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Department of Forest Mycology and Plant Pathology
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Abstract

This study aimed at investigating gene expression patterns in resistant and susceptible clones of *Fraxinus excelsior* L. (European ash) in response to infection with *Hymenoscyphus fraxineus*. A further objective of this study was to investigate the role of *ABA8-hydroxylase genes* (*ABA8H*) in European ash under infection with *H. fraxineus*. To address the first aim, bark of susceptible and resistant clones were infected with *H. fraxineus* and samples were taken after 9 months from the margins of necrosis. Total RNA was extracted and sequenced by next generation Illumina sequencing technique. To identify differentially expressed genes (DEGs), sequences were aligned to the European ash draft genome before analyzing in the Cufflinks pipeline. The DEGs were then annotated in Blast2go. More than 87,000 transcripts were presented in the library. In the inoculated susceptible clones, up-regulation of the *2-hydroxyisoflavanone dehydratase-like (HID)* gene, associated with formation of secondary metabolites, was observed. The over expression of *caffeoyl- o-methyltransferase* (*COMT*) and *farnesyl diphosphate synthase* was also detected in the inoculated susceptible clones. In the inoculated resistant clones, the defense response genes such as *1-aminocyclopropane-1-carboxylate oxidase* (*ACC*), some of the *ethylene-responsive transcriptional factors* (*ERFs*) and WRKY-transcriptional factors (TFs) were up-regulated. The *peroxidase-like* and *pathogen-related protein* (*PR*) genes, *secoisolariciresinol dehydrogenase-like*, *flavonol synthase flavanone 3-hydroxylase-like*, *phenylalanine ammonia-lyase* and *shikimate chloroplastic-like* genes were up-regulated but *ERFs* were down-regulated in both susceptible and resistant clones in response to *H. fraxineus* inoculation. *Shikimate chloroplastic-like* acts in the secondary metabolite pathway just like *flavonol synthase flavanone 3-hydroxylase-like*. To address the second aim, susceptible genotypes of the European ash were subjected to biotic stress which included wounding and inoculation with *H. fraxineus*. RNA was extracted from lesions and expression of candidate genes, i.e. three *ABA8H* genes and two encoding NAC-TFs was quantified. Some *NAC* genes are known to have a role in abscisic acid (ABA) signaling pathway. The European ash *ABA8H* and *NAC* genes were up-regulated 1 day after inoculation but already by day 7, they had been down-regulated. It is possible that the *NAC* genes contribute to the ABA biosynthesis pathway and thereby play roles in the defense mechanism.

Keywords: Hymenoscyphus fraxineus, *Fraxinus excelsior*, *ABA8H*, RNA-seq, *NAC*

Dedication

To my beloved husband and daughter

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Abbreviations

ACC	1-aminocyclopropane-1-carboxylate oxidase
ABA	Abscisic acid
ABA8H	ABA8-hydroxylase
cDNA	complementary DNA
COMT	caffeoyl- o-methyltransferase
dpt	days post treatment
ERFs	ethylene-responsive-TFs
ET	Ethylene
ETI	effector trigger immunity
ETS	effector trigger susceptibility
FPKM	fragments per kilo base of transcript per million mapped reads
HID	2-hydroxyisoflavanone dehydratase-like
HR	hypersensitive response
JA	Jasmonic acid
MAMPs	microbe associated molecular patterns
NAC	NAC-domain
ORF	open reading frame
PCR	Polymerase chain reaction
PP2C	Protein phosphatate 2C
PR	pathogen-related protein
PTI	pattern trigger immunity
qRT-PCR	quantitative polymerase chain reaction

1 Introduction

1.1 The *Hymenoschypus/Fraxinus* pathosystem

European ash, *Fraxinus excelsior* L., is an economically important tree in Europe, widely spread in Russia, Sweden, Ireland, Spain and Italy. European ash has also been found in the northern part of Iran (Fraxigen, 2005). The wood characteristics of the tree, i.e. strength and plasticity, make it suitable for usage in furniture and sport instruments (Fraxigen, 2005). The European ash is an outcrossing wind pollinated species (Fraxigen, 2005; Dobrowolska et al., 2011). Another important species of this genus is *F. mandshurica* known as Asian ash. This species is found in the eastern part of Russia but the main region for its growth is the North-East China (Drenkhan et al., 2014).

