a DUF231 *Arabidopsis* protein was isolated (Ud-11). Although its function is unknown, several members of this family are believed to be co-expressed with other cell wall-related genes, specifically in cellulose biosynthesis (Bischoff et al. 2010). Another putative ribonucleoprotein related protein, Sm-like protein (Ud-12), was identified in *P. pinea*, which are involved in a variety of RNA processing events (Séraphin 1995). Finally, a probable NifU-like protein (Ud-14), a scaffold protein that is a crucial regulator of many key signalling pathways was also detected (Yabe et al. 2004).

Overview of expressed sequences

In order to provide an overview on the type of expressed sequences, these were grouped by origin and function. As presented in Fig. 1, 58% of the sequences were clustered into “no significant homology” and other 21% into “unclassified protein”, as their hit in BlastN was ‘unknown’. However, as can be seen in Fig. 2, 40% of the unclassified proteins were found to be defence related by the analysis of their conserved domains. Also, the most significant hits (30%) showed that the sequences expressed had more homology with *Picea* spp. (Fig. 3).

Validation of differential expression by qRT-PCR

To verify that genes in the libraries truly reflected their differential expression, qRT-PCR assays were developed. Four genes isolated with potential roles in the host’s defence response to nematode infection were selected for confirmation of differential

Table 5 Proteins and respective conserved domains found in Unsubtracted tester (Ut), Unsubtracted driver (Ud) and Subtracted driver (D)

Seq.	Protein	Conserved domain
Ut-1	Unknown	RNA recognition motif, involved in post-transcriptional gene expression processes including mRNA and rRNA processing, RNA export and RNA stability
Ud-4	Unknown	
Ut-2	EST, clone 14–5 JM E7 (20)	Cytokine-induced anti-apoptosis inhibitor 1, Fe-S biogenesis; functions in cytosolic Fe-S protein
Ut-3	Alpha tubulin (TUA1)	Major component of microtubules; can be divided into three regions, the amino-terminal nucleotide-binding region
Ut-5	Cytochrome oxidase subunit I	Heme-copper oxidase subunit I; catalyse the reduction of O2 and simultaneously pump protons across the membrane
Ut-6	Unknown	Xyloglucan endotransglycosylase; cleave and relegate xyloglucan polymers in plant cell walls via transglycosylation mechanism
Ut-7	Unknown	Non-specific lipid-transfer protein type 1; facilitate the transfer of fatty acids, phospholipids, glycolipids and steroids between membranes; also play a key role in the defence of plants against pathogens
Ut-8	Translation elongation factor-1 alpha	Eukaryotic elongation factor 1 alpha; interacts with the actin of the eukaryotic cytoskeleton and may thereby play a role in cellular transformation and apoptosis
Ut-9	Phenylalanine ammonia-lyase	Member of the Lyase class I-like superfamily of enzymes; catalyzes the conversion of L-phenylalanine to E-cinnamic acid
Ut-10	Unknown	TRX family; plays an important role in the defence against oxidative stress by directly reducing hydrogen peroxide and certain radicals and by serving as a reductant for peroxiredoxins
Ut-11	Unknown	FMN-dependent alpha-hydroxyacid oxidizing enzymes; key enzyme in photorespiration where it oxidizes glycolate to glyoxylate
Ud-1	Unknown	4 F5 protein family; short proteins that are rich in aspartate, glutamate, lysine and arginine; found to be ubiquitously expressed
Ud-3	Clavata-like receptor	Protein kinases, catalytic domain; PK regulate many cellular processes including proliferation, division, differentiation, motility, survival, metabolism, cell-cycle progression, cytoskeletal rearrangement and immunity
Ud-8	Unknown	Class-II DAHP synthetase family; aldolase enzymes that catalyse the first step of the shikimate pathway
Ud-9	s-adenosyl methionine synthetase 2	S-adenosylmethionine synthetase, C-terminal domain; responsible for polyamines and ethylene production
Ud-10	CopC	Copper resistance protein CopC; bacterial blue copper protein that binds 1 atom of copper per protein molecule. Along with CopA, CopC mediates copper resistance by sequestration of copper in the periplasm.
Ud-11	Unknown	Duf231 Arabidopsis proteins of unknown function
Ud-12	Unknown	Sm-like protein; associate with RNA to form the core domain of the ribonucleoprotein particles involved in a variety of RNA processing events;
Ud-14	Unknown	NifU-like domain; alignment of the carboxy-terminal domain; biochemical function unknown
D1	Histone H4	Histone H4; bind to DNA and wrap the genetic material into “beads on a string” in which DNA is wrapped around small blobs of histones at regular intervals.
D2	Histone H4	
D11	Histone H4	
D12	Histone H4	
D14	Histone H4	

