Tree Genetics & Genomes (2015) 11: 6
DOI 10.1007/s11295-014-0829-7

ORIGINAL PAPER

Castanea root transcriptome in response to *Phytophthora cinnamomi* challenge

Susana Serrazina · Carmen Santos · Helena Machado · Catia Pesquita · Renato Vicentini · Maria Salomé Pais · Mónica Sebastiana · Rita Costa

Received: 30 September 2014 / Revised: 12 December 2014 / Accepted: 16 December 2014 / Published online: 21 January 2015

Abstract The European chestnut, an important forest species for the economy of Southern Europe, covers an area of 2.53 million hectares, including 75,000 ha devoted to fruit production. *Castanea sativa* is declining due to ink disease caused by *Phytophthora cinnamomi*. To elucidate chestnut defense mechanisms to ink disease, we compared the root transcriptome of the susceptible species *C. sativa* and the resistant species *C. crenata* after *P. cinnamomi* inoculation. Four cDNA libraries were constructed, two of them included root samples from *C. sativa*, inoculated and non-inoculated and the other two libraries comprised samples from *C. crenata* at identical conditions. Pyrosequencing produced 771,030 reads and assembly set up 15,683 contigs for *C. sativa* and 16,828 for *C. crenata*. GO annotation revealed terms related to stress as "response to stimulus", "transcription factor activity" or "signaling" for both transcriptomes. Differential gene expression analysis revealed that *C. crenata* involved more genes related with biotic stress upon pathogen inoculation than *C. sativa*. Those genes for both species are involved in regulation of plant immune response and stress adaptation and recovery. Furthermore, it is suggested that both species recognize the pathogen attack; however, the resistant species may involve more genes in the defense response than the susceptible species. RNA-seq enabled the selection of candidate genes for ink disease resistance in *Castanea*. The present data is a valuable contribution to the available *Castanea* genomic resources and constitutes the basis for further studies.

Keywords *Castanea sativa* · *Castanea crenata* · *Phytophthora cinnamomi* · RNA-seq · Differentially expressed genes · Biotic stress

Communicated by R. Sederoff.

Mónica Sebastiana and Rita Costa contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11295-014-0829-7) contains supplementary material, which is available to authorized users.

S. Serrazina (✉) · M. S. Pais · M. Sebastiana
Plant Molecular Biology and Biotechnology Lab, Center for Biodiversity, Functional and Integrative Genomics (BioFIG), Edifício C2, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal
e-mail: smserrazina@fc.ul.pt

C. Santos · H. Machado · R. Costa
Forestry Systems, Molecular Biology Laboratory, INIAV I.P, Av da República, 2780-159 Oeiras, Portugal

C. Pesquita
Informatics Department, Edifício C6, Faculdade de Ciências da Universidade de Lisboa, Campo Grande, 1749-016 Lisbon, Portugal

R. Vicentini
Systems Biology Laboratory, CBMEG, Universidade Estadual de Campinas, CEP: 13083-875, Campinas, SP, Brazil

Introduction

European chestnut (sweet chestnut, *Castanea sativa* Miller) has great economic value due to fruit production, and ecological value including forest diversity and soil stability. In the last 100 years, ink disease caused by the soil oomycete *Phytophthora cinnamomi* Rands has contributed to a drastic reduction of *C. sativa* distribution area in Europe. Oomycetes (eukaryotic heterokonts) show strategies of plant infection that are similar to many fungal pathogens (Latijnhouwers et al. 2003). Sweet chestnut groves have also been affected since the 1930s by the chestnut blight fungus [*Cryphonectria parasitica* (Murril) Barr], causal agent of the American chestnut [*Castanea dentata* (Marshall) Borkh.] decimation.

Ink disease was introduced to Europe from the USA through the Azores islands (Fernandes 1955; Anagnostakis 2001). The first records on its appearance in northern Portugal date from 1838. It has since been reported in many European

countries, including Spain, Italy, France, and the UK. The progression of the disease in grove areas with high humidity has limited the establishment of new groves and impeded the conservation of old ones (Vannini and Vettraino 2001). Presently, the greatest impact of ink disease is limited to the warm southwestern and southern regions of central Europe [reviewed by (Brasier and Jung 2006)]. In the nineteenth century, ink disease was partially responsible for a decline of *C. dentata* in the Southeastern USA (Anagnostakis 2001), prior to its broad decimation by chestnut blight. Ink disease is currently re-emerging in the USA and constitutes a serious threat to the American chestnut reintroduction (Jacobs et al. 2013).

Common woody hosts of *P. cinnamomi* include *Eucalyptus*, *Quercus*, *Juglans*, *Betula*, and *Castanea*, and the mycelia also persist saprophytically in soil. In the presence of water, oospores and chlamydospores differentiate sporangia that form and release zoospores. Zoospores are motile and are able to penetrate non-lignified root tissue and the base of stems or trunks: both scenarios result in local tissue rot. Growth, reproduction, and dissemination of the pathogen are favored under compacted and water saturated soils with poor aeration. Symptoms on the adult trees include leaf chlorosis, thinning of the crown, and the persistence of immature fruits on the trees after leaf-fall. Larger roots are mainly affected, producing a black exudate which increases during spring and fall. Infected seedlings undergo a rapid or gradual leaf wilting, depending on the severity of the infection. The root system suffers extensive necrosis of the tap root that extends to the lateral roots and up the lower stem (Vannini and Vettraino 2001). Oßwald et al. (2014) explain the primary physiological, biochemical, and molecular reactions described on infected roots of susceptible *Phytophthora*-host interaction, summarized as follows: (1) The pathogen releases elicitins into the rhizosphere, facilitating root penetration; (2) Downregulation of defense genes in the host, facilitating pathogen growth; (3) Destruction of roots and impairment of water and nutrient uptake; (4) Increase of the abscisic acid phytohormone in roots; (5) Decrease in leaf water potential; (6) Stomata closure and decrease in photosynthesis; (7) Probable release of toxins and effectors into the host tissue during biotrophic growth of the pathogen and transport into the canopy via xylem sap flow; (8) Upregulation of genes of the ethylene pathway and release of the phytohormone by leaves; (9) Decrease in cytokinin content in roots during the necrotrophic growth of the pathogen; (10) Chlorosis and wilting of leaves resulting from the changed water and hormonal status of the host caused by root infection.

Progression of ink disease depends on environmental conditions, pathogen virulence and plant susceptibility. One strategy to control the disease is through breeding with resistant species. Soon after the introduction of Asian chestnuts to Europe it was verified that *C. crenata* (*Castanea crenata* Siebold & Zucc., the Japanese chestnut) has a high level of resistance to *Phytophthora* (Vannini and Vettraino 2001). Since the 1950s, breeding programs with the European and Japanese chestnut were established in Portugal, France, and Spain to obtain hybrids tolerant to ink disease, while maintaining fruit production and quality traits to satisfy commercial demands (Vannini and Vettraino 2001; Martins et al. 2009). However, fruit quality produced by these hybrids is below current market standards, so there is demand from both researchers and producers, for developing genomic tools to understand resistance mechanisms against *P. cinnamomi*. Barakat et al. (2009; 2012) described the generation of more than 1.5 million cDNA sequences for the American and Chinese chestnuts that have been used to analyze chestnut resistance to *C. parasitica*. The data are available through the Fagaceae Genomics Web (http://www.fagaceae.org/), and represent the first public resource on chestnut transcriptomes. The data we present here contribute to this resource by identifying Japanese chestnut genes involved in the reaction to ink disease, another critical threat to *Castanea*.

To compare the response of the resistant Japanese chestnut with the response of the susceptible European chestnut to *P. cinnamomi* challenge, four cDNA libraries of *C. sativa* (Cs) and *C. crenata* (Cc) root tissues, inoculated (i) and non-inoculated (n) with the pathogen were prepared for 454 pyrosequencing. Contig annotation and analysis of transcript abundance supported the quantification of transcript expression on inoculated and non-inoculated roots in each species, as well as identifying differentially expressed genes upon pathogen inoculation. This allowed a preliminary comparison of each species' response to the pathogen and the selection of candidate genes for resistance to ink disease.

Materials and methods

Plant material and pathogen inoculation

The TRAGSA nursery (Grupo TRAGSA-SEPI, Maceda, Spain) provided 36 micropropagated plants at 5 years of age, 18 of *C. sativa* (Cs, susceptible) and 18 of *C. crenata* (Cc, resistant). Four treatments were set, corresponding to *C. sativa* and *C. crenata* inoculated and non-inoculated with *P. cinnamomi* (Supplementary material 1). The plants were distributed in 15-l containers with turf soil.

A hypervirulent isolate of *P. cinnamomi* (IMI 340340) provided by Trás-os-Montes and Alto Douro University was grown at 20 °C on Potato Dextrose Agar. For soil infestation, *P. cinnamomi* inoculum was prepared by growing mycelia on sterilized millet seeds (*Ponicium mileaceum*), which were thoroughly moistened with vegetable juice (V8®) broth [20 % (*v*/*v*) with 3 g/L of $CaCO_3$]. The mixture was incubated for 3 weeks in darkness at 24 °C.

At the time of inoculation (0 h), *P. cinnamomi* was carefully added to each container substrate at a concentration of 5 % (*v*/*v*), in order to minimize root disturbance and wounding. No pathogen was added to non-inoculated plants. After inoculation, all containers were flooded for 3 h to stimulate zoospore release and to promote disease development. At 2, 4, and 7 days after inoculation, six plants per treatment (three of *C. sativa* and three of *C. crenata*, Supplementary material 1) were removed from containers and root samples were collected. After rinsing, roots were frozen in liquid nitrogen and stored at −80 °C.

RNA isolation

Total RNA from root tissue was isolated based on le Provost et al. (2007). RNA integrity and purity was determined with a 2100 Bioanalyser with the RNA 6000 Pico kit (Agilent Technologies, Palo Alto, CA, USA). In order to compare gene expression between the two chestnut species after pathogen inoculation, four RNA pools were prepared, based on the experimental design described by Barakat et al. (2009; 2012): Cci, Ccn, Csi, and Csn (i: inoculated; n: non-inoculated). Each pool included the RNA from nine plants, collected at three time points after inoculation (2, 4 and 7 days, Supplementary material 1).

Poly(A) RNA enrichment, cDNA library construction, and pyrosequencing

The procedures described in this section were provided by the Next Gen Sequencing Unit at Biocant (Cantanhede, Portugal).

The integrity of all RNA pools was verified on a 2100 Bioanalyzer as above and the quantity assessed by fluorometry with the Quant-iTRiboGreen RNA kit (Invitrogen, CA, USA). Poly(A)RNA was enriched from total RNA using two rounds of the MicroPoly(A) Purist Kit (Applied Biosystems, Ambion, CA, USA), according to the manufacturer's instructions. The RNA quality was again assessed on a 2100 Bioanalyser and the quantity determined by fluorometry as described above.

A fraction of 200 ng of Poly(A)+RNA of each isolate was used as starting material for cDNA library construction using Multiplex Identifiers (MIDs) according to the cDNA Rapid Library Preparation Method Manual, "GS FLX Titanium Series, October 2009" (Roche-454 Life Sciences, Brandford, CT, USA). The four dscDNA libraries were quantified by fluorescence, pooled in equimolar amounts and pyrosequenced in a single plate with GS FLX Titanium chemistry (Roche-454 Life Sciences, Brandford, CT, USA), according the standard manufacturers' procedures.