European ash is currently listed as an endangered species by the Swedish Species Information Centre (http://www.artfakta.se) due to a wide-spread of ash dieback disease. The disease is caused by *Hymenoscyphus fraxineus*, an ascomycete fungus (Baral et al., 2014). The *H. fraxineus* was first found in mid-1990s in Poland and Lithuania (Kowalski, 2006). The disease causes necrotic lesion in barks and wilting of leaves, which eventually results in the death of tree (Kowalski, 2006; Kowalski and Holdenrieder, 2009a; Timmermann et al., 2011). At early stages of infection, black necroses can usually be detected on leaf rachies and petioles (Bakys et al., 2009). The germination of ascospores from ascocarps results in appressoria formation on the leaf surface. The appressoria penetrates through the leaf cuticle (Cleary et al., 2013; Baral and Bemman, 2014), resulting in colonization of fungus in rachises and phloem and thereafter, appearance of disease symptoms (Schumacher et al., 2010).

It is believed that *H. fraxineus* was introduced from Asia to Europe (Zhao et al., 2012). Zhao et al. (2012) by phylogenetic analysis showed that *Lambertella albida*, found on the *F. mandshurica* with no pathogenic effects, and *H. fraxineus* are indeed conspecific. However, the *Lambertella albida* has a higher genetic di-

versity than the *H. fraxineus* (Zhao et al., 2012). Other studies also suggested that *H. fraxineus* was most likely originated from East Asia (Queloz et al., 2011).

H. albidus, a saprophyte, is widely spread in Europe (Kowalski and Holdenrieder, 2009b). This species is morphologically similar but genetically different from *H. fraxineus* and cannot cause any disease on European ash (Kowalski and Holdenrieder, 2009b; Bengtsson et al., 2012). Both *H. albidus* and *H. fraxineus* form ascocarp as a fruit body on necrotic petiole and rachises (Queloz et al., 2011; Baral and Bemmann, 2014). However, the ascocarps formed by *H. fraxineus* are bigger than those formed by *H. albidus* (Queloz et al., 2011; Baral and Bemmann, 2014). Another interesting species of the *Hymenosyphus* genus is *H. albidoides* which was first found in East China (Zheng and Zhuang, 2014). This species is morphologically different from *H. albidus* and H. *fraxineus* but has a close genetic similarity to *H. fraxineus* (Zheng and Zhuang, 2014).

1.2 Molecular control of plant-pathogen interaction

Plant pathogens are generally classified into three classes namely necrotrophs, biotrophs and hemibiotrophs. The necrotrophic pathogens obtain their nutrients though killing the host cells, often by producing toxins (Smith et al., 2014). On the contrary, biotrophic pathogens obtain nutrients from the living cells (Smith et al., 2014). The hemibiotrophs have an initial biotrophic phase but switch to necrotrophic later during infection (Smith et al., 2014).

Plant defense system against pathogens, so called Zigzag model, was first described by Jones and Dangl (2006). The first stage includes recognition of microbe associated molecular patterns (MAMPs), which is initiated by a signal sent from the infection site. This activates the pattern trigger immunity (PTI) (Jones and Dangl, 2006). Most biotrophic pathogens have an ability to deactivate PTI by producing effectors, which results in effector trigger susceptibility (ETS). In resistant plants, however, these effectors can be recognized by specific proteins known as receptor proteins (R-proteins), which lead to effector trigger immunity (ETI) (Jones & Dangl, 2006). The ETI results in necrosis and death of cells, which are adjacent to the infection site. By this hypersensitive response (HR), the biotrophic pathogen is isolated (Jones & Dangl, 2006). However, the molecular interaction between necrotrophic pathogens, as *H. fraxineus* is presumed to be, and host plants (*Fraxinus excelsior*) does not necessarily rely on the production or recognition of effectors (Mengiste et al., 2012). In the case of host-specific necrotrophs, inherited resistance factors will confer protection in a manner that may be somewhat reminiscent of the Zigzag model, whereas, resistance to broad host-range necrotrophs is multifaceted (Mengiste et al., 2012).

1.3 Plant hormone regulation in response to pathogen

Plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) have vital roles in plant defense signaling (Robert-Seilaniantz et al., 2007). There is a partly antagonistic relationship between JA/ET and SA in response to a pathogen (Robert-Seilaniantz et al., 2007). In short, up-regulation of JA/ET is generally associated with resistance against necrotrophic pathogens (Robert-Seilaniantz et al., 2007). However, responses mediated by SA often promote susceptibility against necrotrophic pathogens but are tightly linked to HR and resistance against biotrophic pathogens (Robert-Seilaniantz et al., 2007).

Abscisic acid (ABA) is also known to be involved in the plant defense mechanism (Lim et al., 2015). Pathogens invade plants via stomata, inter- or intracellularly (Lim et al., 2015). Closure/opening of stomata is regulated by ABA. We also found that inoculating the European ash with *H. fraxineus* resulted in the down-regulation of *ABA8-hydroxylase* (*ABA8H*) genes (Eshghi Sahraei et al., 2015). These enzymes degrade the ABA to phaseic acid (Saito et al., 2004; Jensen et al., 2013; Lim et al., 2015). Other roles of ABA include plant growth and development, seed dormancy and seed germination (Saito et al., 2004; Lim et al., 2015).