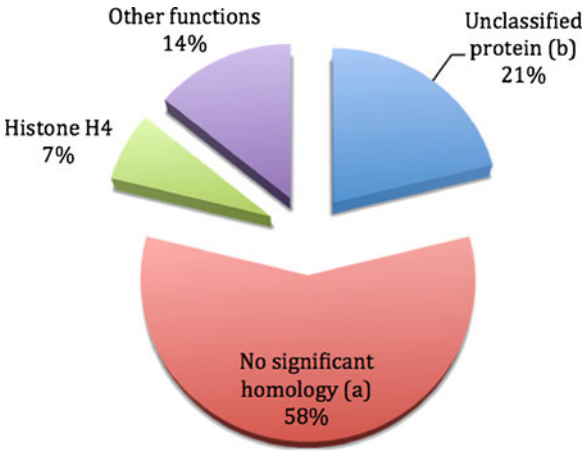

Fig. 1 Functional categorization of induced genes. All 71 genes were functionally annotated using BlastN and BlastX at NCBI, and categorizated based on GO annotation. Percentages of genes in each category are also presented. **a** These genes had no significant hits in BlastN and BlastX searches of NCBI. **b** These genes matched proteins annotated as 'unknown protein'

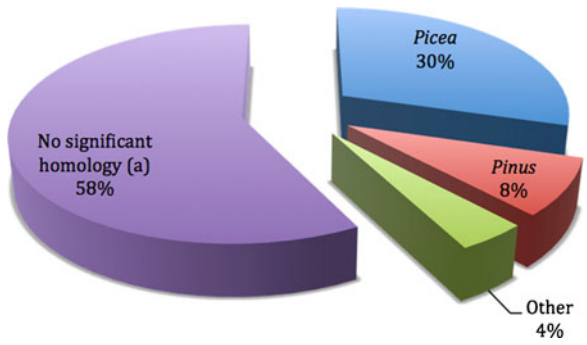

Fig. 3 Genus characterization of induced genes. **a** These genes had no significant hits in BlastN and BlastX searches of NCBI

expression. The results from qRT-PCR are presented in Fig. 4 (A-D). As expected, Histone H4 was expressed differentially by *P. pinea*. The other three genes—PAL, *P. pinaster* mRNA up-regulated during drought stress and an unknown gene isolated from *P. pinaster* subtracted library—had higher levels of expression in *P. pinaster* samples.

General discussion

When using SSH technique is advisable to use confirmation tests since one of its drawbacks is the occurrence of false positives. Background molecules in the subtracted library can arise via unspecific annealing during the ligation of the adaptor-molecules or represent redundant cDNA fragments that were not subtracted during the two hybridizations, giving positive results in the first screening process (Boengler et al. 2003). For this reason, the differential expression of 4 sequences that were identified using the SSH technique was conducted using qRT-PCR (see Fig. 4A-D). The fact that H4 histone protein was the differentially expressed gene by *P. pinea* lead us into hypothesizing that this gene may be involved in the lower susceptibility of *P. pinea* when compared to more susceptible species *P. pinaster*. Histone H4 is a chromatin protein, which is present in the nucleosome, contains flexible N-terminal tails, and undergoes diverse post-translational modifications (Yan et al. 2007). It has been reported that cyst nematode feeding-site construction interferes with the normal nuclear biology of the host cell, by triggering repeated cycles of DNA endoreduplication (Elling et al. 2007). Several histone-related enzymes have been reported to have a particularly important role in plant defence against pathogens (Mach 2008).