Transcript assembly and functional annotation

After 454 sequencing, the raw reads were processed to remove sequences with less than 100 nucleotides and low quality regions. Ribosomal, mitochondrial, and chloroplast reads were identified through BLASTx against the non-redundant NBCI database and any hits with an *E* value of 0.0 were removed from the data set. All remaining reads were then assembled into contigs using 454 Newbler 2.6 (Roche, Branford, CT, USA) with the default parameters (40 bp overlap and 90 % identity). A three step analysis was carried out to identify genes. First, the translation frame of each contig was assessed through BLASTx searches against Swissprot (*E* value<1E−6) and the corresponding amino acid sequence was translated using an in-house script. Then, any contigs without translation were submitted to FrameDP (Gouzy et al. 2009) software with default parameters. Finally, all remaining contigs were analyzed with ESTScan (Lottaz et al. 2003) with default parameters. Transcripts identified by FrameDP or ESTScan were searched using BLASTp against the non-redundant NBCI database (*E* value<1E−2) to translate putative proteins. The functional annotation of all translated amino acid sequences was predicted through assignment into protein families and identification of protein domains using InterProScan version 4.6 (Hunter et al. 2009). Gene Ontology (GO) terms identified by InterProScan results for each translated amino acid sequence were additionally retrieved and added to classify each transcript product. The procedures above described were provided by the Next Gen Sequencing Unit at Biocant.

All contigs were taxonomy annotated in order to separate the sequences belonging to the *Streptophyta* phylum for further analysis. To obtain the taxonomical assignments, we uploaded the contigs to MG-RAST (Meyer et al. 2008) (with default parameters), an automated analysis platform for metagenomes based on sequence similarity to both protein and nucleotide databases.

Identification of differentially expressed genes related to *P. cinnamomi* resistance

Differentially expressed genes were identified as genes showing significant higher/lower expression levels in inoculated root tissue versus non-inoculated root tissue. The number of reads mapping to each transcript (contig) in the two treatments (inoculated and non-inoculated) was counted and used as an approximate estimation of gene expression level in the corresponding tissues. First, the contigs from the different samples were clustered at 90 % similarity and 95 % identity by CD-Hit 454 (Niu et al. 2010) to eliminate redundant sequences and generate reference contigs. The reads from each sample were then mapped to those references with Newbler mapping 2.6 (Roche, Branford, CT, USA) using the default parameters, and the number of reads contributed by each sample counted.

Reads with multiple hits were discarded. The number of reads per reference contig per sample was used to build a contingency table, which was analyzed with the Myrna statistical analysis package (Langmead et al. 2010), with the normalization factor set to 95th percentile. Statistical significance of the differential expression was evaluated using a linear regression model based on a Gaussian distribution, and using only contigs with a minimum of eight mapped reads. All results were compiled into a SQL database developed as an information management system. The procedures described above were provided by the Next Gen Sequencing Unit at Biocant.

For the selection of Differentially Expressed Genes (DEG), contigs with a *P* value<1E−03 were considered. *P* value describes the probability that differences in counts between the two sets in comparison are due to chance (Langmead et al. 2010). Fold expression changes were calculated for the inoculated vs. non-inoculated comparisons, Csi-Csn and Cci-Ccn. Contigs with a fold change greater than 1 were classified as upregulated genes and contigs with a fold change less than to 1 were classified as downregulated genes. For further analysis of DEG (*P. cinnamomi* resistance-related genes), additional criteria were applied: *Q* value<1E−2 and $\log_2$ of fold change>|1|. *Q* value is a false discovery rate analog of *P* value (Storey and Tibshirani 2003).

DEG were also analyzed for the two inoculated species in the comparison Csi-Cci (*P* value<1E−03) to reveal the genes that were significantly induced in both species after pathogen challenge.

The application Blast2GO (Conesa and Götz 2008), namely the Enrichment Analysis, was used to statistically analyze GO annotation in the comparisons Csi-Csn, Cci-Ccn, and Csi-Cci for DEG. It employs a Fisher's exact test with multiple testing correction of FDR (Benjamini and Hochberg). Upon selection of a single test and *P* value <5E−3, all GO terms were tested if they are enriched in the DEG group when compared to a reference group (all contigs in the comparison).

454 sequencing validation by real-time PCR

The relative expression of a subset of genes was achieved by quantitative real-time PCR (qRT-PCR) to validate RNA-sequencing (RNA-seq). The four RNA pools used for sequencing (Cci, Ccn, Csi, and Csn) were prepared for qRT-PCR as follows: RNA was treated with DNase (Turbo DNase-free kit Ambion, Inc., USA), according to manufacturer's instructions. cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Gene specific primers were designed for six target genes (Supplementary material 2) using Primer Express (version 1.0, Applied Biosystems, Sourceforge, USA). *Actin-7* was selected as a reference gene after verifying a similar number of reads for all cDNA libraries and used for normalization of expression. A final concentration of 0.2 μM of each primer was used in 25 μL reactions, together with cDNA as template and Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Ontario, Canada), on a StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Thermal cycling for all genes started with a denaturation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing temperatures for 30 s. Three technical replicates were used per reaction set, including template and no template controls. Non-specific PCR products were analyzed by dissociation curves. The relative expression value and mean absolute deviation values were calculated for the pool comparisons Cci-Ccn and Csi-Csn according to the ΔΔCT method (Livak and Schmittgen 2001).

Results

454 sequencing and assembly summary

Two Japanese chestnut cDNA libraries were constructed, one from a RNA pool of inoculated root tissue (Cci) and the other from a RNA pool of non-inoculated root tissue (Ccn). A half plate of sequencing was used, resulting in 220,412 reads for Cci and 182,314 reads for Ccn, with an average read length of 350 nt (Table 1). Approximately 77 and 64 megabases of cDNA were generated for Cci and Ccn respectively. After assembly, 8528 contigs were generated for Cci and 8300 contigs were generated for Ccn, with an average length of 885 nt. 2712 Cci contigs and 2214 Ccn contigs had more than 1000 nt, corresponding to 32 and 27 % of all respective contigs.

Two cDNA libraries were also constructed for European chestnut, Csi (inoculated roots) and Csn (non-inoculated roots). A half sequencing plate resulted in 181,384 reads for Csi and 186,920 reads for Csn, with an average read length of 362 nt (Table 1). Csi and Csn libraries contained approximately 65 and 69 megabases, respectively. Assembly resulted in 7208 contigs for Csi and 8475 contigs for Csn, with an average length of 840 nt; 1943 Csi contigs and 2065 Csn contigs had more than 1000 nt, corresponding to 27 and 24 % of total respective contigs.

Functional annotation

BLASTX against Swissprot, FrameDP, and ESTScan tagged 8149 Cci contigs and 7969 Ccn contigs as putative proteins. Likewise, 6852 contigs were tagged for Csi and 8073 contigs were tagged for Csn. This indicates that 95 % of the contigs could be tagged as putative proteins, comprising highly informative transcripts on a plant genome that has not yet been sequenced.

Table 1 Summary of 454 sequencing for *Castanea crenata* and *Castanea sativa* root transcriptomes

cDNA library	Cci	Ccn	Csi	Csn
Tissue sampled	*C. crenata* inoculated roots	*C. crenata* non-inoculated roots	*C. sativa* inoculated roots	*C. sativa* non-inoculated roots
No. of plates	1/4	1/4	1/4	1/4
No. of reads	220,412	182,314	181,384	186,920
Average read length (nt)	350	350	357	367
No. of bp	77,175,000	63,823,300	64,884,000	68,672,896
No. of contigs	8528	8300	7208	8475
Average contig length (nt)	915	854	856	823
No. of large contigs[a]	2712	2214	1943	2065
No. of putative proteins	8149	7969	6852	8073
AA sequences assigned to InterPro terms	6373	6279	5350	6213
AA sequences assigned to GO terms	4885	4790	4090	4691

[a] Greater than 1000 nt

The *Castanea-P. cinnamomi* system in the present report was analyzed on plants in non-sterile soil containers and comprises several other organisms, mostly soil-borne. To analyze *C. sativa* and *C. crenata* metagenomes concerning taxonomic hit distribution, all contigs from the four libraries were uploaded to MG-RAST server. Results were similar for all four root transcriptomes (Cci, Ccn, Csi, and Csn) and are combined in Fig. 1. Sequence alignments to the M5 non-redundant protein database (M5NR) revealed that *C. crenata* and *C. sativa* contigs have the highest similarities with plant proteomes: *Arabidopsis thaliana* (24 %), *Vitis vinifera* (19 %), *Ricinus communis*, and *Populus trichocarpa* (both 15 %). Presently, the sequenced genome of *A. thaliana* is the one with the highest level of information among the dicotyledonous. *V. vinifera*, *R. communis*, and *P. trichocarpa* are woody/semi-woody plants, all with sequenced genomes with similar coverage and taxonomically close to *Castanea*. Barakat et al. (2012) obtained comparable similarities with their proteomes for Chinese and American chestnut transcripts. MG-RAST attributed 13 % of the annotated contigs to "unknown proteins" that may contain chestnut specific proteins. Additionally, 1 % of the contigs were classified as "unknown sequences" that had no homology to predicted proteins or rRNA and may correspond to 5′ or 3′ untranslated regions and small RNAs. The very low percentage of "non Streptophyta" sequences (4 %) may be correlated to the presence of other organisms in the soil as bacteria or fungus (Ascomycota and Basidiomycota).

The contigs of all four libraries were uploaded to InterProScan, which provided Gene Ontology (GO) terms within the three GO categories (Molecular Function, Biological Process, and Cellular Component) for functional annotation. As exposed in Table 1, GO terms were attributed to 57 % of the contigs: 4885 for Cci, 4790 for Ccn, 4090 for Csi, and 4691 for Csn. When the annotation sets of *C. crenata* (Cci, Ccn) and *C. sativa* (Csi, Csn) were combined on Blast2GO, the distribution of GO terms was similar for the two species' root transcriptomes (Fig. 2). "Membrane part" and "membrane-bounded organelle" are well represented on Cellular Component (CC) category and include genes related to biotic stress regulation and recovery. The Molecular Function (MF) terms "binding" and "catalytic activity" include "hydrolase activity" (with 949 and 200 contigs, respectively) and genes involved in the synthesis of enzymes that degrade pathogenic fungal cell walls. Moreover, "transporter activity" and "nucleic acid binding transcription factor activity" enclose genes involved in biotic stress signaling and regulation. The Biological Process (BP) "metabolic process" includes "oxidation-reduction process" (587 contigs) and "cellular process" includes "cell communication" (229 contigs), pointing to genes related to anti-fungal metabolite synthesis and stress signaling, respectively. We also highlight the BP terms "biological regulation" and "response to stimulus", which contains "response to stress" (230 contigs). This GO terms distribution indicates that *C. crenata* and *C. sativa* transcriptomes are suitable for stress physiology studies and for the selection of candidate genes to ink disease resistance.

Differentially expressed genes after pathogen inoculation

RNA-seq read count is directly related to gene expression levels between the two experimental conditions (Langmead et al. 2010). 454 Newbler Mapping 2.6 quantified the reads in each *Castanea* library for a specified contig. Then Myrna attributed statistical significance to the difference between the reads or a *P* value, which was used to select Differentially Expressed Genes after pathogen inoculation (DEG). *C. crenata* DEG derive from a comparison of the reads in

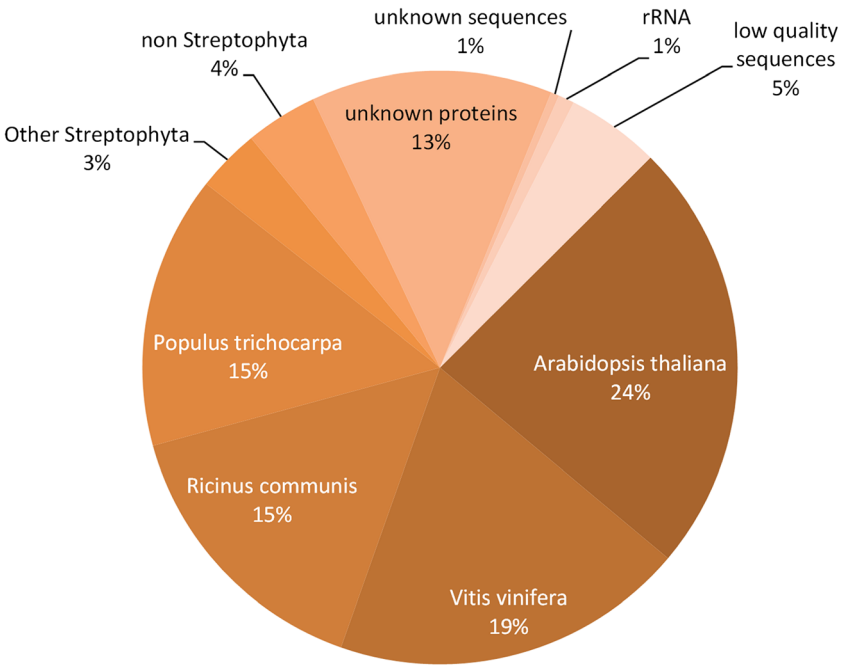

Fig. 1 Taxonomic hits of *Castanea crenata* and *Castanea sativa* contigs at the proteome level. All contigs were uploaded to MG-RAST and annotated

the pathogen inoculated library to the reads in non-inoculated library (Cci-Ccn). Likewise, *C. sativa* DEG derive from the comparison Csi-Csn. With a *P* value<1E−3, 283 DEG could be identified in the Cci-Ccn comparison and 305 DEG in Csi-Csn comparison (Supplementary material 3 and Supplementary material 4). In inoculated *C. crenata* 229 and 54 contigs were respectively up- and downregulated relative to non-inoculated roots (Fig. 3). After analysis of BLAST best hits, we identified 34 % of upregulated contigs and 6 % of downregulated contigs related with a response to pathogens. In inoculated *C. sativa* 117 contigs were upregulated and 188 genes were downregulated relative to non-inoculated root tissue. Eighteen percent of the upregulated and 10 % of the downregulated contigs were related with biotic stress response.