There are several proteins involved in the upstream regulation of ABA biosynthesis pathway including Snf1-related protein kinases (SnRK2) and group A protein phosphatate 2C (PP2C) (Cutler et al., 2010). A simplified schematic model of ABA biosynthesis is shown in Figure 1. The SnRK2 stimulate the ABA production contrary to PP2C which down-regulate it. The PP2C does this by binding to SnRK2 which in turn, deactivates the SnRK2 (Cutler et al., 2010). Under stress conditions which demand high levels of ABA for the plant, ABA is bound to PYR/PYL/RCAR family receptors and this unit is attached to PP2C (Cutler et al., 2010). This results in the release of SnRK2 and activation of downstream ABA biosynthesis (Cutler et al., 2010). Another protein that has a role in regulating ABA biosynthesis is *Arabidopsis* transcription factor ATAF1, belonging to the super family of NAC-domain (NAC) transcriptional factors (TFs) (Nuruzzaman et al., 2013). ATAF1 regulates the expression of *9-cis-epoxycarotenoid dioxygenase* (*NCED*) gene in upstream ABA biosynthesis (Miyazono et al., 2009; Yu et al., 2012; Jensen et al., 2013). *NCED* triggers the production of ABA by catalysing 9-cis-epoxycarotenoids to xanthoxin (Jensen et al., 2013). Genes involved in ABA degradation are *P450 CYP707A* (I-IV), which regulate the production of ABA8H enzymes (Saito et al., 2004; Jensen et al., 2013; Lim et al., 2015).

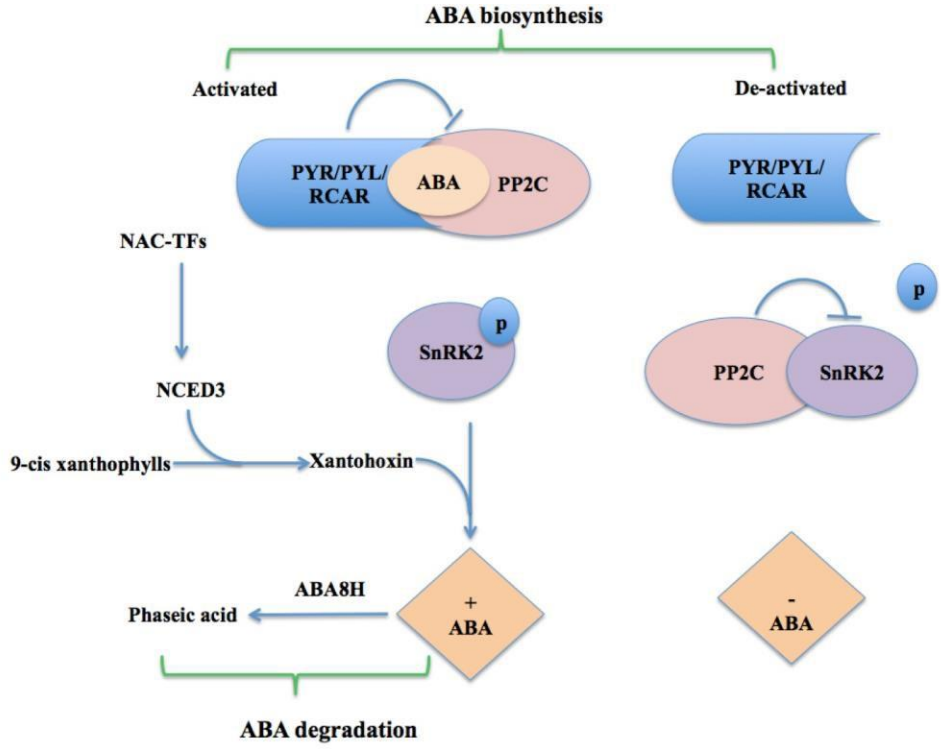

Figure 1. A simplified schematic model of ABA biosynthesis. ABA=abscisic acid; PYR/PYL/RCAR=Pyrabactin-resistant and related; PP2C=group A protein phosphatate 2C; SnRK2=Snf1-related protein kinases; ATAF1= NAC domain transcriptional factors; NCED=9-cis-epoxycarotenoid dioxygenase; ABA8H=ABA8-hydroxilase.

1.4 Controversy over virulence factor of *H. fraxineus*

Andersson et al. (2010) isolated phytotoxin viridiol from *H. fraxineus*. When viridiol was applied on the European ash, symptoms similar to the infection by the fungus itself were observed (Anderson et al., 2010; Cleary et al., 2014). Cleary et al. (2014) also observed the accumulation of ABA precursors such as ABA cysteine and xanthoxin in response to viridiol treatment. Applying viridiol obtained from the *H. albidus* on the European ash, however, did not result in disease symptoms, suggesting that the viridiol is possbily not pathogenicity factor of *H. fraxineus* (Junker et al., 2014). It seems more research is needed to resolve this issue.

