Gene Expression in European Ash in response to inoculation with *Hymenoscyphus pseudoalbidus*

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Summary

European Ash is one of the economically important trees in Europe. This tree has a wide application in industry (e.g. furniture), as the wood made from this tree has elastic property. However, it has recently been included as an endangered species in Sweden as it is threatened by an invasive pathogenic fungus, *Hymenoscyphus pseudoalbidus,* causing Ash dieback disease. The symptoms of this disease are necrosis in the leaves, leading to dieback in the shoot and eventually death. Few studies have been done to investigate the defensive mechanisms employed by the plant against this disease. It was found that the toxin (viridiol) produced by *H. pseudoalbidus* gives the fungus an ability to overcome the host defensive mechanism. When viridiol applied to the Ash leaves, similar necrotic symptoms as infection by the fungus were observed. In another study, after application of viridiol, abscisic acid (ABA) precursor components were identified. In our experiment, RNA-sequence data were generated by Illumina sequencing in three conditions namely freezing (I), inoculation with this fungus (II) and control (III). The data were then aligned to the Ash reference genome and analyzed by computer softwares. 70083 genes were represented in the library. However, 204, 151 and 97 transcripts were significantly regulated in comparisons between I-III, I-II and II-III respectively. By bioinformatic analysis, it was found that the expression of some ABA responded genes was increased after inoculation. However, the expression of ABA 8-hydroxylase genes, which is an enzyme with role in ABA hormone degradation, were decreased. The same result of gene expression was also observed in inoculation condition with using real time quantitative polymerase chain reaction (qPCR). This may suggest that levels of the abiotic stress hormone ABA was increased two weeks after inoculation. In the clustering analysis of Two-way Hierarchical clusters, it was also found that genes associated with ABA were up regulated during inoculation but the expression of genes associated with cell wall biosynthesis was increased in freezing condition. A phylogenetic tree was formed from ABA 8-hydroxylase amino acid sequenses in different Asterids. Four ABA 8-hydroxylase amino acid sequence were observed in each family (*Solanaceae, Phrymaceae*) but only three Ash sequences were detected in our experiment.

Abstract

European Ash is one of the economically important tree in Europe which is threatened by an invasive pathogen namely *Hymenoscyphus pseudoalbidus,* causing Ash dieback disease. Our objective was to investigate genes that are expressed after inoculation with this fungus and with freezing injury. In this experiment, high-throughput cDNA sequencing (RNA-seq) data was obtained from Ash bark subjected to different experimental conditions namely control, freezing and inoculation with fungus. RNA-seq data were generated by Illumina sequencing and analyzed by TopHat and Cufflinks. 70083 transcripts were represented in the libraries which all of them belonged to Ash. 97 transcripts were significantly differentially expressed in the control and freezing comparisons, 204 transcripts in the control and inoculation comparison and 151 transcripts in the comparison between freezing and inoculation. It was found that genes responded to abscisic acid (ABA) were up regulated by inoculation. But the expression of ABA 8-hydroxylase genes, which play roles in ABA degradation, was decreased.

1. Introduction

European Ash or common Ash (*Fraxinus excelsior L.*) found in Europe and in Asia only in Iran, belongs to the Oleaceae family in the Asterids clade. Ash is an economically important tree as it has elastic characteristics, which makes it suitable for many purposes such as producing sport instruments (e.g. tennis racquets and billiard cues), furniture, windows and doors particularly in Scandinavian countries (http://www.kew.org/plants-fungi/Fraxinus-excelsior.htm). This tree is considered as an endangered species by the Swedish species information centre (http://www.artfakta.se/GetSpecies.aspx?SearchType=Advanced). This is because Ash is threatened by a disease known as Ash dieback, caused by an ascomycete fungus. The incidence of Ash dieback has rapidly increased in Europe (Bengtsson et al., 2012;

Queloz et al., 2011). The fungus was first named *Chalara fraxinea* by T. Kowalski in 2006. It was later found that *C. fraxinea* is only an asexual form (anamorph) of the fungus. After the sexual state was found, the fungus was given its teliomorph name *Hymenoscyphus pseudoalbidus* V. Queloz (Queloz et al., 2011). *H. pseudoalbidus* penetrates in Ash trees through the leaves and causes necrosis in the bark and xylem, leading to dieback (Kowalski, 2006; Timmerman et al., 2011). It is believed that the fungus was introduced to Europe from Asia (Queloz et al., 2011). Interestingly, Asian Ash is not infected by *H. pseudoalbidus* and the defensive mechanism of the plant is still unknown (Kowalski and Holderieder, 2009b; Queloz et al., 2011).