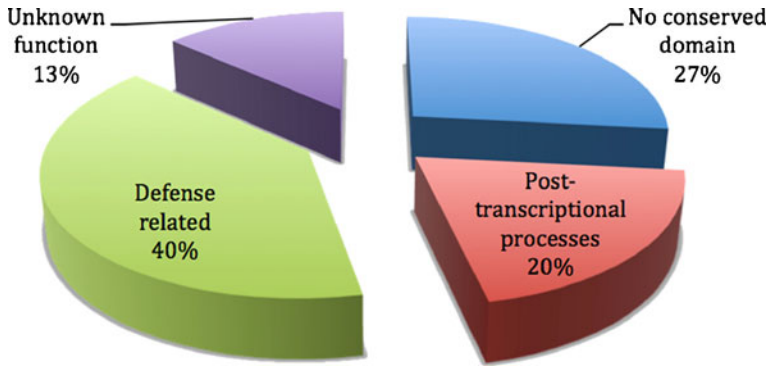

Fig. 2 Conserved domain information for the 15 genes annotated as "unknown protein" in NCBI databases

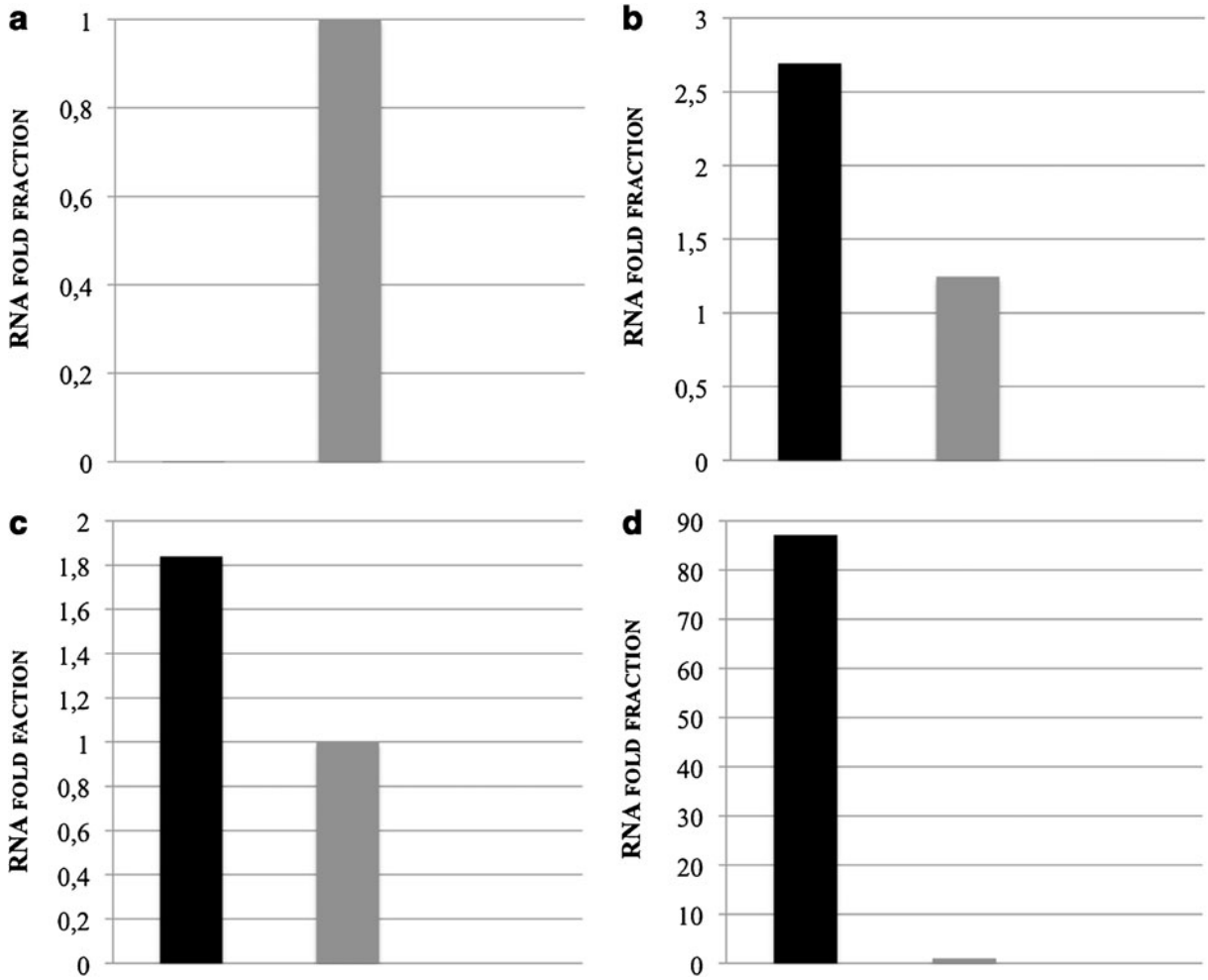

Fig. 4 (A-D)—qRT-PCR analysis for transcript quantification and confirmation of differential expression in *P. pinaster* (black) and *P. pinea* (grey): **a** histone H4 (D14); **b** unknown gene from *P. pinaster* subtracted library; **c** mRNA up-regulated during drought stress (Ud-5), and **d** phenylalanine ammonia-lyase (Ut-9). Abundance of transcripts was normalized using the housekeeping gene 18S-rRNA. Milli-Q water was used as control (red, which is not visible in the figure because there was no amplification)

All genes from the *P. pinaster* subtracted library had no homology in the databases. In order to verify the differential expression of the isolated sequences, a quantification of one of those sequences was performed in both tester and driver samples. As can be seen in Fig. 4B, the unknown gene was expressed in more than double by *P. pinaster*, when compared to *P. pinea*.

With regards to the genes preferentially expressed by *P. pinaster* (unsubtracted library), an alfa tubulin related sequence was cloned, and although there is still no published data that makes a direct correlation between this gene and nematode infestation one can theorize that, as nematodes feed off parenchyma cells and undifferentiated cambial cells, the xylem starts to suffer from cavitation and disrupt wood formation, requiring newly synthesised tubulin to re-synthesise nematode-affected feeding sites.

Several genes were found to be related to oxidative stress, like one belonging to the TRX family, which plays an important role in the defence against oxidative stress and acts as a signalling molecule in plants through its redox-regulation properties (Jacquot et al. 1997) and an elongation factor, EF1α, which overexpression might favour expression of glycolytic enzymes, whose activity must be sustained for the surviving of plant cells with O_2 lack (Hochachka et al.

1996). Oxidative stress is an important response of pine trees when infested by the PWN and is a result of, for example, release of metabolites by the action of enzymes in nematode saliva and the host's secondary metabolites, that exposes plant cells to highly toxic oxygen species (Shin et al. 2009).