We analyzed GO annotation of DEG in the comparison sets Cci-Ccn and Csi-Csn and the enrichment analysis revealed divergences for the two chestnut species (Fig. 4 and Supplementary material 5). Upon inoculation, genes upregulated in *C. crenata* but not in *C. sativa* included transcription factors (TF) assigned to the GO terms "DNA binding" (MF) and "Sequence-specific DNA binding transcription factor activity" (MF), related to the regulation of the host response to the pathogen (e.g., *WRKY transcription factor 31*) (Zhang et al. 2008a). The term "Cell wall" (CC) also was only revealed for *C. crenata*, with transcripts involved in cellulose synthesis (e.g., *Xyloglucan endotransglucosylase/hydrolase protein 23*) (Yokoyama and Nishitani 2001). The term "Carbon-carbon lyase activity" (MF) was common to upregulated DEG in both species, with genes involved in glycolysis (e.g., *Fructose-bisphosphate aldolase*, *cytoplasmic isozyme 2*). The enrichment analysis of GO annotation for downregulated DEG upon inoculation (Fig. 4) revealed a common BP term for the two species, "Cellular polysaccharide metabolic process", which includes genes related with starch and cellulose synthesis (e.g., *Granule-bound starch synthase 1*, *chloroplastic/amyloplastic*). *C. sativa* had a preponderance of downregulated genes included in the term "Catalytic activity" (MF), including genes related with the response to oxidative stress (e.g., *Glutamate decarboxylase 1*) (Bouché and Fromm 2004), secondary metabolite synthesis (e.g., *Chalcone-flavonone isomerase 1*) (Hartmann et al. 2005), general defense proteins (e.g., *Pleiotropic drug resistance protein 2*) (Yazaki 2006), receptor-like protein kinases responsive to pathogen infection (e.g., *Cysteine-rich receptor-like protein kinase 10*) (Chen et al. 2004) and jasmonic acid (JA) synthesis (e.g., *Allene oxide synthase*) (Sivasankar 2000). The GO term "Oxidation-reduction process" (BP) was the most significant for *C. crenata* downregulated DEG, comprising genes related to secondary metabolite synthesis (e.g., mevanolate biosynthesis, *3-hydroxy-3-methylglutaryl-coenzyme A reductase*) (Leivar et al. 2011).

We also analyzed the most significant GO terms for DEG in the comparison Cci-Csi, which shows the similar set of genes that were induced in both inoculated species (Supplementary material 6 and 7). The most represented GO term "Catalytic activity" (MF) includes genes of hydrolases related with plant defense to fungi (e.g., *Glucan endo-1,3-beta-glucosidase*, *basic vacuolar isoform*) (Subroto et al. 2001) and genes related with cell wall synthesis (e.g., *UDP-glucose:protein transglucosylase 2*) (Bocca et al. 1999). These genes are also represented in the GO terms "Carbohydrate

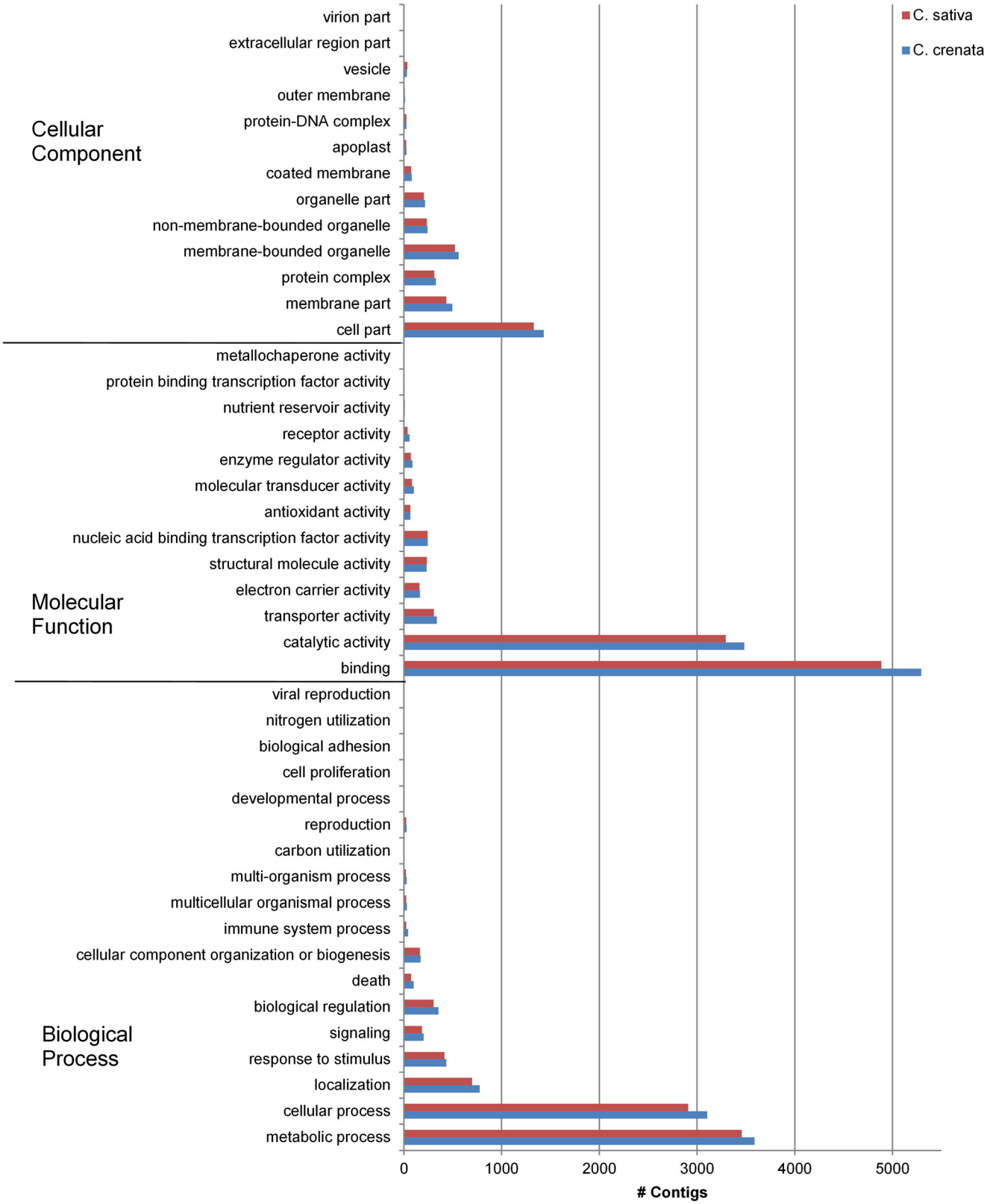

Fig. 2 Distribution of *Castanea crenata* and *Castanea sativa* contigs into functional sub-categories of Gene Ontology. Only contigs corresponding to putative proteins were considered in this analysis. Cut-off on GO levels: 1 for Biological Process and Molecular Function and 2 for Cellular Component

metabolic process" and "Cell wall organization and biogenesis" (both BP). The term "Cofactor binding" (MF) comprises genes involved in the generation of ROS during incompatible interactions with pathogens (e.g., *Respiratory burst oxidase homolog protein D*) (Torres et al. 2002) and the term "Iron ion binding" (MF) contains genes involved in oxylipin synthesis (e.g., *Lipoxygenase 3, chloroplastic*) (Vellosillo et al. 2007) and genes concerned with secondary metabolite synthesis (e.g., *Taxane 13-alpha-hydroxylase*) (Sun et al. 2013).

The comparison of *C. crenata* and *C. sativa* DEG (Supplementary materials 3, 4 and 6) revealed common upregulated genes after inoculation that may be involved in *P. cinnamomi* response (Supplementary material 8). We associated those genes into functional categories and highlight (a) Regulation of plant immune response, with four genes including *Lipoxygenase A*, related to signaling during pathogen attack and induction of cell death (Porta and Rocha-Sosa 2002), and (b) four genes related to drought stress, e.g., *Dehydrin DHN2* (Yang et al. 2012). In the comparison of Cci-Ccn with Csi-Csn we also identified one common downregulated DEG, *Zinc finger protein1 WZF 1*, a probable transcriptional repressor (Ohta et al. 2001).

Further analysis of the data found in Supplementary materials 3, 4 and 6 revealed *C. sativa* downregulated genes that correspond to upregulated genes in *C. crenata*. These transcripts were associated in the functional categories Regulation, Lipid signaling, Anti-fungal metabolite synthesis, Cell wall synthesis, and Stress recovery (Supplementary material 9).

P. cinnamomi resistance-related genes

We consider that the most significant candidate genes of resistance to *P. cinnamomi* are *C. crenata* (resistant species) genes upregulated at least two times after inoculation that are not present among *C. sativa* DEG and are putatively related to stress response. A selection of *C. crenata* candidate genes with *Q* value <1E−2 was inferred from the Cci-Ccn DEG list in Supplementary material 3 and assigned to the functional categories in Table 2.

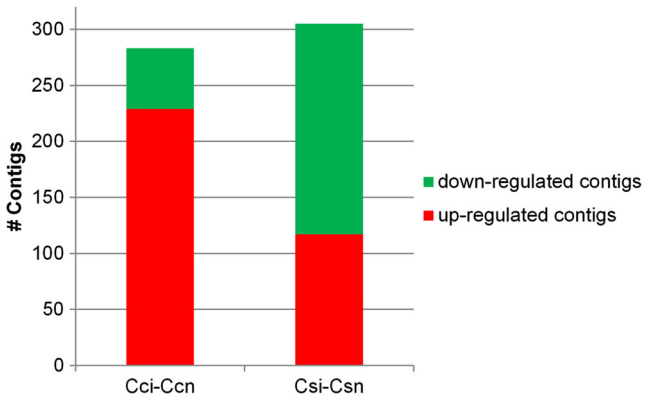

Fig. 3 Differentially expressed genes in the root transcriptomes of *Castanea crenata* (Cc, resistant) and *Castanea sativa* (Cs, susceptible), P value<1E−3. A contig is upregulated when the ratio i/n (number of reads in inoculated library/number of reads in non-inoculated library) is >1 and downregulated when the same ratio is <1

Especially noteworthy are the kinase receptor genes that may be involved in pathogen recognition (as the *Probable LRR receptor-like serine/threonine-protein kinase*) (Diévart and Clark 2003) and genes corresponding to TF involved in the regulation of host response after pathogen perception (as *WRKY* TF) (Yang et al. 2009). The putative involvement of JA and salicylic acid (SA) signaling pathways was inferred from the categories "Regulation of host response after pathogen perception" and "Lipid signaling". Two genes in the "Regulation of plant immune response" category are responsive to the plant hormone ethylene (e.g., *Ocs element-binding factor 1*) (Zhang and Singh 1994).