Up to now only a hand full of attempts has been made to understand the defensive mechanisms in Ash. For instance, Andersson et al. (2010) studied viridiol, a phytotoxin produced by *H. pseudoalbidus,* to investigate the *H. pseudoalbidus* pathogenicity. Application of this phytotoxin directly on the Ash leaf tissues resulted in similar symptoms as infection by *H. pseudoalbidus* (Andersson et al., 2010). In another study, the abscisic acid (ABA)-cycteine and xantoxin were identified after treatment of Ash clones with viridiol (Clearly et al., 2014). These compounds are known as precursors in ABA pathway (Milborrow, 2001).

ABA is an important plant hormone that plays important roles in seed development and dormancy. The concentration of this hormone increases during stress such as drought or salinity (Saito et al., 2004). One of the enzymes that has essential role in the ABA homeostasis is ABA 8'-hydroxylase. This enzyme belongs to the Cytochrome P450 superfamily (Krochko et al., 1998; Saito et al., 2004). ABA 8-hydroxylase belongs to the CYP707As gene family, which in the model plant species Arabidopsis (*Arabidopsis thaliana)* comprises of four genes. The level of ABA increases when ABA 8-hydroxylase is repressed. Therefore, it is expected that stressful conditions result in ABA 8-hydroxylase repression (Saito et al., 2004).

Recently, two methods have been widely used to profile the expression of genes namely microarrays and high-throughput cDNA sequencing (RNA-seq). In microarray, the amount of RNA is quantified by hybridization of the RNA on a solid surface containing single strand DNA (Lesk, 2012). Then, the expression profile between different states such as healthy and disease conditions are compared (Lesk, 2012). RNA-seq has also been used to find expression of unknown genes. In this technique, the extracted RNA is turned to cDNA. Then, it is sequenced and aligned to the reference genome (Lesk, 2012). However, for species with no reference genome, the sequenced reads have to be *de novo* assembled (You et al., 2011). Pair-end-sequencing which reads both ends of the fragment is best used for *de novo* sequencing (Morozova and Marra, 2008). In microarrays, a probe set is designed according to the possible sequences (Lesk, 2012). This is a disadvantage for this technique in comparison with RNA-seq as the latter does not need any probe preparation. RNA-seq has high accuracy because it indicates the frequency of the sequence in the pool (Lesk, 2012). The RNA-seq data is obtained by using next generation sequencing (NGS) techniques such as 454, Illumina and SOLiD (Morozova and Marra, 2008). Sanger sequencing was the technology widely used before the NGS. However, NGS techniques are both cost and time effectives (Morozova and Marra, 2008). The resulting data from NGS are analyzed by computer softwares like TopHat and cufflinks (Trapnell et al., 2012).

The aim of this study was to identify specific transcriptional responses in Ash in interactions with *H. pseudoalbidus*. To achieve this, we compared the gene expression patterns in Ash after inoculation with *H. pseudoalbidus* and freezing injury. The candidate genes identified after RNA-seq were then analyzed by real time quantitative polymerase chain reaction (qPCR) to confirm the obtained results.

2. Materials and methods:

2.1. Biological material:

The experiment was conducted with 2-year-old bare-root seedlings of *F. excelsior* (European Ash) (n=40). They were 30-50 cm in height and were obtained from commercial nursery near Helsingborg, Sweden in March 2011. Two-week-old cultures of *H. pseudoalbidus* (isolate nf4) were used for inoculation.

2.2. Experimental set up:

Ash seedlings were planted in plastic pots (20 cm diameter) filled with light peat sieved, black peat and sand at the proportion of 60: 25: 15 (Hasselfors garden, Örebro, Sweden). The plants were kept in a greenhouse at 16 h photoperiod and at 20/15°C (day/night) and watered as needed for 7 weeks before being subjected to one of the following treatments:

I)   Freezing with cryospray (superficial wounding of the bark) (n=16)

II)   Inoculation with *H. pseudoalbidus* (no wounding of the tissue) (n=16)

III)   Healthy control (n=8)

Sterile woody plugs were added to 2-week-old cultures of *H. pseudoalbidus* (nf4). After 4 weeks, the plugs fully colonized by *H. pseudoalbidus* (nf4) were used to inoculate the unwounded Ash by sterilized forceps before sealing with Parafilm^TM.