1.5 Profiling and quantifying gene expression

Several methods can be used to study expression of mRNA such as high-throughput sequencing (RNA-seq) and real time quantitative polymerase chain reaction (qRT-PCR). In the RNA-seq method, sequences of short fragment mRNA (30-400 bp), known as reads, are obtained and data are subjected to bioinfomratic analysis (Trapnell et al., 2013). The reads can be aligned to a reference genome or assembled by the *de-novo* assembling method (Wang et al., 2009). The development of next generation sequencing techniques such as Illumina, which are fast and inexpensive, has resulted in popularity of the RNA-seq method (Wang et al., 2009). A further advantage of this method compared to other techniques is that low amounts of RNA are needed. The disadvantage of the RNA-seq method is however that, heavy bioinformatic analyses of data are required (Wang et al., 2009). The qRT-PCR can also be used for studying gene expression. In this method, the extracted RNA is converted to complementary DNA (cDNA), primers are designed for genes of interest and expression of these genes is quantified in relation to a reference gene (Pfaffl, 2001).

1.6 Objectives

The overall aim of this study was to increase understanding of transcriptional responses in *F. excelsior* to infection by *H. fraxineus*. The study addressed the following specific research questions:

1. How genetically controlled resistance or susceptibility to *H. fraxineus* is reflected in *F. excelsior* genes expression patterns
2. How *F. excelsior ABA8H* genes and NAC-TFs (*NAC2* and *NAC72*) are regulated in responses to *H. fraxineus.*

In addition, the sequences of *ABA8H* genes obtained from cloning were compared and validated against the reference genome.

2 Materials and Methods

2.1 Inoculation on defined genotype

In this part of the study, the RNA sequenceing data obtained from susceptible (genotypes) and resistant (genotypes) clones of the *Fraxinus excelsior* growing in a common garden at Trolleholm in southern Sweden were analyzed. The experimental design and sampling procedure was as follows: healthy branches of susceptible and resistant clones were wounded and inoculated with agar plugs from two weeks old *H. fraxineus* (nf4). After nine months, lesion length was measured (cm) and three biological samples were collected from necrotic parts of each clone. Samples from uninfected branches of the same clones were taken as control. After RNA extraction (see section 2.2.4), cDNA was synthesized and samples were sent to SciLifeLab (Stockholm, Sweden) for sequencing on an Illumina (1.8) HiSeq2500 (HiSeq Control Software 2.0.12.0/RTA 1.17.21.3).

2.1.1 Bioinformatic analysis

Data were filtered by Nesonito and it included removal of adaptor sequences, low quality bases and reads shorter than 55 bp (Harrison and Seemann, http://www.vicbioinformatics.com/software.nesoni.shtml). Data were then aligned to the European ash draft genome (Harper et al., 2016) using the TopHat softwar. After this step, Cufflinks pipeline (http://cufflinks.cbcb.umd.edu/) was used to assemble the transcripts (Trapnell et al., 2012). Comparisons were made in cuffdiff to identify differentially expressed genes (DEGs). These included comparisons between healthy and inoculation conditions within each clone, comparisons between resistant and susceptible clones. Thereafter, data were plotted with cummeRbund and DEGs were identified in R statistical software. Candidate genes in each condition were identified in venny (http://bioinfogp.cnb.csic.es/tools/venny/) and annotated in Blast2Go (Conesa et al., 2005). The expression pattern of fragments per kilo base of transcript per million mapped reads (FPKM) data from

DEGs were visualized in JMP10 software using two-way hierarchical clustering technique (Ward method). The same procedure was applied to align the data to the *H. fraxinus* draft genome.

2.2 Expression of ABA8H genes in response to biotic stress

2.2.1 Experimental set-up

An amount of 800 ml of fungal growth medium was prepared (14 g malt extract, 2 g peptone, 16 g agarose gel and milli-Q water). The autoclaved medium was poured in petri dish in a sterile-hood. The *H. fraxinus* (nf 4) was cultured on solidified growth medium at 20 °C. Sterile woody plugs (approximately 2×2×7 mm) were then placed in two weeks old *H. fraxinus* cultures and after 3 weeks, the plugs were fully covered with mycelium

Ash seedlings were planted in peat (Hasselfors garden, Örebro, Sweden). They were kept in a greenhouse on 16 h photoperiod at 20/15°C (day/night) for 7 weeks before being subjected to inoculation or wounding (Table 1). In short, the colonized woody plugs were placed on wound made in stem with sterile scalpels before sealing with parafilm. The same procedure, except inoculation, was done for the wounding treatment. Samples were taken at 1, 7, 14 and 21 days post-treatment (dpt). For each treatment, five plants were used at each sampling time point. For the control treatment, sampling was only done at days zero and 21. The samples from sampling days 0, 1 and 7 were subjected to RNA extraction and thereafter qRT-PCR.