Due to its possible connexion with water-deficit the expression of an mRNA up-regulated during drought stress was analysed using qRT-PCR (please see Fig. 4C). This gene is expressed almost 1-fold higher in *P. pinaster* than in *P. pinea*, although both species were subjected to the same watering conditions. Therefore, this difference in expression might be related to the water stress to which plants were subjected in consequence of nematode infection.

Putative defence related genes were identified, namely PAL that is activated in the incompatible interaction between pathogen-host trees as a defence gene and was proposed to be involved in synthesizing precursors for lignification, a possible nematode resistance process (Mauch-Mani and Slusarenko 1996). Using qRT-PCR we were able to confirm that, although both species were expressing this gene, *P. pinaster* had over 80-fold higher expression, when compared to *P. pinea* (see Fig. 4D). This difference in expression can be related to the fact that *P. pinaster* is more susceptible to the infection, hence activating this defence gene.

Also, the identified Class-II DAHP synthetase is involved in the shikimate pathway, which has as resulting product chorismate, the common precursor for phenylalanine/tyrosine or tryptophan (Hu et al. 2009; Zucko et al. 2010). Shikimate is also involved in the phenylpropanoid pathway, crucial in lignin production (Wagner et al. 2007). It is possible that the amount of lignin in the tissues may serve as an obstacle for nematode progression.

Enhanced ethylene production during expression of PWD is another known physiological symptom that appears to result from nematode movement that ultimately results in tracheid embolism (Fukuda 1997). Also, as the pathogenic infection of plants increases the respiration rate, ethylene levels also increase, and the ethylene-induced increase is further potentiated by increased oxygen, acting as a defence mechanism (Ecker and Davis 1987).

Similarly to what Verica et al. (2004) identified in applying the SSH technique to the study of gene expression in cacao leaves, several genes encoding a variety of transcription factors, transcriptional regulators and biosynthesis of secondary metabolites related to defence response were isolated. These can participate in the induction of wound response, antimicrobial and antifungal defence and antioxidant defence.

Our results showed that defence-related genes are expressed at an early stage of infestation of pine trees by PWN. Particularly, genes involved in PWD, like ethylene production and oxidative stress related genes were triggered in the first 3 h after infestation, in both *P. pinaster* and *P. pinea*.