At day 14 seedlings were destructively sampled; 4 samples from treatments I and II and 2 samples from treatment III were examined. The bark at freezing injury or point of inoculation was removed by a sterile scalpel.

2.3. RNA extraction and Illumina sequencing

After total RNA extraction (Chang et al., 1993), samples were treated with DNaseI (SIGMA) to remove the genomic DNA before being stored at -70°C. The RNA concentration was then measured on the BioAnalyzer 2100 (Agilent). Then, the Dynabeads® mRNA Purification Kit (Invitrogen) was used according to the protocol described by the manufacturer, to extract poly (A) + RNA from samples. The mRNA amplification was conducted according to the manufacturer's instruction by using MessageAmpIII kit (Ambion). Then, from the amplified RNA (aRNA), cDNA was first synthesized according to the protocol described by the manufacturer using iScriptcDNA Synthesis Kit (Bio-Rad) except the RT-reaction that was continued for approximately 50 minutes. The second strand cDNA was synthesized according to the protocol described by Sambrook and Russel (2001). This was followed by pooling enough quality of double stranded cDNA according to genotype and the treatments. 2-5 µg from each cDNA samples was then sent to SciLifeLab (Stockholm) for sequencing on an Illumina HiSeq 2000 instrument.

2.4. Bioinformatics

The RNA-seq obtained from Illumina sequencing was filtered with Nesonito remove low quality bases (quality below 20), adaptor sequences, and reads shorter than 55 bp after trimming (Harrison and Seemann, http://www.vicbioinformatics.com/software.nesoni.shtml). The Illumina data was first aligned to the Ash genome (Richard Buggs, unpublished data) by means of TopHat before assembling the transcripts with Cufflinks (http://cufflinks.cbcb.umd.edu/). Thereafter, Cuffdiff was used to identify genes that were differentially regulated in each treatment, using reads and merged assemblies. Further, analyses of differentially expressed genes were done in CumeRbund (Trapnell et al., 2012). Differentially expressed transcripts were annotated with blast2go (Conesa et al., 2005) to find the candidate genes in each condition. To visualize expression patterns, a Two-way cluster analysis, of significantly differentially expressed genes was done. For the clustering *fragments*

per kilo base of transcript per million mapped reads (FPKM) were clustered using Wards hierarchical algorithm in the JMP10 software.

2.5. Real time qPCR:

The parts of the gene sequence that were specific for the gene were chosen and inserted into the Primer 3 site (http://primer3.ut.ee) to design primers according to standard setting. The fragment length was 75-200 bp. The list of primers is found in Table 1. To prepare a linear standard curve, PCR product was then prepared for each amplicon (TEF, ACO, 49542, 28188, 39609, 40775 and 64552) and the PCR products were verified on gel and then purified. Thereafter, the DNA concentration was determined by Nanodrop and a linear standard curve was then recalculated for each gene according to the concentration of the DNA using http://www.thermoscientificbio.com/webtools/copynumber/.

The extracted RNA from treatment replicates (4 in freezing and inoculation; 2 in control) were pooled before sampling, 1 µg of the pooled RNA for cDNA synthesis. A calculated amount of RNA was mixed with RNase-free water to reach the volume of 15 µl. For each sample, 4 µl of 5x iScript Reaction mix was then mixed with 1 µl iScript Reverse Transcriptase. Thereafter, 5 µl of Master Mix was added to each tubes containing 15 µl of RNA. All steps were done on ice. The PCR machine was run to synthesize cDNA according to the following program: 25°C, 5 minutes; 42°C, 30 minutes and 85°C, 5 minutes.