Table 1. Treatment and the number of the samples in each sampling time point.

Treatment	Sampling time & number of samples				
	0	1d	7d	14d	21d
Control	5	-	-	-	5
H. fraxinus inoculation	-	5	5	5	5
Wounding	-	5	5	5	5

2.2.2 qPCR primer design

The open reading frame (ORF) was obtained from http://www.bioinformatics.org/sms2/orf_find.html for the following genes: *FeABA8H1* (XLOC-020479), *FeABA8H2* (XLOC-040776), *FeABA8H3* (XLOC-064553), *NAC 2* (XLOC-048803) and *NAC 72* (XLOC-039047), all identified previously in the *F. excelsior* genome assembly (Eshghi Sahraei et al., 2015). The nucleotide sequences residing in the ORF were imported to Primer3

(http://bioinfo.ut.ee/primer3-0.4.0/). The forward and reverse primers were designed for qRT-PCR according to the standard setting of the program. The fragment length was 70-120 bp and optimal annealing temperature was 60°C. Detailed information on primers used are presented in Appendix 1. Primers were also design for α-tubulin as the reference gene as above (Appendix 1).

2.2.3 RNA extraction

The bark samples were milled in a mortar and pestle in liquid Nitrogen after which, 3 ml extraction buffer was added to the ground materials. The suspension was incubated at 65°C for 15 min. Thereafter, 3 ml CHISAM comprising of chloroform: isoamylalcohol at 24:1 was added before centrifuging at 6793 × g for 10 minutes at room temperature. The upper phase was transferred into a falcon tube and ¼ volume of 8M lithium chloride was added and the mixture was thoroughly shaken before storing at 4°C overnight. The samples were then centrifuged at 6793 × g for 40 min at 4°C. The supernatant was saved for DNA extraction and stored in a refrigerator. The pellet was dried at room temperature for 10 minutes before dissolving in 100 µl of RNA free water. After this step, 200 µl of cold pure ethanol was added before adding 10 µl of NaAc (3M). Samples were kept at -20°C overnight after which, they were centrifuged at 4700 × g for 20 minutes at 4°C. The supernatant was discarded and the pellet was washed by 70% ethanol before being centrifuged again at 1200 × g for 10 min at 4°C. The pellet was dissolved in 16 µl RNA free water and treated with DNase I (SIGMA-ALDRICH, St. Louis, Missouri USA) according to the manufacturer's instruction. The quality and quantity of extracted RNA was measured by BioAnalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). Samples were stored at -70°C.

2.2.4 cDNA synthesis

The synthesis of cDNA was done using iScript cDNA Synthesis kits (Bio-Rad, Sundbyberg, Sweden). The PCR program was as follows: annealing at 25°C for 5 min; elongation at 42°C for 30 min; a final elongation at 85°C for 5 min. The cDNA was diluted with RNase free water to a concentration of 20 ng/µl.

2.2.5 Standard curve and polymerase chain reaction (PCR)

The cDNA from 5 randomly chosen samples was used to construct a standard curve. A Master mix was prepared by mixing 5 µl green buffer, 5 µl dNTP, 36.35 µl pure water and 0.25 µl Dream taq buffer (ThermoFisher Scientific, Sweden). For each 50 µl PCR, 1.2 µl forward and revers primers and 1 µl of cDNA template were used. The PCR program used is as follows: initial denaturation at 95°C for 5

min; 35 cycles of denaturation (95°C for 30 sec), annealing (57 °C for 30 sec) and elongation (72°C for 30 sec); a final elongation at 72°C for 7 min.

Three PCR products for each primer-pair were pooled and purified by E.Z.N.A. Cycle pure kit (VWR (OMEGA bio-tek), Radnor, PA, USA). DNA concentration was measured by Nano drop. Concentration and length of genes obtained for each primer-pair were then fed to an online copy number calculator available at ThermoFisher Scientific, Sweden.

2.2.6 qRT-PCR

The qRT-PCR was done in duplicate for each gene. Master mix was prepared by mixing 10 µl Eva-green SsoFast (Bio-Rad, Sweden), 8 µl RNase free water and 0.5 µl of 10 mM forward and reverse primers. An amount of 19 µl of Master mix was loaded to each well before adding 1 µl cDNA (20 ng/µl). For each primer-pair, 1 µl of the serial dilutions, used to construct the standard curve, and samples was loaded in wells in duplicate. Then, the qRT-PCR was run according to the following protocol: 95 °C for 30 sec; 40 cycles of 95 °C for 5 sec, 60°C for 20 sec. The specificity of the primers was checked by obtaining melting curve. The data was analyzed by the delta-delta CT method (Livak and Schmittgen, 2001).