Certain *C. crenata* upregulated genes may prevent pathogen progress, such as the precursor of *Cationic peroxidase 1* (Reimers et al. 1992) [category "Hypersensitive response" (HR)], and *Pectinesterase 2* (Wen et al. 2013) (category "Cell wall strengthening"). Three genes, e.g., *Probable glutathione S-transferase*, were associated in the category "HR recovery" (Ryu et al. 2009).

Genes involved in "Anti-fungal metabolite synthesis" (such as *UDP-glycosyltransferase 85A2*) (Woo et al. 2007) and "Anti-fungal enzymes" (such as *Probable carboxylesterase 120*) (Marshall et al. 2003) may take part in the host response to enhance the defense to *P. cinnamomi*.

Genes in the categories "Regulation of drought stress", "Response to drought stress" and "Stress recovery" are also represented in the *C. crenata* candidate list, such as *NAC domain-containing protein 72* (Singh et al. 2013), *Phosphoprotein ECPP44* (Tan and Kamada 2000), and *Lon protease homolog 2 peroxisomal* (Lingard and Bartel 2009), respectively.

C. sativa DEG after *P. cinnamomi* inoculation are presented in Supplementary material 4. In order to identify the susceptible species' response to the pathogen, we selected upregulated genes in inoculated *C. sativa* (at least two times) that are not present among *C. crenata* DEG and are putatively related to stress response (*Q* value<1E−2). Those genes were distributed in a series of functional categories in Table 3. In the "Regulation of plant immune response" category we emphasize the gene *REF/SRPP-like protein At1g67360* (Taki et al. 2005), induced by a precursor of JA. *C. sativa* also invests in genes related to "Stress recovery" (e.g., *Aminophospholipid flippase 9*) (López-Marqués et al. 2012) and "HR recovery" (e.g., *4-hydroxyphenylpyruvate dioxygenase*) (Sandorf and Hollander-Czytko 2002). Genes involved in "Anti-fungal

Fig. 4 Distribution of *Castanea crenata* and *Castanea sativa* differentially expressed genes (DEG) into functional sub-categories of Gene Ontology. Enriched Analysis was applied separately to upregulated and downregulated DEG and compared with the reference sets of all contigs in the comparison, to obtain significant GO terms. The selected *P* value on Fisher's Exact Test was lower than 5E−3

metabolite synthesis" (e.g., *Flavonoid 3-hydroxylase*) (Sharma et al. 2012) and "Cell wall strengthening" (e.g., *UPF0497 membrane protein At3g06390*) (Roppolo et al. 2011) may prevent *P. cinnamomi* proliferation in the host. Finally, *C. sativa* upregulated genes linked to drought stress regulation, such as the TF *Homeobox-leucine zipper protein HAT5* (Henriksson et al. 2005) may play a role in host recovery from pathogenicity.

Validation of RNA-seq

The RNA-seq approach allowed for the quantification of gene expression levels by sequence read depth. DEG were identified by estimating the ratio between reads in inoculated libraries and non-inoculated controls (Cci-Ccn, Csi-Csn). To validate the differential expression levels observed by RNA-seq, qRT-PCR was used to obtain the expression level of DEG in

Table 2 *Castanea crenata* (Japanese, resistant) candidate genes to *Phytophthora cinnamomi* resistance. Genes were associated into the functional categories in the left column (bold)

Predicted function / Gene	Reported function, notes	Cci/Ccn Log2 (fold change)	References
Pathogen recognition			
Disease resistance protein At4g27190		2.41	1 S10
Probable LRR receptor-like serine/ threonine-protein kinase	Precursor. Accession: At1g07650	1.81	Diévart and Clark 2003
Probable serine/threonine-protein kinase	Accession At1g54610	1.53	2 S10
Regulation of host response after pathogen perception			
C2 domain-containing protein	Accession: At1g53590	2.15	3 S10
Uncharacterized protein At3g61260	Homology with remorins	2.08	4 S10
Probable WRKY transcription factor 31		1.71	Zhang et al. 2008b
Putative receptor protein kinase ZmPK1	Precursor	1.55	5 S10
WRKY transcription factor 22	Induction of JA pathway	1.33	Yang et al. 2009
GRAS family protein 4	TF	1.20	6 S10
Calcium-dependent protein kinase isoform 3 CDPK3	SA and phytoalexin synthesis. Induced by JA	1.14	Chung et al. 2004
Lipid signaling			
Clathrin interactor EPSIN 3		2.23	7 S10
IST1-like protein		2.02	8 S10
Patatin-05	Precursor. Triggering of JA pathway during HR. May be upregulated by SA and ethylene	1.27	Dhondt et al. 2000, 9 S10
Regulation of plant immune response			
RING finger protein 5	Ubiquitin/proteasome system. JA-dependent signaling	2.97	Hondo et al. 2007
Putative *syntaxin-24*	Probable SNARE complex-mediated immunity to pathogenic fungi at the plant cell wall	2.58	10 S10
Sulfate transporter 3,1	Upregulated in response to pathogens, MeJA and SA. Brassinosteroid hormone regulation	2.25	Marsolais et al. 2007
Ethylene-responsive transcription factor 4	Regulation of JA-responsive defense genes' expression to fungal pathogens	1.50	11 S10
Ocs element-binding factor 1	TF. Increased activity by SA	1.13	Zhang and Singh 1994
Regulation of drought stress			
NAC domain-containing protein 72	TF	2.66	Singh et al. 2013
Myb-related protein 21	TF. Responsive to JA	1.97	9 S10
Cysteamine dioxygenase	Mediates the response to hypoxia allied to ERF-VII TFs	1.77	12 S10
Hypersensitive response			
Cationic peroxidase 1	Precursor. Induces cell wall lignification	3.29	Reimers et al. 1992
Arginine decarboxylase	May be induced by MeJA and SA. Induces polyamine synthesis	1.19	Nakane et al. 2003
S-adenosylmethionine decarboxylase alpha chain	Precursor. Induces cell wall strengthening and polyamine synthesis	1.05	13 S10, 14 S10
Hypersensitive response recovery			
Probable glutathione S-transferase	Regulated by SA	2.93	Ryu et al. 2009
B-cell receptor-associated protein 31	Regulation of apoptosis	1.90	Szczesna-Skorupa and Kemper 2006
Pto-interacting protein 1	Induced by SA	1.42	15 S10
Anti-fungal enzymes			
Probable carboxylesterase 120	Serine hydrolase involved in plant-pathogen interactions	5.91	Marshall et al. 2003
Glucan 1,3-beta-glucosidase	Precursor. Induced by JA. Exported to apoplast	2.67	16 S10
Acidic endochitinase	Precursor. Induced by SA	2.44	17 S10
Basic 7S globulin 2	Precursor. May act on pathogen secreted enzymes upon host infection	1.97	18 S10
Glucan endo-1,3-beta-glucosidase 11	Family 17 glycosyl hydrolases	1.68	19 S10

Table 2 (continued)

Predicted function / Gene	Reported function, notes	Cci/Ccn Log2 (fold change)	References
Anti-fungal metabolite synthesis			
UDP-glycosyltransferase 85A2 and *85A5*	Flavonoid pathway	3.19-2.49	Woo et al. 2007
Flavin-containing monooxygenase FMO GS-OX5	Glucosinolate pathway	2.45	20 S10
Taxadiene 5-alpha-hydroxylase	Taxol (terpene pathway). May be induced by JA	2.42	21 S10
Taxane 13-alpha-hydroxylase	Taxol (terpene pathway). May be induced by JA	2.06	Sun et al. 2013
Squalene monooxygenase	Saponin (terpene pathway). Induced by JA	1.76	46, 63
L-allo-threonine aldolase	Saponins (terpene pathway) and isoflavonoids (flavonoid pathway). May be induced by JA	1.40	Broeckling et al. 2005
Reticuline oxidase-like protein	Precursor. Alkaloid pathway. Induced by MeJA	1.30	46, 64
3-ketoacyl-CoA synthase 11	Cuticle. May be induced by SA	1.07	22 S10
Cell wall strengthening			
Pectinesterase 2	Precursor. Responsive to JA. Acts on homogalacturan	2.98	23 S10, 24 S10, Wen et al. 2013
Pyruvate kinase, cytosolic isozyme	Lignin synthesis upon fungal infection	1.39	Mutuku and Nose 2012
Extensin-2	Precursor. Responsive to fungal infection	1.30	Benhamou et al. 1991
6-phosphofructokinase 3	Lignin synthesis	1.26	25 S10
Cell wall synthesis			
UDP-glucose 4-epimerase GEPI48		2.39	26 S10
COBRA-like protein 7	Precursor	1.43	27 S10
UDP-glucose 6-dehydrogenase	Reponsive to pathogen. May be induced by SA	1.20	28 S10
Response to drought stress			
Phosphoprotein ECPP44	Homology with a dehydrin gene	1.97	Tan and Kamada 2000
Stress recovery			
Probable *arylformamidase*	Alternate pathway for NAD biosynthesis	3.30	29 S10
Equilibrative nucleoside transporter 2	Nucleotide synthesis rescue pathway	2.67	Li et al. 2003
Protein translation factor SUI1 homolog 2		2.44	30 S10
Lon protease homolog 2, peroxisomal	Lateral root formation	2.29	Lingard and Bartel 2009
Glutamate decarboxylase 1		2.17	Bouché and Fromm 2004
Ethanol tolerance protein GEKO1		2.15	31 S10
Prolyl 4-hydroxylase subunit alpha-2	Precursor. Root hair formation	1.86	Vlad et al. 2007

S10 references in Supplementary material 10

inoculated libraries (Cci, Csi) relative to non-inoculated libraries (Ccn, Csn). The selected DEG (Fig. 5) are putatively related to *Castanea* response to the pathogen and to host recovery, and include: *Ethylene-responsive TF 4*, *Disease resistance protein At4g27190*, *Ethylene-responsive TF ABR1*, *Precursor of glucan 1,3-beta-glucosidase* (family 5), *Pectinesterase 2*, and *C2 domain-containing protein At1g53590*.

The differential gene expression for the comparisons Cci-Ccn and Csi-Csn acquired with the 454 sequencing was compared with the relative expression levels obtained with qRT-PCR for the selected DEG. The results presented in Fig. 5 reveal differences in the expression levels of *C. crenata* transcripts upon inoculation when compared to *C. sativa* transcripts upon inoculation. Those differences are in accordance with read data obtained by RNA-seq and may reflect *Castanea* root transcriptome in response to *P. cinnamomi*.