The concentration of cDNA obtained was estimated to 50 ng/µl; the concentration needed was 20 ng/µl and therefore it was diluted. The qPCR was done in triplicates for each gene. The translation elongation factor alpha gene (TEF) was used as the reference for internal control. For each sample, 0.5 µl of 5 mM reverse primer and forward primer were added to 10 µl Eva-green SsoFast. The Eva-green SsoFast is light sensitive and hence, it was kept in dark during Master Mix preparation. Master Mixes were prepared by using forward and reverse primer of

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ACO, 28188, 36905, 49542 and TEF as a reference gene (Table 1). 19 µl of Master Mix was added to each tube before adding 1 µl of diluted cDNA (20 ng/µl). Milli-Q water was used as no template control. At the same time, for each gene standard curve was run. Then, real time qPCR was run according to the protocol and the data was analyzed by Pfaffl method (Pfaffl, 2001) to obtain the expression of the gene in different treatments.

Table 1. Primers used for real time qPCR.

primers	sequences	Primer length(bp)	Primer Tm(°C)	Product size(bp)
49542 F1_2571	cactcgttccttcagcctgt	20	59.97	155
49542 R1_2725	aagggctcgatcaaaaccga	20	59.68	
Fe_ACO_F	agaggtacgatcaagcccttat	23	59.03	109
Fe_ACO_R	cggaccaatggagaaacttttgt	23	59.68	
28188_F1	accccgttgccctttgtaaa	20	60.11	113
28188 R1	aagaacagctgggtcggtac	20	59.68	
36905 F1	tcgaaggtttgttctggtcca	21	59.51	147
36905 R1	cgacgtggttttgtgtgtgt	20	59.56	
40775 F2_1501	catgaatgcgcgcagaacta	20	59.35	101
40775 R2_1501	tccctgcaagcaaagagaga	20	58.94	
64552 F2_1501	gcatgatactcgccctgatga	20	60.00	80
64552 R2_1501	ttcaagcccacattccctgc	20	60.90	
FTEF-F[*]	accagcaagtcccagttgagatg	-	59.2	77
FTEF-R[*]	tgagccaggttcagcttccaatg	-	59.7	

*Obtained from Rivera Vega et al. (2012).

2.6. Phylogenetic tree:

Ash belongs to the Asterid clade. Consequently, we collected the amino acid sequences of ABA 8-hydroxylase from the three sequenced genomes in the Asterid clade, *Solanum tuberosum, Solanum lycoperiscon* and *Mimulus guttarus* from the Phytozome 9.0 site (http://www.phytozome.net/search.php). The Arabidopsis and *Thellungiella halophila* ABA 8-hydroxylase sequences were also collected from the Phytozome and ABA 8-hydroxylase sequences of Ash obtained from bioinformatics analysis were fed to the MEGA6 software

(Tamura et al., 2013). The sequences were then aligned by Clustal W and a phylogenetic tree was constructed using the neighbor joining method and a Bootstrap=500 in MEGA 6. The missing data were dealt with partial deletion option.

3. Results

In this study, 70083 transcripts were represented in the libraries which all of them belonged to Ash because the alighnment was done to the Ash reference genome. 97 transcripts were significantly differentially expressed in the control and freezing comparisons, 204 transcripts in the control and inoculation and 151 transcripts in the freezing and inoculation.