2.2.7 Statistical analysis of the lesion length

The effect of wounding and inoculation with *H. fraxinus* on lesion length was tested by the General Linear Model of Minitab 16 (Minitab Inc., State College, PA, USA). The significant level was declared at $P < 0.05$. Data are presented as least square mean ± SEM.

2.3 Validating the sequences of *ABA8H* genes

2.3.1 Primer design

The ORF were identified for *FeABA8H1, FeABA8H2* and *FeABA8H3* with http://www.bioinformatics.org/sms2/orf_find.html. The nucleotide sequences residing in the ORF were imported into the MEGA6 software (Tamura et al., 2013) together with the sequences of the predicted *FeABA8H1, FeABA8H2* and *FeABA8H3* gene models. The sequences were aligned using the clustal W and data were exported as mega format. The variable sites of the sequences were exported into Excel. The predicted ORFs were fed to Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/). The forward primer was manually designed based on the variable sites identified in the clustal W alignment and the reverse primer was designed accord-

ing to the standard setting of the program. The fragment length was 1000-1200 bp and the optimal annealing temperature was 57°C (Appendix1).

2.3.2 PCR

The 25 µl reaction consisted of 1 µl DNA template, 2.5 µl Green buffer, 2.5 µl dNTP, 1 µl DreamTaq-polymerase (ThermoFisher Scientific, Sweden), 16.8 µl of milli-Q water and 0.6 µl of the forward and reverse primers. The PCR program was as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation (95°C for 30 sec), annealing (55°C or 50 °C for 30 sec) and elongation (72°C for 30 sec) and a final elongation at 72°C for 7 min. An amount of 20 µl of PCR products was visualized in 1% agarose gel. The PCR products were harvested from the gel and purified by the Gene JET Gel Extraction Kit (ThermoFisher Scientific, Sweden) before cloning.

2.3.3 Cloning

Cloning was done by the TOPO-TA kit (ThermoFisher Scientific, Sweden). Accordingly, an amount of 0.5 µl of vector was added to 1 µl of salt solution before adding 4.5 µl of the purified DNA. The suspension was incubated at room temperature for 30 min before storing at -20°C overnight.

2.3.4 Plasmid preparation

An amount of 2 µl of the cloning reaction was gently mixed with one shot®chemically component *E.coli* (ThermoFisher Scientific, Sweden). The mixture was incubated in ice for 30 min before heating at 42°C for 30 sec. Samples were then immediately placed in ice and 250 µl of S.O.S medium was added. The tubes were shaken at 37°C for 1 h and 100 µl was spread on pre-warmed LB plates containing kanamycin at 50µg/ml. The plates were incubated at 37 °C overnight after which they were stored at 4°C. From each plate, 8 white colonies were randomly chosen and cultured on LB plates. The colonies were then subjected to PCR using M13 forward (5´-GTAAAACGACGGCCAG-3´) and reverse (5´-CAGGAAACAGCTATGAC-3´) universal primers. The PCR was run according to the program stated above. The colonies with strongest bands in the gel electrophoresis assay were chosen, grown into bacterial culture and purified by E.Z.N.A. Cycle pure kit (VWR (OMEGA bio-tek)), Radnor, PA, USA) before sequencing at Macrogen (Humanizing Genomics Macrogen, Seoul, South Korea).

2.3.5 Bioinformatics

Vector sequences were removed by DNASTAR: Seq Man PRO software (http://www.dnastar.com/t-seqmanpro.aspx) before aligning the sequences by the

clustal W in MEGA 6 software (Tamura et al., 2013). The conserved sites of the sequences were identified. Thereafter, the amino acid sequences of the ORF were obtained (http://www.bioinformatics.org/sms2/orf_find.html). These amino acid sequences were then blasted (blastp, database Non-redundant protein sequences (nr)) in the National Centre for Biological information (NCBI: http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the amino acid sequences of *Solanum tuberosum*, *Solanum lycoperiscon* and *Erythrantha guttatus* which were in a same clade as the *F. excelsior*, i.e. Astrid clade, were also obtained. Thereafter, these amino acid sequences were aligned by the clustal W in MEGA 6 software and a phylogenetic tree was formed using the maximum likelihood method and bootstrap of 1000. The partial deletion option (95%) of the MEGA 6 was used to deal with missing data. The alignment length, identity and similarity of the comparisons conducted between amino acid sequences of *FeABA8H1*, *FeABA8H2* and *FeABA8H3* were obtained from http://www.bioinformatics.org/sms2/ident_sim.html**.**

3 Results

3.1 Inoculations on defined genotypes

3.1.1 Lesion length

The necrotic length after inoculation with *H. fraxinus* for each clone is presented in Table 2. As it was expected the lesion length was greater in the susceptible clones than in the resistant clones.