Discussion

Functional annotation

GO annotation comparison of expressed genes after inoculation between Japanese (*C. crenata*, resistant to pathogen) and

Table 3 *Castanea sativa* (European, susceptible) upregulated genes upon *Phytophthora cinnamomi* inoculation related to stress response. Genes were associated into the functional categories in the left column (bold)

Predicted function / Gene	Reported function, notes	Csi/Csn Log2 (fold change)	References
Regulation of host response after pathogen perception			
Serine/threonine-protein phosphatase PP1 isozyme 4		1.90	Lin et al. 1999
SNF1-related protein kinase regulatory subunit γ 1	May interact with LRR-rich proteins	1.44	32 S10
Regulation of plant immune response			
DEAD-box ATP-dependent RNA helicase 56		1.57	33 S10
REF/SRPP-like protein At1g67360	Induced by the precursor of JA pathway OPDA	1.28	Taki et al. 2005
Histone deacetylase 6	Interacts with Ethylene and JA pathways related with plant defense to necrotrophic pathogens	1.28	Zhu et al. 2011
Regulation of drought stress			
Homeobox-leucine zipper protein HAT5	TF. Responsive to ABA and water deficit stress	2.27	Henriksson et al. 2005
Zinc finger A20 and AN1 domain-containing stress-associated protein 11	May act in protein targeting to the ubiquitin/ proteasome pathway	1.44	Giri et al. 2011
Myb-related protein 44	TF. Modulates SA and JA signaling	1.30	34 S10
Ethylene-responsive transcription factor RAP2-4	Induced by JA. Related to alkaloid metabolism control	1.01	Lin et al. 2008, 35 S10
Hypersensitive response			
Autophagy-related protein 9		2.44	36 S10
Hypersensitive response recovery			
RNA-binding post-transcriptional regulator csx1	Regulatory protein, pathogen-responsive, acting during oxidative signaling	1.75	37 S10
4-hydroxyphenylpyruvate dioxygenase	Plastoquinone synthesis. Upregulated by MeJA	1.49	Sandorf and Hollander-Czytko 2002
Phosphoserine aminotransferase, chloroplastic	Precursor. JA-responsive gene. Pyridoxal synthesis	1.38	38 S10
Late embryogenesis abundant protein Lea5		1.22	39 S10
Anti-fungal enzymes			
Oxalyl-CoA decarboxylase	Oxalate catabolism	2.96	Foster et al. 2012
Protein TAR1	Inhibition of fungal laccase	1.67	Jiang et al. 2009
Anti-fungal metabolite synthesis			
Flavonoid 3-hydroxylase	Flavonoid pathway	2.34	Sharma et al. 2012
Uncharacterized membrane protein At4g09580	Homology with SNARE associated Golgi protein. Probable secretion of antimicrobial molecules and cell wall components into the apoplast	1.18	40 S10
Cell wall strengthening			
UPF0497 membrane protein At3g06390	Casparian strip membrane proteins' family	2.11	Roppolo et al. 2011
Alcohol dehydrogenase	Probable cinnamyl-alcohol dehydrogenase involved in lignin synthesis	1.11	41 S10
Cell wall synthesis			
Probable galacturonosyltransferase-like 9	May be involved in pectin and/or xylan biosynthesis	1.55	42 S10
Stress recovery			
Aminophospholipid flippase 9	Establishment of phospholipid asymmetry in plasma membrane	4.35	López-Marqués et al. 2012, 43 S10
Universal stress protein MJ0531		2.21	44 S10
Auxin-repressed 12.5 kDa protein	Probably related to drought stress recovery	1.11	45 S10
E3 ubiquitin-protein ligase BRE1-like 2	Involved in root growth regulation through histone ubiquitination	1.05	46 S10
High affinity cationic amino acid transporter 1	Drought stress recovery. Induced by JA and ABA	1.03	47 S10

S10 references in Supplementary material 10

European chestnut (*C. sativa*, susceptible to pathogen) revealed a correlation of gene ontology, suggesting a convergent response after pathogen inoculation. However, among DEG, GO annotation revealed differences that suggest distinct host susceptibility to the pathogen as well as variations in gene expression and timing. *C. crenata* inoculated with the pathogen upregulated genes with the functional GO annotation "Oxidation reduction process" (BP), disclosing genes involved in the synthesis of anti-fungal secondary metabolites (6 in 27) and in stress recovery (10 in 27). Examples are *Squalene monooxygenase* (Belchí-Navarro et al. 2013) and *Prolyl 4-hydroxylase subunit alpha-2* (Vlad et al. 2007), respectively. On other hand, "Sequence-specific DNA binding transcription factor activity" (MF) point to genes coding for TF related to pathogen recognition and biotic stress regulation (10 in 14, examples in Table 2). Contrasting with *C. crenata*, GO annotation for inoculated *C. sativa* revealed that the downregulated genes in "Catalytic activity" (MF) were involved in the synthesis of secondary metabolites, protein kinases, and receptor-like protein kinases (24 in 84). In the same term are also included genes related to stress recovery (9 in 84). Inoculated *C. sativa* upregulated genes are significantly annotated to "Carbon-carbon lyase activity" (MF) and almost all genes are involved in glycolysis (4 in 5). The predominance of downregulated genes in *C. sativa* upon pathogen inoculation is in accordance with the species' higher susceptibility to *P. cinnamomi*. A review by Oßwald et al. (2014) states that the reactions of susceptible woody plant hosts to *Phytophthora* spp. include a broad downregulation of defense-related genes in infected roots, promoting pathogen growth.

Pathogen response in resistant Japanese chestnut

Upon infection, Japanese chestnut upregulated twice the number of DEG when compared with the susceptible European chestnut. Among these, we selected a set of Japanese chestnut candidate resistance genes, taking into consideration the regulation (all upregulated), the best hit description (Interpro and BLAST), the protein function, and the relevance to pathogen defense and host recovery. Candidate genes included those putatively involved in pathogen recognition, such as *LRR receptor-like serine/threonine-protein kinase At1g07650*, coding for a transmembrane protein involved in the recognition of pathogen elicitor complexes that locally activate defense-related pathways (Diévart and Clark 2003). Japanese chestnut also upregulated TF which are reportedly active early in the elicitor-induced defense response (e.g., *WRKY transcription factors 22* and *31*) (Zhang et al. 2008b; Yang et al. 2009). One Japanese chestnut upregulated gene involved in lipid signaling corresponds to *Patatin-05*, a Phospholipase A2-related gene. This gene shows a rapid transcriptional activation in virus-infected leaves preceding the increase of Phospholipase A2, which may provide precursors for the synthesis of the JA class of oxylipins during HR (Dhondt et al. 2000).

Japanese chestnut genes which are upregulated upon pathogen inoculation and are involved in plant immune response regulation include *RING finger protein 5*, which may contribute to elicitor-activated response via a JA-dependent signaling pathway (Hondo et al. 2007).

Recognition of a pathogen often triggers a localized resistance reaction known as the hypersensitive response (HR), which is characterized by the production of reactive oxygen species (ROS), leading to accelerated cell death and inhibition of pathogen spread (Hammond-Kosack and Jones 1997). Six Japanese chestnut candidate genes for *P. cinnamomi* resistance may be involved in HR. *Cationic peroxidase 1* induces cell wall reinforcement through lignin deposition (Reimers et al. 1992) and is potentially involved in the defense of *Quercus suber* and avocado to *P. cinnamomi* (Coelho et al. 2011; Reeksting et al. 2014). *Probable glutathione S-transferase* may be related with the reduction of glutathione and consequent protection against the adverse effects of oxidative reactions, such as membrane lipid peroxidation during pathogen infection (Ryu et al. 2009). Rookes et al. (2008) observed an elevation of *Glutathione S-transferase 1* expression in *P. cinnamomi*-inoculated roots of *A. thaliana* Col-0, an ecotype which is considered tolerant to the pathogen.

Most of the Japanese chestnut candidate genes classified in the category "Anti-fungal enzymes" (Table 2) code for glycoside hydrolases. For example, *Acidic endochitinase* was reported to be a putative defense-related gene in the response of avocado to *P. cinnamomi* (Reeksting et al. 2014).

Eight Japanese chestnut candidate genes may be related to anti-fungal metabolite synthesis. Specifically, *UDP-glycosyltransferase 85A2* and *85A5* and *L-allo-threonine aldolase* may act in flavonoid synthesis (Schopfer and Ebel 1998; Broeckling et al. 2005; Woo et al. 2007). Flavonoids are synthesized by the phenylpropanoid pathway. Many phenylpropanoid compounds are stress-induced, comprising physical and chemical barriers against pathogen infection (such as lignin and suberin) and signal molecules involved in local and systemic signaling for defense gene induction (Dixon and Paiva 1995). Four candidate genes related to anti-fungal metabolite synthesis are induced by the JA pathway: *Taxane 13-α-hydroxylase* (involved in taxol synthesis, an oomycete defense compound in *Taxus*) (Sun et al. 2013), *Squalene monooxygenase* (Hu et al. 2003), a precursor of *Reticuline oxidase-like protein* (Shoji and Hashimoto 2011; Belchí-Navarro et al. 2013), and the above-mentioned *L-allo-threonine aldolase*.

Plant disease resistance depends partially on the host's ability to restrict pathogen development at the cell-surface level (Cantu et al. 2008). Structural changes in the host cell wall related to defense mechanisms include deposition of lignin-like material, production of callose and phenolic

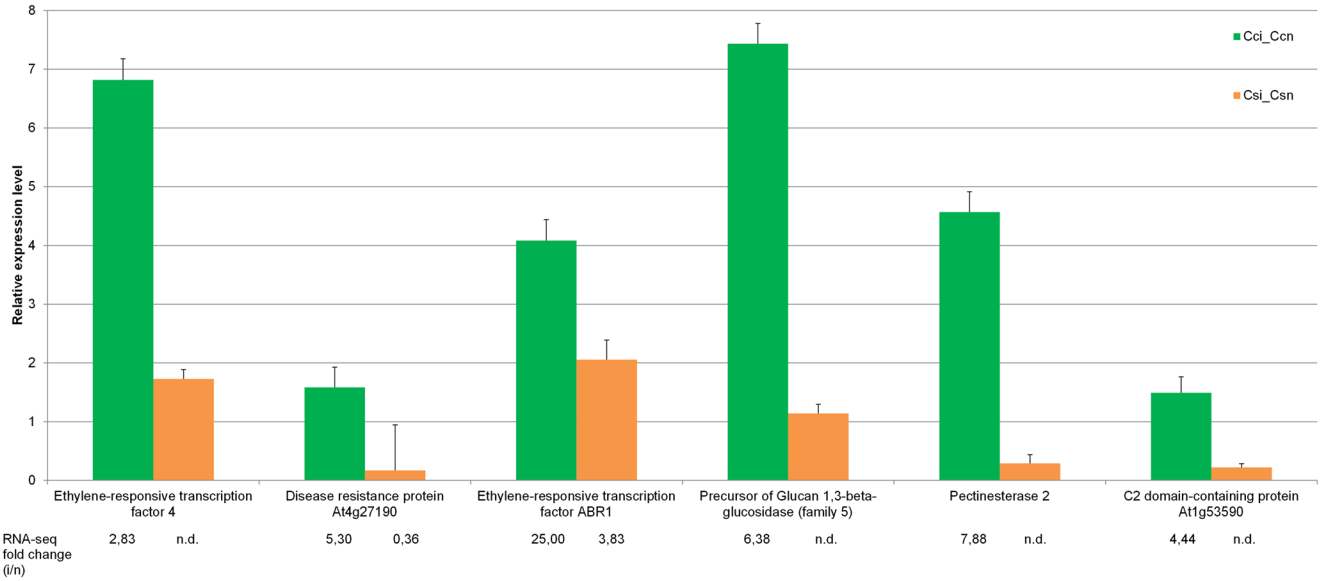

Fig. 5 Relative expression of DEG by quantitative real-time PCR and comparison with 454 sequencing read ratio. Transcript quantitative expression was normalized with the reference gene *Actin-7* in the pools Cci (inoculated Japanese), Ccn (non-inoculated Japanese), Csi (inoculated European) and Csn (non-inoculated European). Cci was calibrated against Ccn and Csi was calibrated against Csn to determine relative expression level, following the ΔΔCT method described in "Material and methods". *Error bars* correspond to the standard deviation of the mean of three technical replicates. Fold change between inoculated libraries and non-inoculated libraries (i/n, *P* value< 1E−3) is shown below the chart for the respective gene and species. *N.d.* non-detected

compounds, and accumulation of hydroxyproline-rich glycoproteins (Benhamou et al. 1991). Four Japanese chestnut candidate genes were associated in the "Cell wall strengthening" category (Table 2). Two genes encode glycolysis enzymes that may be involved in cell wall lignification: cytosolic *Pyruvate kinase* and *6-phosphofructokinase 3*. Mutuku and Nose (2012) reported that those enzymes were highly expressed in *Rhizoctonia solani*-infected rice plants and suggested that the regulation of glycolysis in those conditions was involved in carbon allocation for other pathways such as the phenylpropanoid pathway for lignin synthesis. *Extensin-2* encodes a hydroxyprolin-rich glycoprotein that increases in resistant cultivars of tomato infected by *Fusarium oxysporum* (Benhamou et al. 1991).