After bioinformatics analysis, it was found that by inoculation, some genes that respond to ABA were up regulated in response to inoculation with *H. pseudoalbidus*. The expression of these genes was then validated by real time qPCR (Figure 1). For all of the candidate genes namely *aminocyclopropane-1-carboxylate oxidase (ACO), homeoboxleucine zipper (49542), plasma membrane protein (28188)* and *translocator protein homolog (36905)*, similar results were observed with both techniques (RNA-seq and real time qPCR). A significant increase in expression was observed in I_14dpi condition compared to the unharmed control.
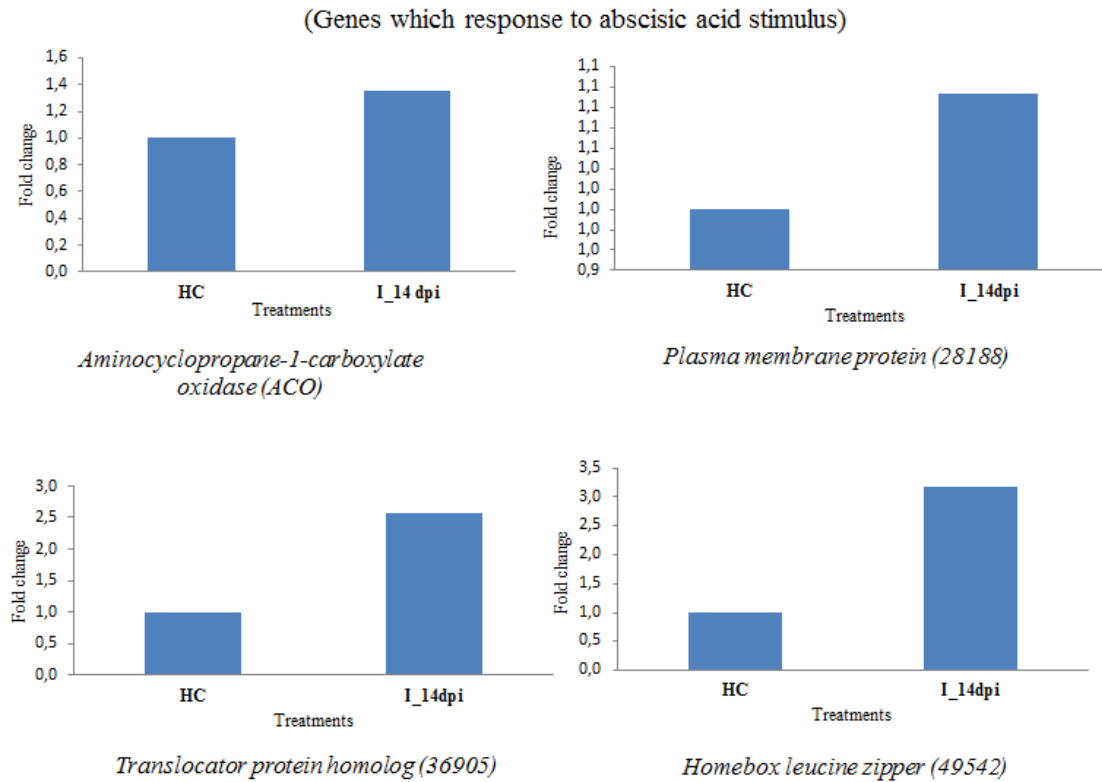
Figure 1. Expression of ABA responsive genes analyzed by real time qPCR. HC: healthy control, I_14dpi: 14 days post inoculation. The TEF was used as the reference gene.

From the bioinformatics analysis, it was found that there was a two-fold repression in ABA *8-hydroxylase (40775)* expression in I_14dpi. The expression level of ABA *8-hydroxylase (64552)* decreased about three times but it was not significant after correction for multiple testing, but both genes were still analyzed by real time qPCR. Both genes were repressed in the I_14dpi condition (Figure 2).

ABA 8-hydroxylase genes

Abscisic acid 8-hydroxylase (40775)

Abscisic acid 8-hydroxylase (64552)
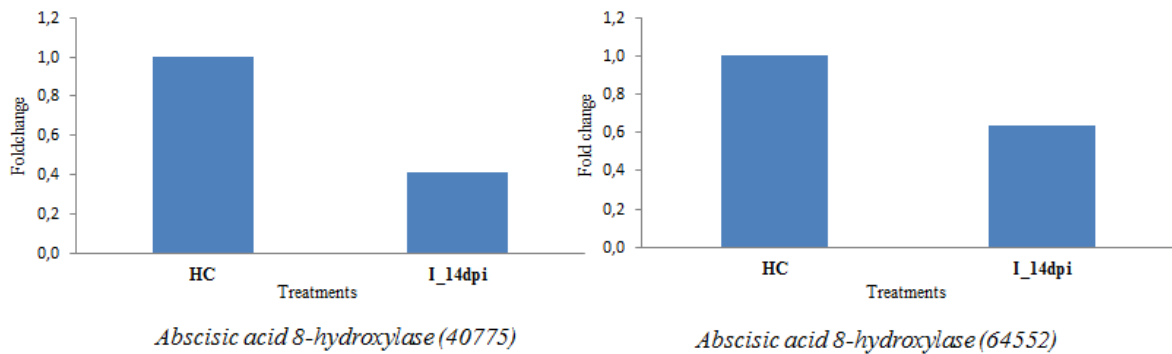
Figure 2. Relative expression level of *abscisic acid 8-hydroxylase 40775 and 64552* analyzed by real time qPCR. HC: healthy control, I_14dpi: 14 days post inoculation. The TEF was used as the reference gene.