Table 2. The lesion length in the barks of the European ash clones 9 months after inoculation with *H. fraxinus*.

Table 2. The lesion length in the barks of the European ash clones 9 months after inoculation with *H. fraxinus*.

ID	clones	Lesion length (cm)
100	Resistant	7
8	Resistant	8.5
76	Susceptible	32
9	Susceptible	70

3.1.2 Identification of DEGs

In the bioinformatics analysis, after aligning the samples to the ash reference genome, 87,413 transcripts were presented in the library. The average read mapping frequency was 80% and the average of aligned pairs was 7,086,883. After aligning the RNA sequencing data to the *H. fraxinus* reference genome, the read mapping frequency varied from 0.1% to 6.6%.

The resistant clones had the highest number of DEGs. A total number of 6,039 DEGs was presented in clone 8 from which, 3,579 were up-regulated and 2,460 were down-regulated in the inoculation condition with *H. fraxinus* (Table3). A total number of 3,709 DEGs was also presented in clone 100 from which, 2,061 and 1,648 were up- and down-regulated respectively in the inoculation condition

(Table3). However, in the susceptible clones, 1,216 and 1,070 DEGs were presented in clone 9 and 76, respectively. The number of DEGs up-regulated in clone 9 and 76 were 640 and 727 respectively while, 576 and 343 DEGs were down-regulated in clone 9 and 76 in the inoculation condition respectively.

The bioinformatics analysis of inoculation treatment identified more than 3,500 DEGs in the resistant clones. The numbers of up-regulated and down-regulated DEGs were 2,097 and 1,418 respectively. However, only 193 DEGs were identified in the susceptible clones of which, 139 were up- and 54 were down-regulated, respectively (Table3).

Table 3. The number of differentially expressed genes (DEGs) expressed in each clone after infection with *H. fraxinus*

	Total DEGs	Up-regulated	Down-regulated
Clone 8 (R)	6,039	3579	2460
Clone 100 (R)	3,709	2061	1648
Clone 9 (S)	1,216	640	576
Clone 76 (S)	1,070	727	343
Resistant clones (8 & 100)	3,515	2,097	1,418
Susceptible clones (9 &76)	193	140	54

R=resistant; S=susceptible. ID 8 and 100 are resistant clones and 9 and 76 are susceptible clones

3.1.3 Two-way hierarchical clusters

The two-way hierarchical clustering of the FPKM data from DEGs between inoculation and healthy conditions in each clone (the first four rows in Table3) is shown in Figure 2. The green and red colors indicate the lowest and highest FPKM values, respectively. In the cluster 3c, 6 and 11, higher gene expression was observed in the inoculation condition compared to healthy condition whereas; in cluster 4 and 7, higher gene expression was found in the healthy condition. None of the clusters showed clear differences in gene expression between resistant and susceptible clones. Genes associated with defense response and JA were expressed in the inoculation treatment. However, genes associated with transport and protein metabolic processes were expressed in cluster 4 for the control treatment. Clone 9 showed a clustering pattern different from the other 3 clones. The inoculated samples in this clone were clustered with the healthy samples rather than with the inoculated samples of the other 3 clones. Genes presented in the cluster 4, 6, 7 and 11 were then annotated with Blast2go.