Since *P. cinnamomi* infection results in drought stress symptoms, we identified four Japanese chestnut candidate genes involved in the response to drought stress (Table 2). One of them, *Phosphoprotein ECPP44,* encodes a protein that specifically protects cells from desiccation damage (Tan and Kamada 2000).

After pathogen attack and host response towards disease tolerance or resistance, plants then attempt to return to a normal status. *C. crenata* upregulated seven genes classified in the functional category "Stress recovery". *Equilibrative nucleoside transporter 2*, for example, is related to a rescue pathway of nucleotide synthesis (Li et al. 2003). Root damage by *P. cinnamomi* probably resulted in the upregulation of three genes associated with the root system, including *Lon protease homolog 2* and *Prolyl 4-hydroxylase subunit alpha-2*, involved in root formation (Vlad et al. 2007; Lingard and Bartel 2009).

Pathogen response in susceptible European chestnut

Upon inoculation, about half the number of DEG were upregulated in the European chestnut plants when compared to Japanese chestnut, while about two thirds as many DEG were downregulated. After analysis of the best hit description (Interpro and BLAST), European chestnut DEG putatively linked to a pathogen response and host recovery were associated to the functional categories shown in Table 3. Examples include *Serine/threonine-protein phosphatase PP1 isozyme 4*, which codes for a phosphatase that may regulate signal transduction initiated by pathogen elicitors (Lin et al. 1999), and *Histone deacetylase 6*, which codes a for a protein that interacts with enzymes of the ET and JA pathways related to plant defense to necrotrophic pathogens (Zhu et al. 2011).

Fungal pathogens can use oxalate as a phytotoxin to promote plant infection. European chestnut significantly upregulated *Oxalyl-CoA decarboxylase* upon inoculation that may take part of an oxalate catabolism pathway (Foster et al. 2012). Transgenic potato and taro plants transformed with a wheat oxalate oxidase gene demonstrated increased resistance to the oomycete pathogens *Phytophthora infestans* and *Phytophthora colocasiae*, respectively (Schneider et al. 2002; He et al. 2013), which comes in accordance with the putative role of the oxalate catabolism in the host defense to *Phytophthora*. The American chestnut restoration program is

partially based on genetic transformation with the same oxalate oxidase gene, so the host can degrade the oxalate secreted by the fungus *C. parasitica* during infection (Zhang et al. 2013). The *Protein TAR1* gene upregulated in inoculated European chestnut is reported to be involved in fungal laccase inhibition upon host infection (Jiang et al. 2009). Fungi can adopt Cu-containing polyphenol oxidases or laccases to degrade plant lignin during infection. Feng and Li (2012) identified laccase genes in *Phytophthora* spp., providing the hypothesis of a similar role of laccases in oomycete pathogenicity.

Five upregulated European chestnut genes were associated in the "Stress recovery category". Two of them are likely related to drought stress recovery, such as the *High affinity cationic amino acid transporter 1* (*CAT1*). According to Liu and Bush (2006), the promoter of the homologous gene *AtCAT1* has motifs responsive to methyl JA and drought. In birch, Cu-induced oxidative stress may induce the CAT1-like transporter, which then delivers amino acids used in cellular repair processes (Keinänen et al. 2007).

When the set of European chestnut genes downregulated upon inoculation was analyzed in terms of best hit description (DEG in Supplementary material 4, Interpro and BLAST columns), several of them were found to correspond to upregulated genes in inoculated *C. crenata* (DEG in Supplementary material 3). This suggests that infection of European chestnut plants by *P. cinnamomi* had a significant influence on host gene regulation, resulting in damage to the host. For example, *Myb-related protein 306* (Supplementary material 9) corresponds to an activator of anthocyanin synthesis genes (Jackson et al. 1991) and its inhibition could constrain the synthesis of some anti-fungal metabolites. The downregulation of *Peroxisomal-coenzyme A synthetase* may repress the JA pathway activation (Schneider et al. 2005). Repression of *Expansin-like A1* may affect cell wall synthesis (Irshad et al. 2008) and therefore may contribute to the spread of the pathogen spread in the host. Downregulation of *Pleiotropic drug resistance protein 2*, an ABC transporter of plant secondary metabolites (Yazaki 2006), probably prevents anti-fungal metabolites from reaching the pathogen. Repression of *Probable methyltransferase PMT2* may reduce synthesis of nicotine (Shoji and Hashimoto 2011), which could otherwise act as a fungicide. *Glutamate decarboxylase 1* codes for root-specific calcium/calmodulin-regulated GAD1, which plays a major role in GABA synthesis in plants responding to stress, thereby helping maintain plant homeostasis (Bouché and Fromm 2004). Thus, the repression of *GAD1* may affect host recovery from pathogen attack. Glutaredoxins are candidates for mediating redox regulation of transcriptional regulators that target genes associated with detoxification and pathogen defense (Ndamukong et al. 2007). The *Glutaredoxin-C9* gene was highly upregulated in Japanese chestnut; its downregulation in European chestnut may also affect host recovery.

Comparison between Japanese and European chestnut response

DEG annotation analyses revealed that Japanese and European chestnuts show many common features in their responses to *P. cinnamomi*. However, this evaluation must be carefully regarded as it is not supported by transcript profiling or functional analysis. Upon *P. cinnamomi* inoculation, both species upregulated genes involved in HR/HR recovery, genes related to the regulation of JA pathway and genes induced by JA related to anti-fungal metabolite synthesis and anti-fungal enzymes. The presence of HR and JA signaling upon pathogen inoculation indicates that both host species were able to recognize the pathogen attack. According to Thomma (1998), the JA-dependent defense response pathway is required for resistance to necrotrophic pathogens. Eshragui et al. (2013) suggest that a *P. cinnamomi* challenge activates JA-related plant defense responses in leaves of *A. thaliana* Col-0. HR is suggested to be associated with all forms of resistance to *Phytophthora* (Kamoun et al. 1999) and is believed to constitute one of the primary mechanisms of resistance to plant pathogens. Induction of HR is often associated with synthesis of anti-microbial compounds and cell wall thickening (Hammond-Kosack and Jones 1996). We identified *C. crenata* and *C. sativa* DEG related to cell wall strengthening and anti-fungal metabolite synthesis. HR also induces several genes involved in cellular protection (Jabs et al. 1996). We also identified DEG related to HR recovery in both species. The suggested occurrence of HR in inoculated European chestnut, the susceptible species, potentially points to partial resistance within this genotype. Partial resistance to *P. infestans* is common in wild *Solanum* species, which may reveal HR-like necrotic reactions and, occasionally, late or trailing HR. This suggests a weak R gene-Avr gene interaction or a gene-dosage effect resulting in ineffective HR and partly resistant phenotypes (Kamoun et al. 1999).

Japanese chestnut resistance to ink disease may in part result from a set of upregulated genes during *P. cinnamomi* attack involved in pathogen recognition, regulation of host response after pathogen perception, and signaling through lipids. When compared to Japanese chestnut, European chestnut upregulated much less genes in those functional categories. Current knowledge describes the plant immune response as starting with the recognition of pathogen elicitors by plant receptors, followed by induction of resistance genes (R genes) that initiate signal transduction cascades leading to (a) HR and rapid cell death and (b) the activation of phytohormone signaling pathways [reviewed in (Bari and Jones 2009)]. In our study, inoculated Japanese chestnut induced DEG involved in the SA pathway regulation (e.g., *Calcium-dependent protein kinase isoform 3*) (Chung et al. 2004) as well as DEG induced by SA related to the regulation of plant immune response (e.g., *Sulfate transporter 3,1*) (Marsolais et al. 2007) and HR (e.g.,

Arginine decarboxylase) (Nakane et al. 2003). García-Pineda et al. (2009) observed that SA inhibited avocado root colonization in the interaction between *Persea americana* and *P. cinnamomi*. In the complex web of defense responses JA, SA, ethylene, and abscisic acid are essential players (Bari and Jones 2009). SA is activated during and following HR (Jabs et al. 1996) and is generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens, as well as in the establishment of systemic acquired resistance (Bari and Jones 2009). Vleeshouwers et al. (2000) studied the *P. infestans*-*Solanum* interaction using wild species and reported that in fully resistant genotypes, the HR was faster and resulted in smaller lesions than in partially resistant clones. The authors suggest that the difference between compatibility (non-resistant host response) and incompatibility (resistant host response) is quantitative rather that qualitative. In our study, Japanese chestnut regulated a higher number of genes involved in biotic stress upon *P. cinnamomi* inoculation when compared to the European chestnut. The identified DEG are not only related to HR but also with cell wall strengthening, anti-fungal metabolite synthesis and anti-fungal enzyme synthesis, and may account for the Japanese chestnut's adequate resistance to ink disease.

Castanea response to *P. cinnamomi* and *C. parasitica*: brief comparison

The reports of Barakat et al. (2009; 2012) provided the first insights into chestnut resistance to *C. parasitica* using high-throughput RNA-seq. The response of chestnut to *C. parasitica* and *P. cinnamomi* may be comparable, as fungi and oomycetes share similar infection mechanisms (Latijnhouwers et al. 2003). When comparing Chinese and American chestnut responses to *C. parasitica* with the Japanese and European chestnut responses to *P. cinnamomi*, we found similar DEG that fall in the following functional categories: (a) Regulation of biotic stress response (*ATPase transporter*, *Pyridine nucleotide-disulphide oxidoreductase*), (b) HR and cell wall lignification (*Peroxidase*), (c) HR recovery (*Arginine decarboxylase*, *Manganese superoxide dismutase*), (d) Anti-fungal enzymes (*Thaumatin-like protein*, *β-1,3-glucanase*, *Chitinase*), (e) Anti-fungal metabolite synthesis (family 1 *Cytochrome P450 glycosyltransferase*, *Abscisic acid 8'-hydroxylase*, *Squalene monooxygenase*, *UDP-glucosyltransferase*), (f) Cell wall synthesis (*β-expansin*), and (g) Stress recovery (*ABC transporter family*, *Glyceraldehyde 3-phosphate dehydrogenase*). Other shared *Castanea* responses to both pathogens include DEG related to kinase genes involved in pathogen recognition and JA pathway activation, gene regulation by Myb TF and ethylene-responsive TF, and genes of the 26S proteasome regulatory unit. The response of all four species to both pathogens further includes genes from the flavonoid pathway that promote phytoalexin synthesis.

In summary, the DEG analysis of *C. sativa* and *C. crenata* root transcriptomes after *P. cinnamomi* inoculation revealed similarities among the four *Castanea* species response to both pathogens, namely genes related to systemic acquired resistance, HR that may prevent pathogen spread and the putative involvement of JA pathway. Some of these DEG may also promote cell wall strengthening through lignification and synthesis of flavonoids as anti-fungal metabolites.

Final considerations

RNA-seq using 454 platform was adequate for comparing the root transcriptomes of two Fagaceae species, *C. sativa* and *C. crenata* when either inoculated or non-inoculated with the pathogen *P. cinnamomi*. The four sequenced transcript libraries allowed a draft comparison of both species' responses to the pathogen in terms of gene regulation and pathways, together with the selection of candidate genes for host resistance to *P. cinnamomi*. Although further research is required on gene expression at specific time points after inoculation, in silico analysis has shown that Japanese and European chestnut, despite the association of expressed genes in similar functional categories, differ in the distribution of DEG after pathogen inoculation. The most noteworthy result from DEG analysis was the overall downregulation of genes in susceptible *C. sativa*, which may facilitate the pathogenicity of *P. cinnamomi*. On the other hand, in the resistant *C. crenata* there was the regulation of a higher number of genes related with biotic stress when compared to *C. sativa*, mostly upregulated. Analysis of homology and functional annotation revealed associations between many of those upregulated genes with pathogen response in other plant species, and suggests involvement in pathogen recognition, regulation of the host immune response, signaling, hypersensitive response, cell wall strengthening and encoding of enzymes and synthesis of metabolites against oomycetes and fungal pathogens. The regulation of DEG in *C. crenata* and many of the specific transcripts we identified may account for the adequate resistance level of this species to *P. cinnamomi*.