The results from clustering analysis are shown in Figure 3.The expression of the genes was different in different treatments. The blue colour shows the lowest and the red colour the highest expresion of the genes. Therefore, genes in cluster 8 showed lowest expression in compared with cluster 11 which shows highest expression. Genes associated with growth were gathered in cluster 1. The genes associated with cell wall biosynthesis were also expressed more during freezing (cluster 5, 8b and 10). However, genes that responded to ABA and abiotic stress were up-regulated in I_14 dpi (cluster 2a, 6 and 11).
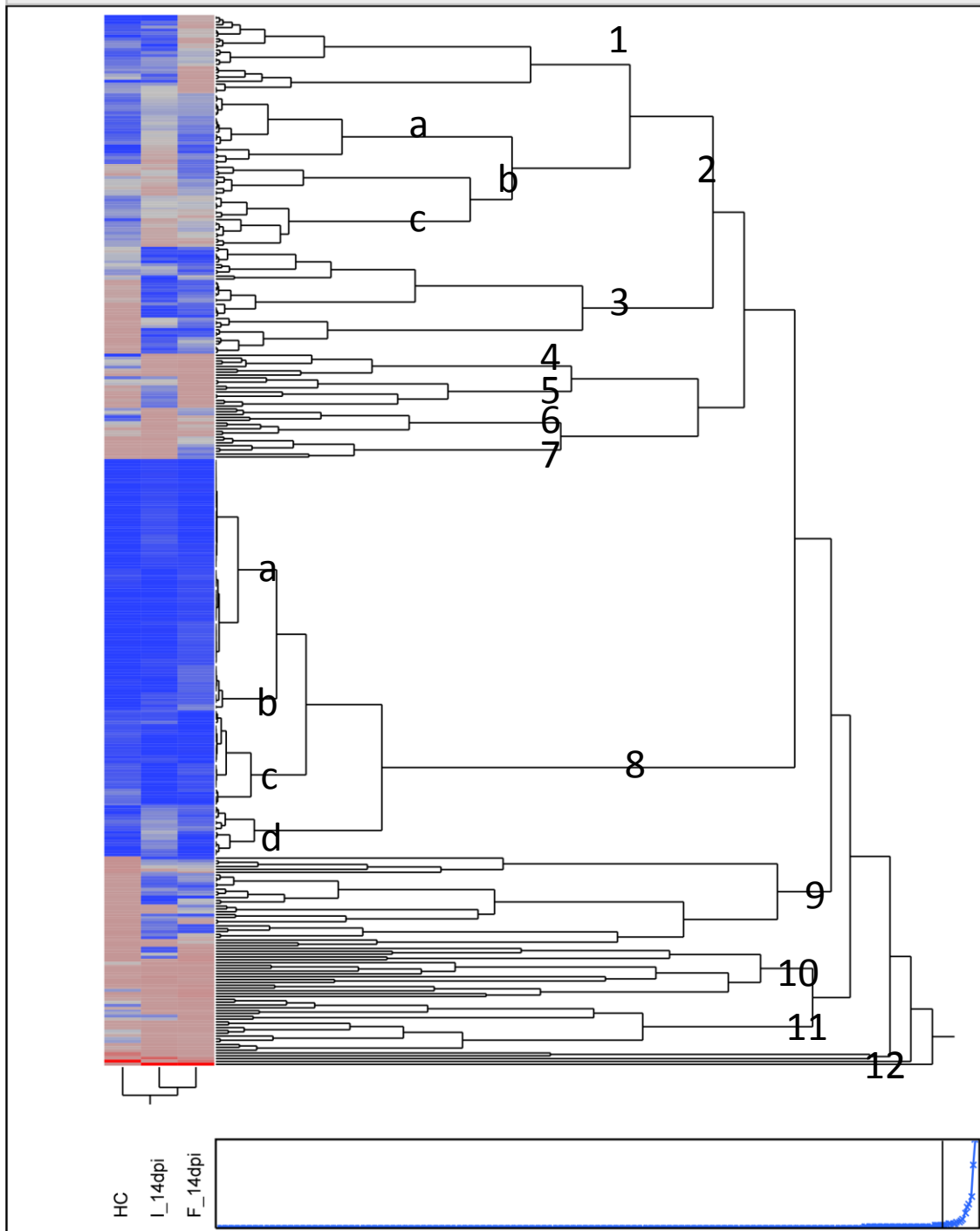
Figure 3. Hierarchical clusters of the genes that were up or down regulated during three different conditions: control (HC), freezing after 14 days (F_14dpi) and inoculation after14 days (I_14dpi). Low, moderate and high expressions are respectively shown with blue, gray and red colours.
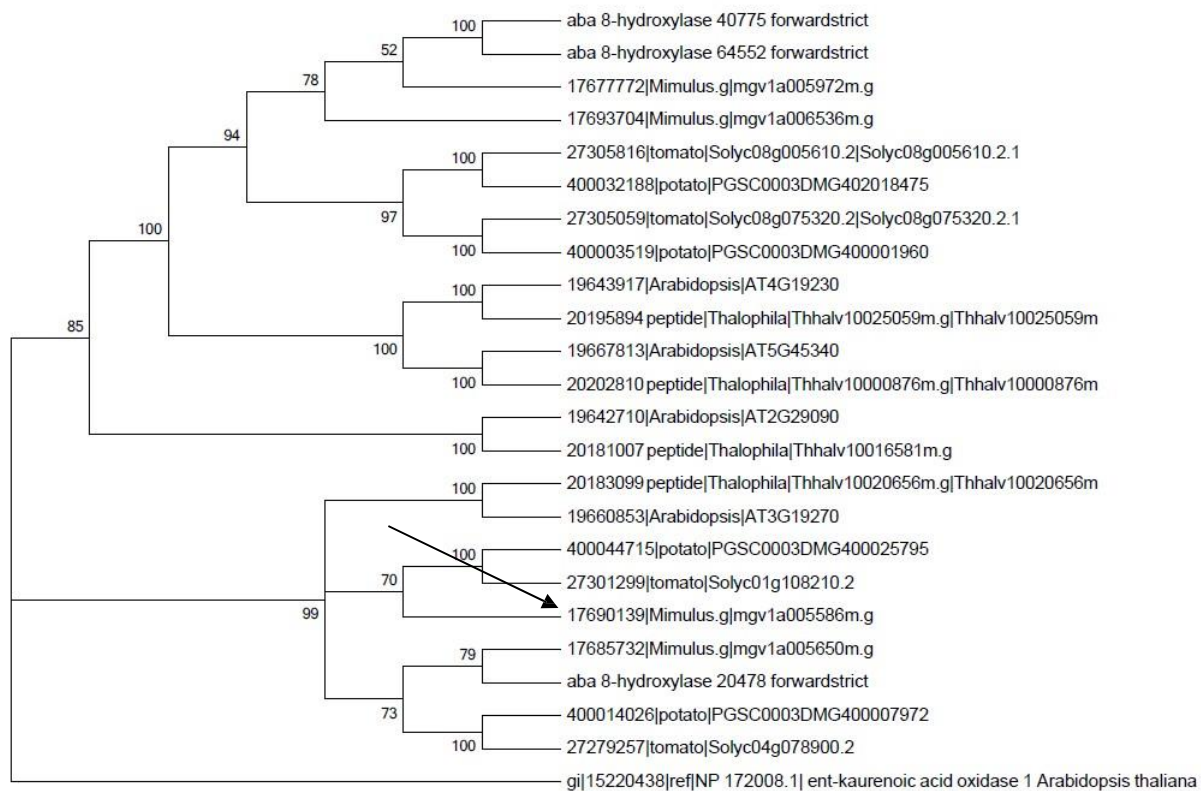
Figure 4. Neighbor joining Phylogenetic tree of ABA 8-hydroxylase amino acid sequences of potato, tomato, Ash, Arabidopsis and *Thellungiella halophile.*