As indicated above, little is known about the *Pinus* spp. genome. In Results we mentioned that the most significant hits (30%) showed that the sequences expressed had more homology with *Picea* spp. (Figure 3) such as *P. abies*, *P. glauca* and *P. sitchensis*, all of them reported to be susceptible to the disease or an alternative PWN host, when environmental conditions are suitable (Kuroda 2008). This can be explained by the fact that *Picea* spp. are much better known than *Pinus* spp. as seen, for example, in the work of Ralph et al. (2008) who described more than 200,000 EST from *Picea* spp.. Moreover, this difference can be seen in KEGG databases, where 13,223 EST of *P. sitchensis* are available, whereas only 4,071 EST of *P. pinaster* have been reported.

Because of the high concentrations of polyphenols polysaccharides and other secondary metabolites in pine tissues (Wang et al. 2010), it was hard to optimize and implement the SSH technique in this study. Only nematode-inoculated plants were used for library construction due to the high cost implication of the SSH technique, but it would be interesting to construct a new library with water inoculated plants and look for basal differences in gene expression.

Even though the subtraction did not result as expected, since most of the sequences identified were isolated from unsubtracted samples, the results show some interesting differences between the samples under study, and high throughput transcriptome sequencing studies of infested and non infested *P. pinaster* and *P. pinea* are under way, which should provide us sequencing data that will help us identify the 'unknown' sequences obtained in our study.

The resistance or susceptibily to infestation of a particular plant genotype within a species is determined

by intertwined layers of defence, including both constitutive barriers and inducible reactions (Thordal-Christensen 2003). Also, the control of inducible reactions emphasizes the importance of understanding the mechanisms by which plants both perceive environmental signal and transmit them through the cellular machinery to activate adaptive responses (Afzal et al. 2008), in particular at an early stage of the disease.

Acknowledgements This work was supported by the National Forest Authority, Agriculture Ministry, and Rural and Fisheries Development. The authors would like to thank Dr. Manuel Mota and Dr. Pedro Barbosa for providing the nematode strain HF.

References

Afzal, A. J., Wood, A. J., & Lightfoot, D. A. (2008). Plant receptor-like serine threonine-kinases: roles in signaling and plant defence. *MPMI, 21*, 507–517.

Baermann, G. (1917). Eine einfache Methode zur Auffindung von Ankylostomum (nematoden) Larven in Erdproben. *Geneesk Tijdschr Ned-Indie, 57*, 131–137.

Baldo, A., Norelli, J. L., Farrell, R., Jr., Basset, C. L., Aldwinckle, H. S., & Malnoy, M. (2010). Identification of genes differentially expressed during interaction of resistant and susceptible apple cultivars (*Malus* x *domestica*) with *Erwinia amylovora*. *BMC Plant Biology, 10*. doi:10.1186/1471-2229-10-1.

Bischoff, V., Nita, S., Neumetzler, L., Schindelasch, D., Urbain, A., Eshed, R., et al. (2010). *Trichome birefringence* and its homolog *At5g01360* encode plant-specific DUF231 proteins required for cellulose biosynthesis in *Arabidopsis thaliana*. *Plant Physiology, 153*(2), 590–602. doi:10.1104/pp.110.153320.

Boengler, K., Pipp, F., Schaper, W., & Deindl, E. (2003). Rapid Identification of Differentially Expressed Genes by Combination of SSH and MOS. *Laboratory Investigation, 83*(5), 759. doi:10.1097/01.LAB.0000069520.16101.73.

Bray, E. A. (2002). Classification of genes differentially expressed during water-deficit stress in *Arabidopsis thaliana*: an analysis using microarray and differential expression data. *Annals of Botany, 89*, 803–811.

Chisholm, S. T., Coaker, G., Day, B., & Staskawicz, B. J. (2006). Host-Microbe interactions: shaping the evolution of the plant immune response. *Cell, 124*, 803–814.

Diatchenko, L., Lau, Y.-F., Campbell, A. P., Chenchik, A., Moqadam, F., Huang, B., et al. (1996). Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA, 93*, 6025–6030.

Dryková, D., Cenklová, V., Sulimenko, V., Volc, J., Dráber, P., & Binarová, P. (2003). Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes. *The Plant Cell, 15*, 465–480.

Eckard, N. A. (2004). Inside the matrix: crystal structure of a xyloglucan endotransferase. *The Plant Cell, 16*, 792–793.