SSR markers are already being developed from the sequences of these candidate genes in order to improve the mapping approach for identification of QTLs related to pathogen resistance in Japanese and European chestnut (Costa et al. 2011). In a future approach, we will analyze if the candidate genes map to disease resistance QTLs, which will provide further support for a major role in chestnut resistance to the pathogen.

Acknowledgments We acknowledge Dr. Beatriz Cuenca (TRAGSA-SEPI) for providing the plant material used in this study. The authors are

also grateful to Dr. Andreia Figueiredo (BioFIG) and Dr. Filipa Monteiro (BioFIG) for help on qRT-PCR, Dr. Conceição Egas (Next Gen Sequencing Unit, Biocant) for submitting raw data to NCBI, Dr. Dana Nelson (USDA Forest Service, MS, Unit of Forest genetics and Ecosystems Biology) for arrangements on submitting data to Fagaceae.org, and Prof. William Powell and Andrew Newhouse (SUNY College of Environmental Science and Forestry, NY, Department of Environmental and Forest Biology) for critical review of the manuscript. This work was funded by the Portuguese Foundation for Science and Technology (www.fct.pt) in the frame of the project Understanding Resistance to Pathogenic Fungi in Castanea sp (PTDC/AGR-CFL/101707/2008). Grant to SS (Investigador Ciência 2008) was also supported by FCT. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Archiving Statement Raw data files can be accessed in the Short Read Archive at NCBI (http://www.ncbi.nlm.nih.gov/) with the reference PRJNA215368. Nucleotide and aminoacid sequences are publicly available in the Fagaceae Genomics Web (http://www.fagaceae.org/).

References

Anagnostakis SL (2001) The effect of multiple importations of pests and pathogens on a native tree. Biol Invasions 3:245–254. doi:10.1023/A:1015205005751

Barakat A, DiLoreto DS, Zhang Y et al (2009) Comparison of the transcriptomes of American chestnut (*Castanea dentata*) and Chinese chestnut (*Castanea mollissima*) in response to the chestnut blight infection. BMC Plant Biol 9:51. doi:10.1186/1471-2229-9-51

Barakat A, Staton M, Cheng C-H et al (2012) Chestnut resistance to the blight disease: insights from transcriptome analysis. BMC Plant Biol 12:38. doi:10.1186/1471-2229-12-38

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol 69:473–488. doi:10.1007/s11103-008-9435-0

Belchí-Navarro S, Almagro L, Sabater-Jara AB et al (2013) Induction of trans-resveratrol and extracellular pathogenesis-related proteins in elicited suspension cultured cells of *Vitis vinifera* cv Monastrell. J Plant Physiol 170:258–264. doi:10.1016/j.jplph.2012.10.003

Benhamou N, Mazau D, Grenier J, Esquerré-Tugayé M-T (1991) Time-course study of the accumulation of hydroxyproline-rich glycoproteins in root cells of susceptible and resistant tomato plants infected by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Planta 184:196–208. doi:10.1007/BF01102419

Bocca SN, Kissen R, Rojas-Beltrán JA et al (1999) Molecular cloning and characterization of the enzyme UDP-glucose: protein transglucosylase from potato. Plant Physiol Biochem 37:809–819. doi:10.1016/S0981-9428(99)00117-5

Bouché N, Fromm H (2004) GABA in plants: just a metabolite? Trends Plant Sci 9:110–115. doi:10.1016/j.tplants.2004.01.006

Brasier CM, Jung T (2006) Recent developments in *Phytophthora* diseases of trees and natural ecosystems in Europe. Prog. Res. Phytophthora Dis. For. Trees. Proceedings, 3rd Int. IUFRO Work. Party. pp 5–16

Broeckling CD, Huhman DV, Farag MA et al (2005) Metabolic profiling of *Medicago truncatula* cell cultures reveals the effects of biotic and abiotic elicitors on metabolism. J Exp Bot 56:323–336. doi:10.1093/jxb/eri058

Cantu D, Vicente AR, Labavitch JM et al (2008) Strangers in the matrix: plant cell walls and pathogen susceptibility. Trends Plant Sci 13:610–617

Chen K, Fan B, Du L, Chen Z (2004) Activation of hypersensitive cell death by pathogen-induced receptor-like protein kinases from *Arabidopsis*. Plant Mol Biol 56:271–283. doi:10.1007/s11103-004-3381-2

Chung E, Park JM, Oh S-K et al (2004) Molecular and biochemical characterization of the *Capsicum annuum* calcium-dependent protein kinase 3 (CaCDPK3) gene induced by abiotic and biotic stresses. Planta 220:286–295. doi:10.1007/s00425-004-1372-9

Coelho AC, Horta Jung M, Ebadzad G, Cravador A (2011) *Quercus suber–Phytophthora cinnamomi* interaction: a hypothetical molecular mechanism model. N Z J For Sci 41S:S143–S157

Conesa A, Götz S (2008) Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int J Plant Genom. doi:10.1155/2008/619832

Costa R, Santos C, Tavares F et al (2011) Mapping and transcriptomic approaches implemented for understanding disease resistance to *Phytophthora cinammomi* in *Castanea* sp. BMC Proc 5:O18. doi:10.1186/1753-6561-5-S7-O18

Dhondt S, Geoffroy P, Stelmach BA et al (2000) Soluble phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves and is contributed by patatin-like enzymes. Plant J 23:431–440. doi:10.1046/j.1365-313x.2000.00802.x

Diévart A, Clark SE (2003) Using mutant alleles to determine the structure and function of leucine-rich repeat receptor-like kinases. Curr Opin Plant Biol 6:507–516

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085–1097. doi:10.1105/tpc.7.7.1085

Eshraghi L, Anderson JP, Aryamanesh N et al (2013) Defence signalling pathways involved in plant resistance and phosphite-mediated control of *Phytophthora cinnamomi*. Plant Mol Biol Report 32:342–356. doi:10.1007/s11105-013-0645-5

Feng B, Li P (2012) Genome-wide identification of laccase gene family in three *Phytophthora* species. Genetica 140:477–484. doi:10.1007/s10709-012-9696-z

Fernandes CT (1955) A luta contra a doença da tinta nos soutos do norte de Portugal e ensaios diversos para a sua maior eficiência e economia., Direcção-G. 61 p

Foster J, Kim HU, Nakata PA, Browse J (2012) A previously unknown oxalyl-CoA synthetase is important for oxalate catabolism in *Arabidopsis*. Plant Cell 24:1217–1229. doi:10.1105/tpc.112.096032

García-Pineda E, Benezer-Benezer M, Gutiérrez-Segundo A et al (2009) Regulation of defence responses in avocado roots infected with *Phytophthora cinnamomi* (Rands). Plant Soil 331:45–56. doi:10.1007/s11104-009-0225-5

Giri J, Vij S, Dansana PK, Tyagi AK (2011) Rice A20/AN1 zinc-finger containing stress-associated proteins (SAP1/11) and a receptor-like cytoplasmic kinase (OsRLCK253) interact via A20 zinc-finger and confer abiotic stress tolerance in transgenic *Arabidopsis* plants. New Phytol 191(3):721–732. doi:10.1111/j.1469-8137.2011.03740.x

Gouzy J, Carrere S, Schiex T (2009) FrameDP: sensitive peptide detection on noisy matured sequences. Bioinformatics 25:670–671. doi:10.1093/bioinformatics/btp024

Hammond-Kosack KE, Jones JD (1996) Resistance gene-dependent plant defense responses. Plant Cell 8:1773–1791

Hammond-Kosack KE, Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48:575–607. doi:10.1146/annurev.arplant.48.1.575

Hartmann U, Sagasser M, Mehrtens F et al (2005) Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue-specific activation of phenylpropanoid biosynthesis genes. Plant Mol Biol 57:155–171. doi:10.1007/s11103-004-6910-0

He X, Miyasaka SC, Fitch MMM et al (2013) Taro (*Colocasia esculenta*) transformed with a wheat oxalate oxidase gene for improved resistance to taro pathogen *Phytophthora colocasiae*. HortSci 48:22–27

Henriksson E, Olsson ASB, Johannesson H et al (2005) Homeodomain leucine zipper class I genes in *Arabidopsis*. Expression patterns and

phylogenetic relationships. Plant Physiol 139:509–518. doi:10.1104/pp. 105.063461

Hondo D, Hase S, Kanayama Y et al (2007) The LeATL6-associated ubiquitin/proteasome system may contribute to fungal elicitor-activated defense response via the jasmonic acid-dependent signaling pathway in tomato. Mol Plant Microbe Interact 20:72–81. doi:10.1094/MPMI-20-0072

Hu X, Neill S, Cai W, Tang Z (2003) Hydrogen peroxide and jasmonic acid mediate oligogalacturonic acid-induced saponin accumulation in suspension-cultured cells of *Panax ginseng*. Physiol Plant 118:414–421. doi:10.1034/j.1399-3054.2003.00124.x

Hunter S, Apweiler R, Attwood TK et al (2009) InterPro: the integrative protein signature database. Nucleic Acids Res 37:D211–D215. doi:10.1093/nar/gkn785

Irshad M, Canut H, Borderies G et al (2008) A new picture of cell wall protein dynamics in elongating cells of *Arabidopsis thaliana*: confirmed actors and newcomers. BMC Plant Biol 8:94. doi:10.1186/1471-2229-8-94

Jabs T, Dietrich RA, Dangl JL (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science 273:1853–1856. doi:10.1126/science.273.5283.1853, 80-

Jackson D, Culianez-Macia F, Prescott AG et al (1991) Expression patterns of myb genes from Antirrhinum flowers. Plant Cell 3:115–125. doi:10.1105/tpc.3.2.115

Jacobs DF, Dalgleish HJ, Nelson CD (2013) A conceptual framework for restoration of threatened plants: the effective model of American chestnut (*Castanea dentata*) reintroduction. New Phytol 197:378–393. doi:10.1111/nph.12020

Jiang N, Xiao D, Zhang D et al (2009) Negative roles of a novel nitrogen metabolite repression-related gene, TAR1, in laccase production and nitrate utilization by the basidiomycete *Cryptococcus neoformans*. Appl Environ Microbiol 75:6777–6782. doi:10.1128/AEM. 00708-09

Kamoun S, Huitema E, Vleeshouwers V (1999) Resistance to oomycetes: a general role for the hypersensitive response? Trends Plant Sci 4:196–200

Keinänen SI, Hassinen VH, Kärenlampi SO, Tervahauta AI (2007) Isolation of genes up-regulated by copper in a copper-tolerant birch (*Betula pendula*) clone. Tree Physiol 27:1243–1252

Langmead B, Hansen KD, Leek JT (2010) Cloud-scale RNA-sequencing differential expression analysis with Myrna. Genome Biol 11:R83. doi:10.1186/gb-2010-11-8-r83

Latijnhouwers M, de Wit PJGM, Govers F (2003) Oomycetes and fungi: similar weaponry to attack plants. Trends Microbiol 11:462–469. doi:10.1016/j.tim.2003.08.002

Le Provost G, Herrera R, Paiva JA et al (2007) A micromethod for high throughput RNA extraction in forest trees. Biol Res 40:291–297

Leivar P, Antolín-Llovera M, Ferrero S et al (2011) Multilevel control of *Arabidopsis* 3-hydroxy-3-methylglutaryl coenzyme A reductase by protein phosphatase 2A. Plant Cell 23:1494–1511. doi:10.1105/tpc.110.074278

Li G, Liu K, Baldwin SA, Wang D (2003) Equilibrative nucleoside transporters of *Arabidopsis thaliana*. cDNA cloning, expression pattern, and analysis of transport activities. J Biol Chem 278:35732–35742. doi:10.1074/jbc.M304768200