The phylogenic tree of ABA 8-hydroxylase in different plants (Potato, Tomato, *Mimulus, Thellungiella halophila*, Arabidopsis and Ash) is shown in Figure 4. A CYP450 from a different CYP450 family was used to root the tree. The arrow shows the possible position of other ABA 8-hydroxylase in Ash that was not expressed or detected in our experiment.

4. Discussion

This experiment was started by bioinformatics analysis of the RNA-seq data from Ash in three different treatments. More than 70000 genes were expressed. It was found that the ABA related genes were up regulated in inoculation condition. In the study of defensive mechanism conducted by Cleary et al. (2014), some ABA responses genes were identified. Therefore, these genes were chosen as candidate genes to study in depth by real time qPCR. After real

time qPCR, all of the four candidate genes, which responded to ABA, showed the same expression pattern in the inoculation condition as observed in bioinformatics (Figure 1). However, in the freezing condition, the same pattern was not observed. This might be because the TEF reference gene was differentially regulated during the studied conditions. This reference gene was previously suggested as a suitable gene to study Ash (Rivera Vega et al., 2012), but a closer examination in our RNA-seq data suggested that this gene indeed is regulated in an unexpected way. In addition, we found that α tubulin would have been a more suitable reference gene for this study because it showed consistent expression in all the conditions analyzed by RNA-seq.

It was expected that genes involved in the host defensive mechanism should also be expressed by inoculation. However, very few defensive genes were found to be differentially expressed in the bioinformatics analysis. The zigzag model of defensive mechanism could explain these results (Jones and Dangl, 2006). When plants are encountered with pathogen molecules (PAMPs), the first activated defensive mechanism is PAMP- triggered immunity (PTI) (Jones and Dangl, 2006). However, some pathogens are able to suppress PTI by producing effectors, making the plants susceptible to these pathogens (ETS) (Jones and Dangl, 2006). Although, there is a complementary defensive system in resistance plant known effectors-triggered immunity (ETI), which recognizes the effectors by using the R protein (Jones and Dangl, 2006). In our experiment, two weeks after inoculation with fungus, only expression of the ABA responses genes were observed. The expression of ABA response genes is also increased during abiotic stress, leading to production of ABA hormone (Satio et al., 2004). The symptoms (e.g. necrosis) observed during abiotic stress are the same as the symptoms caused by *H. pseudoalbidus* (Andersson et al., 2010). Therefore, it seems that this fungus has an ability to suppress the first defensive mechanism (PTI), simulating an abiotic stress for the plant that induces an ETS state.

According to the data obtained from Blast2go analysis, those genes that responded to ABA were up regulated during inoculation but *ABA 8-hydroxylase* genes were down regulated. ABA 8-hydroxylase has a role in ABA signaling pathway. Increased concentration of this enzyme leads to the degradation of ABA and vice versa (Satio et al., 2004). Here, the reduced expression of this gene was observed in I_14dpi. This indicates that the level of ABA was increased in this condition. This can be expected, as ABA is a stress hormone. Therefore, these genes were chosen to analysis by real time qPCR and the same pattern of fold change was again observed (Figure 2).

After analyzing data with Blast2go and Two-way Hierarchical clustering, it was found that genes associated with growth and cell wall biosynthesis were up-regulated in the freezing treatment (Figure 3: Cluster 1, 5, 8b and 10) which is expected, as with freezing injury cell walls are damaged. However, genes responded to ABA were upregulated in I_14dpi (Figure 3: Cluster 2a, 6 and 11).

The *Solanaceae* (tomato and potato) and *Phrymaceae* (*Mimulus*) amino acid sequences of ABA 8-hydroxylase were selected to form phylogenetic tree with the ABA 8-hydroxylase sequences because all of these species belong to Asterids. The ABA 8-hydroxylase sequences of Arabidopsis and *Thellungiella* were also used in this phylogenic tree, as Arabidopsis is a model organism and its genes are fully sequenced. According to phylogenetic tree (Figure 4), ABA 8-hydroxylase genes are paralogous for Mimulus and Ash. In each plant, 4 genes for *ABA 8-hydroxylase* were found (Figure 4), however, we found only 3 genes in Ash. The possible position ABA 8-hydroxylase not being detected in our experiment is shown by an arrow in Figure 4. The tree also shows that the sequences in tomato and potato have a common origin for this gene but it is impossible to conclude that Ash and *Mimulus* have a common ancestor. The tree however indicates that gene duplication has most likely occurred within Ash species.

To sum up, it was found that two weeks after inoculation, only the expression of genes associated with ABA hormone was observed. It was also found that duplication was occurred in Ash ABA 8-hydroxylase gene. RNA-seq and bioinformatics is also a useful tool to study the expression of genes in host plant in different conditions.

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