Ecker, J. R., & Davis, R. W. (1987). Plant defence genes are regulated by ethylene. *Proc Natl Acad Sci USA, 84*, 5202–5206.

Elling, A. A., Davis, E. L., Hussey, R. S., & Baum, T. J. (2007). Active uptake of cyst nematode parasitism proteins into the plant cell nucleus. *Int J Parasitol, 37*, 1269–1279.

Evans, S., Evans, H., & Ikegami, M. (2008). Modelling PWN-Induced Wilt Expression: A Mechanistic Approach. In M. M. Mota & P. Vieira (Eds.), *Pine Wilt Disease: A Worldwide Threat to Forest Ecosystems* (pp. 259–278). Netherlands: Springer.

Fry, S. C., Aldington, S., Hetherington, P. R., & Aitken, J. (1993). Oligosaccharides as signal and substrates in the plant cell wall. *Plant Physiology, 103*, 1–5.

Fukuda, K. (1997). Physiological process of the symptom development and resistance mechanism in pine wilt disease. *Journal of Forest Research, 2*, 171–181.

Futai, K., & Furuno, T. (1979). The variety of resistances among pine species to pine wood nematode, *Bursaphelenchus lignicolus*. *Bull Kyoto Uni For, 51*, 23–36.

González-Martínez, S. C., Wheeler, N. C., Ersoz, E., Nelson, C. D., & Neale, D. B. (2007). Association genetics in *Pinus taeda* L. I. wood property traits. *Genetics, 175*, 399–409.

Hein, I., Campbell, E. I., Woodhead, M., Hedley, P., Young, V., Morris, W. L., et al. (2004). Characterisation of early transcriptional changes involving multiple signalling pathways in the *Mla*13 barley interaction with powdery mildew (*Blumeria graminis* f sp *hordei*). *Planta, 218*, 803–813.

Herrmann, K. M., & Weaver, L. M. (1999). The shikimate pathway. *Annu Rev Plant Physiol Plant Mol Biol, 50*, 473–503.

Hochachka, P. W., Buck, L. T., Doll, C. J., & Land, S. C. (1996). Unifying theory of hypoxia tolerance: molecular/metabolic defence and rescue mechanisms for surviving oxygen lack. *Proc Natl Acad Sci USA, 93*, 9493–9498.

Hu, P., Meng, Y., & Wise, R. P. (2009). Functional contribution of chorismate synthase, anthranilate synthase, and chorismate mutase to penetration resistance in barley-powdery mildew interactions. *MPMI, 22*, 311–320.

Jacquot, J.-P., Lancelin, J.-M., & Meyer, Y. (1997). Thioredoxins: structure and function in plant cells. *New Phytologist, 136*, 543–570.

Kispal, G., Csere, P., Prohl, C., & Lill, R. (1999). The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *The EMBO Journal, 18*, 3981–3989.

Kobayashi, F., Yamane, A., & Ikeda, T. (1984). The Japanese pine sawyer beetle as the vector of pine wilt disease. *Ann Rev Entomol, 29*, 115–135.

Kuroda, K. (2008). Host responses and wilting mechanisms. In B. G. Zhao, K. Futai, J. R. Sutherland, & Y. Takeuchi (Eds.), *Pine wilt disease* (pp. 202–222). Netherlands: Springer.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ Method. *Methods, 25*(4), 402–408.

Mach, J. (2008). Basal defence in Arabidopsis: WRKYs Interact with histone deacetylase HDA19. *The Plant Cell, 20*, 2282.

Mauch-Mani, B., & Slusarenko, A. J. (1996). Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. *The Plant Cell, 8*, 203–212.

Mota, M. M., Braasch, H., Bravo, M. A., Penas, A. C., Burgermeister, W., Metge, K., et al. (1999). First report of *Bursaphelenchus xylophilus* in Portugal and in Europe. *Nematology, 1*, 727–734.

Myers, R. F. (1988). Pathogenesis in Pine Wilt caused by Pinewood Nematode, *Bursaphelenchus xylophilus*. *Journal of Nematology, 20*, 236–244.

Peleman, J., Boerjan, W., Engler, G., Seurinck, J., Botterman, J., Alliotte, T., et al. (1989). Strong cellular preference in the expression of a housekeeping gene of *Arabidopsis thaliana* encoding S-Adenosylmethionine Synthetase. *The Plant Cell, 1*, 81–93.