Lin R-C, Park H-J, Wang H-Y (2008) Role of *Arabidopsis* RAP2.4 in regulating light- and ethylene-mediated developmental processes and drought stress tolerance. Mol Plant 1(1):42–57. doi:10.1093/mp/ssm004

Lingard MJ, Bartel B (2009) Arabidopsis LON2 is necessary for peroxisomal function and sustained matrix protein import. Plant Physiol 151:1354–1365. doi:10.1104/pp. 109.142505

Liu X, Bush DR (2006) Expression and transcriptional regulation of amino acid transporters in plants. Amino Acids 30:113–120. doi:10.1007/s00726-005-0248-z

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 25:402–408. doi:10.1006/meth.2001.1262

López-Marqués RL, Poulsen LR, Palmgren MG (2012) A putative plant aminophospholipid flippase, the Arabidopsis P4 ATPase ALA1, localizes to the plasma membrane following association with a β-subunit. PLoS ONE 7:e33042. doi:10.1371/journal.pone.0033042

Lottaz C, Iseli C, Jongeneel CV, Bucher P (2003) Modeling sequencing errors by combining Hidden Markov models. Bioinformatics 19:ii103–ii112. doi:10.1093/bioinformatics/btg1067

Marshall SDG, Putterill JJ, Plummer KM, Newcomb RD (2003) The carboxylesterase gene family from *Arabidopsis thaliana*. J Mol Evol 57:487–500. doi:10.1007/s00239-003-2492-8

Marsolais F, Boyd J, Paredes Y et al (2007) Molecular and biochemical characterization of two brassinosteroid sulfotransferases from Arabidopsis, AtST4a (At2g14920) and AtST1 (At2g03760). Planta 225:1233–1244. doi:10.1007/s00425-006-0413-y

Martins L, Anjos R, Costa R, Gomes-Laranjo J (2009) COLUTAD: um clone de castanheiro resistente à doença da tinta. In: Gomes-Laranjo J, Peixoto F, Ferreira-Cardoso J (eds) Castanheiros, Técnicas e Práticas, UTAD - Vil. Vila Real, pp 135–142

Meyer F, Paarmann D, D'Souza M et al (2008) The metagenomics RAST server—a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinforma 9:386. doi:10.1186/1471-2105-9-386

Mutuku JM, Nose A (2012) Changes in the contents of metabolites and enzyme activities in rice plants responding to *Rhizoctonia solani* Kuhn infection: activation of glycolysis and connection to phenylpropanoid pathway. Plant Cell Physiol 53:1017–1032. doi:10.1093/pcp/pcs047

Nakane E, Kawakita K, Doke N, Yoshioka H (2003) Elicitation of primary and secondary metabolism during defense in the potato. J Gen Plant Pathol 69:378–384. doi:10.1007/s10327-003-0075-6

Ndamukong I, Al Abdallat A, Thurow C et al (2007) SA-inducible Arabidopsis glutaredoxin interacts with TGA factors and suppresses JA-responsive PDF1.2 transcription. Plant J 50:128–139. doi:10.1111/j.1365-313X.2007.03039.x

Niu B, Fu L, Sun S, Li W (2010) Artificial and natural duplicates in pyrosequencing reads of metagenomic data. BMC Bioinforma 11:187. doi:10.1186/1471-2105-11-187

Ohta M, Matsui K, Hiratsu K et al (2001) Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. Plant Cell 13:1959–1968

Oßwald W, Fleischmann F, Rigling D, et al. (2014) Strategies of attack and defence in woody plant- Phytophthora interactions. For Pathol 44:n/a–n/a. doi: 10.1111/efp.12096

Porta H, Rocha-Sosa M (2002) Plant lipoxygenases. Physiological and molecular features. Plant Physiol 130:15–21. doi:10.1104/pp.010787

Reeksting BJ, Coetzer N, Mahomed W et al (2014) De novo sequencing, assembly, and analysis of the root transcriptome of *Persea americana* (Mill.) in response to *Phytophthora cinnamomi* and flooding. PLoS ONE 9:e86399. doi:10.1371/journal.pone.0086399

Reimers PJ, Guo A, Leach JE (1992) Increased activity of a cationic peroxidase associated with an incompatible interaction between *Xanthomonas oryzae* pv *oryzae* and rice (*Oryza sativa*). Plant Physiol 99:1044–1050

Rookes JE, Wright ML, Cahill DM (2008) Elucidation of defence responses and signalling pathways induced in *Arabidopsis thaliana* following challenge with *Phytophthora cinnamomi*. Physiol Mol Plant Pathol 72:151–161. doi:10.1016/j.pmpp.2008.08.005

Roppolo D, De Rybel B, Tendon VD et al (2011) A novel protein family mediates Casparian strip formation in the endodermis. Nature 473:380–383. doi:10.1038/nature10070

Ryu HY, Kim SY, Park HM et al (2009) Modulations of AtGSTF10 expression induce stress tolerance and BAK1-mediated cell death.

Biochem Biophys Res Commun 379:417–422. doi:10.1016/j.bbrc.2008.11.156

Sandorf I, Holländer-Czytko H (2002) Jasmonate is involved in the induction of tyrosine aminotransferase and tocopherol biosynthesis in *Arabidopsis thaliana*. Planta 216(1):173-179. doi:10.1007/s00425-002-0888-0

Schneider M, Droz E, Malnoë P et al (2002) Transgenic potato plants expressing oxalate oxidase have increased resistance to oomycete and bacterial pathogens. Potato Res 45:177–185. doi:10.1007/BF02736113

Schneider K, Kienow L, Schmelzer E et al (2005) A new type of peroxisomal acyl-coenzyme A synthetase from *Arabidopsis thaliana* has the catalytic capacity to activate biosynthetic precursors of jasmonic acid. J Biol Chem 280:13962–13972. doi:10.1074/jbc.M413578200

Schopfer CR, Ebel J (1998) Identification of elicitor-induced cytochrome P450s of soybean (*Glycine max* L.) using differential display of mRNA. Mol Gen Genet 258:315–322

Sharma M, Chai C, Morohashi K et al (2012) Expression of flavonoid 3'-hydroxylase is controlled by P1, the regulator of 3-deoxyflavonoid biosynthesis in maize. BMC Plant Biol 12:196. doi:10.1186/1471-2229-12-196

Shoji T, Hashimoto T (2011) Tobacco MYC2 regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NIC2-locus ERF genes. Plant Cell Physiol 52:1117–1130. doi:10.1093/pcp/pcr063

Singh AK, Sharma V, Pal AK et al (2013) Genome-wide organization and expression profiling of the NAC transcription factor family in potato (*Solanum tuberosum* L.). DNA Res 20:403–423. doi:10.1093/dnares/dst019

Sivasankar S (2000) Expression of allene oxide synthase determines defense gene activation in tomato. Plant Physiol 122:1335–1342. doi:10.1104/pp. 122.4.1335

Storey JD, Tibshirani R (2003) Statistical significance for genomewide studies. Proc Natl Acad Sci U S A 100:9440–9445. doi:10.1073/pnas.1530509100

Subroto T, de Vries H, Schuringa JJ et al (2001) Enzymic and structural studies on processed proteins from the vacuolar (lutoid-body) fraction of latex of *Hevea brasiliensis*. Plant Physiol Biochem 39:1047–1055. doi:10.1016/S0981-9428(01)01325-0

Sun G, Yang Y, Xie F et al (2013) Deep sequencing reveals transcriptome re-programming of Taxus×media cells to the elicitation with methyl jasmonate. PLoS ONE 8:e62865. doi:10.1371/journal.pone.0062865

Szczesna-Skorupa E, Kemper B (2006) BAP31 is involved in the retention of cytochrome P450 2C2 in the endoplasmic reticulum. J Biol Chem 281(7):4142–4148. doi:10.1074/jbc.M509522200

Taki N, Sasaki-Sekimoto Y, Obayashi T et al (2005) 12-oxo-phytodienoic acid triggers expression of a distinct set of genes and plays a role in wound-induced gene expression in *Arabidopsis*. Plant Physiol 139:1268–1283. doi:10.1104/pp. 105.067058

Tan S-K, Kamada H (2000) Initial identification of a phosphoprotein that appears to be involved in the induction of somatic embryogenesis in carrot. Plant Cell Rep 19:739–747. doi:10.1007/s002999900186

Thomma BPHJ (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc Natl Acad Sci 95:15107–15111. doi:10.1073/pnas.95.25.15107

Torres MA, Dangl JL, Jones JDG (2002) Arabidopsis gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc Natl Acad Sci U S A 99:517–522. doi:10.1073/pnas.012452499

Vannini A, Vettraino AM (2001) Ink disease in chestnuts: impact on the European chestnut. For Snow Landsc Res 76:345–350

Vellosillo T, Martínez M, López MA et al (2007) Oxylipins produced by the 9-lipoxygenase pathway in *Arabidopsis* regulate lateral root development and defense responses through a specific signaling cascade. Plant Cell 19:831–846. doi:10.1105/tpc.106.046052

Vlad F, Spano T, Vlad D et al (2007) Involvement of Arabidopsis prolyl 4 hydroxylases in hypoxia, anoxia and mechanical wounding. Plant Signal Behav 2:368–369

Vleeshouwers VG, van Dooijeweert W, Govers F et al (2000) The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta 210:853–864

Wen B, Ström A, Tasker A et al (2013) Effect of silencing the two major tomato fruit pectin methylesterase isoforms on cell wall pectin metabolism. Plant Biol (Stuttg). doi:10.1111/j.1438-8677.2012.00714.x

Woo H-H, Jeong BR, Hirsch AM, Hawes MC (2007) Characterization of *Arabidopsis* AtUGT85A and AtGUS gene families and their expression in rapidly dividing tissues. Genomics 90:143–153. doi:10.1016/j.ygeno.2007.03.014

Yang B, Jiang Y, Rahman MH et al (2009) Identification and expression analysis of WRKY transcription factor genes in canola (*Brassica napus* L.) in response to fungal pathogens and hormone treatments. BMC Plant Biol 9:68. doi:10.1186/1471-2229-9-68

Yang Y, He M, Zhu Z et al (2012) Identification of the dehydrin gene family from grapevine species and analysis of their responsiveness to various forms of abiotic and biotic stress. BMC Plant Biol 12:140. doi:10.1186/1471-2229-12-140

Yazaki K (2006) ABC transporters involved in the transport of plant secondary metabolites. FEBS Lett 580:1183–1191. doi:10.1016/j.febslet.2005.12.009

Yokoyama R, Nishitani K (2001) A comprehensive expression analysis of all members of a gene family encoding cell-wall enzymes allowed us to predict *cis*-regulatory regions involved in cell-wall construction in specific organs of *Arabidopsis*. Plant Cell Physiol 42:1025–1033

Zhang B, Singh KB (1994) ocs element promoter sequences are activated by auxin and salicylic acid in *Arabidopsis*. Proc Natl Acad Sci U S A 91:2507–2511

Zhang J, Addepalli B, Yun K-Y et al (2008a) A polyadenylation factor subunit implicated in regulating oxidative signaling in *Arabidopsis thaliana*. PLoS ONE 3:e2410. doi:10.1371/journal.pone.0002410

Zhang J, Peng Y, Guo Z (2008b) Constitutive expression of pathogen-inducible OsWRKY31 enhances disease resistance and affects root growth and auxin response in transgenic rice plants. Cell Res 18:508–521. doi:10.1038/cr.2007.104

Zhang B, Oakes AD, Newhouse AE et al (2013) A threshold level of oxalate oxidase transgene expression reduces *Cryphonectria parasitica*-induced necrosis in a transgenic American chestnut (*Castanea dentata*) leaf bioassay. Transgenic Res. doi:10.1007/s11248-013-9708-5

Zhu Z, An F, Feng Y et al (2011) Derepression of ethylene-stabilized transcription factors (EIN3/EIL1) mediates jasmonate and ethylene signaling synergy in *Arabidopsis*. Proc Natl Acad Sci U S A 108:12539–12544. doi:10.1073/pnas.1103959108