Provost, G., Herrera, R., Paiva, J., Chaumeil, P., Salin, F., & Plomion, C. (2007). A micromethod for high throughput RNA extraction in forest trees. *Biological Research, 40*, 291–297.

Ralph, S. G., Chun, H. J. E., Kolosova, N., Cooper, D., Oddy, C., Ritland, C. E., et al. (2008). A conifer genomics resource of 200,000 spruce (*Picea* spp.) ESTs and 6,464 high-quality, sequence-finished full-length cDNAs for Sitka spruce (*Picea sitchensis*). *BMC Genomics, 9*, 484.

Rizhsky, L., Liang, H., Shuman, J., Shulaev, V., Davletova, S., & Mittler, R. (2004). When defence pathways collide: the response of *Arabidopsis* to a combination of drought and heat stress. *Plant Physiology, 134*, 1–14.

Scharf, J. M., Endrizzi, M. G., Wetter, A., Huang, S., Thompson, T. G., Zerres, K., et al. (1998). Identification of a candidate modifying gene for spinal muscular atrophy by comparative genomics. *Nature Genetics, 20*, 83–86.

Schiffer, R., Gorg, R., Jarosch, B., Beckhove, U., Bahrenberg, G., Kogel, K.-H., et al. (1997). Tissue Dependence and Differential Cordycepin Sensitivity of Race-Specific Resistance Responses in the Barley-Powdery Mildew Interaction. *MPMI, 10*, 830–839.

Séraphin, B. (1995). Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. *The EMBO Journal, 14*, 2089–2098.

Shin, H., Lee, H., Woo, K.-S., Noh, E.-W., Koo, Y.-B., & Lee, K.-J. (2009). Identification of genes upregulated by pinewood nematode inoculation in Japanese red pine. *Tree Physiology, 29*, 411–421.

Stenberg, K., & Lindqvist, Y. (1997). Three-dimensional structures of glycolate oxidase with bound active-site inhibitors. *Protein Science, 6*, 1009–1015.

Tan, J.-J., Ye, J.-R., Wu, X.-Q., Zhu, Y.-F., & Li, Y. (2005). A study on disease development and early diagnosis of pine wood nematode, *Bursaphenchus xylophilus*, infection of Japanese black pine. *Nematology, 7*, 481–485.

Thordal-Christensen, H. (2003). Fresh insights into processes of non-host resistance. *Current Opinion in Plant Biology, 6*, 351–357.

Torres-Schumann, S., Godoy, J. A., & Pintor-Toro, J. A. (1992). A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. *Plant Molecular Biology, 18*, 749–757.

Verica, J. A., Maximova, S. N., Strem, M. D., Carlson, J. E., Bailey, B. A., & Guiltinan, M. J. (2004). Isolation of ESTs from cacao (*Theobroma cacao L.*) leaves treated with inducers of the defence response. *Plant Cell Reports, 23*, 404–413.

Wagner, A., Ralph, J., Akiyama, T., Flint, H., Phillips, L., Torr, K., et al. (2007). Exploring lignification in conifers by silencing hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase in *Pinus radiata*. *PNAS, 104*, 11856–11861.

Wang, X., Wang, B., Liu, L., Cui, X., Yang, J., Wango, H., et al. (2010). Isolation of high quality RNA and construction of a suppression subtractive hybridization library from ramie (*Boehmeria nivea* L. Gaud.). *Molecular Biology Reports, 37*, 2099–2103.

Yabe, T., Morimoto, K., Kikuchi, S., Nishio, K., Terashima, I., & Nakai, M. (2004). The Arabidopsis chloroplastic NifU-Like Protein CnfU, which can act as an iron-sulfur cluster scaffold protein, is required for biogenesis of ferredoxin and photosystem I. *The Plant Cell, 16*, 993–1007.

Yan, D., Zhang, Y., Niu, L., Yuan, Y., & Cao, X. (2007). Identification and characterization of two closely related histone H4 arginine 3 methyltransferases in *Arabidopsis thaliana*. *Biochemical Journal, 408*, 113–121.

Zucko, J., Dunlap, W. C., Shick, J. M., Cullum, J., Cercelet, F., Amin, B., et al. (2010). Global genome analysis of the shikimic acid pathway reveals greater gene loss in host-associated than in free-living bacteria. *BMC Genomics, 11*, 628.