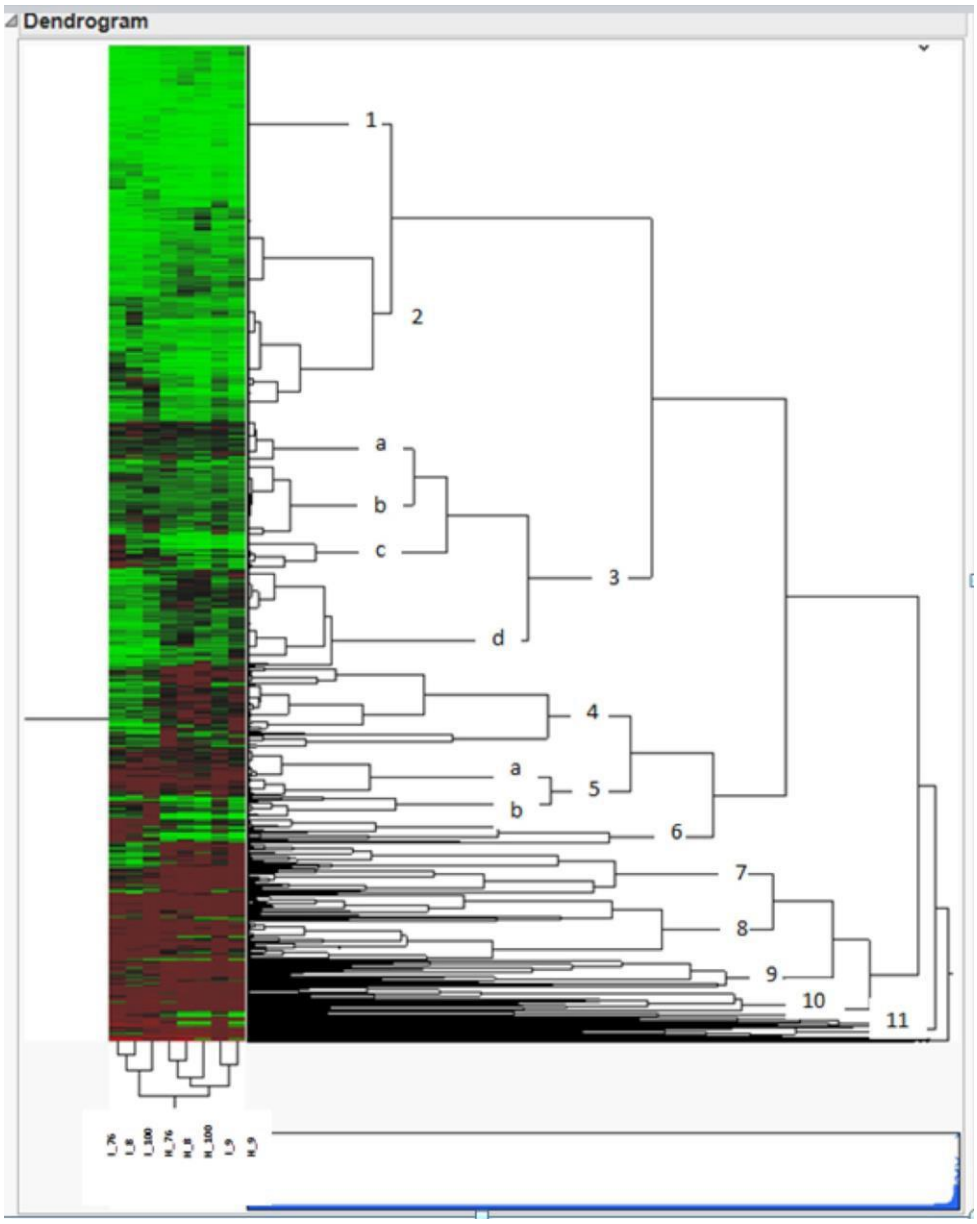

Figure 2. Two-way hierarchical clusters of the FPKM data obtained from deferentially expressed genes of susceptible and resistance clones. Green, black and red colors show low, moderate and high expression, respectively. From left to right 1_76, I_8, I_100, H_76, H_8, H_100, I_9 and H_9. H=Healthy; I= Inoculation. FPKM= *fragments per kilo base of transcript per million mapped reads*.

3.1.4 Annotated DEGs

The Venny diagram was formed from DEGs that were up- and down-regulated in each clones (see Table 3). Those genes presented only in the intercept were chosen to be annotated with Blast2go (Figure 3), as they were more likely regulated in response to infection. After inoculation, 625 genes were up-regulated in the resistant clones compared to only 6 genes in the susceptible clones (Figure 3). The numbers of DEGs down-regulated after inoculation in the resistant and susceptible clones were respectively 527 and 13 (Figure 3). The up-regulation of *1-aminocyclopropane-1-carboxylate oxidase* (*ACC*), some *ethylene-responsive*- TFs (*ERFs*) and WRKY-TFs was detected in the inoculation treatment in resistant clones (Appendix 2). However, *photosystem i subunit o* (PS I) as well as *ERFs* were up-regulated in healthy condition meaning that, these genes were down-regulated in the inoculation treatment in resistant clones (Appendix 3). The expression of *2-hydroxyisoflavanone dehydratase-like* (*HID*), *caffeoyl- o-methyltransferase* (*COMT*) and *farnesyl diphosphate synthase* was observed in the inoculated susceptible clones (Appendix 5). However, *ERFs* were down-regulated in the inoculation treatment in susceptible clones (Appendix 6). In both resistant and susceptible clones, the up-regulation of *peroxidase like* and *pathogen-related protein* (*PR*), *secoisolariciresinol dehydrogenase-like*, *flavonol synthase flavanone 3-hydroxylase-like*, *phenylalanine ammonia-lyase*, *shikimate chloroplastic-like* (Appendix 4) and down-regulation of *ERFs* (Appendix 7) were observed. The description of some genes with the highest fold change and best blast hit is presented in Appendixes 2-7.

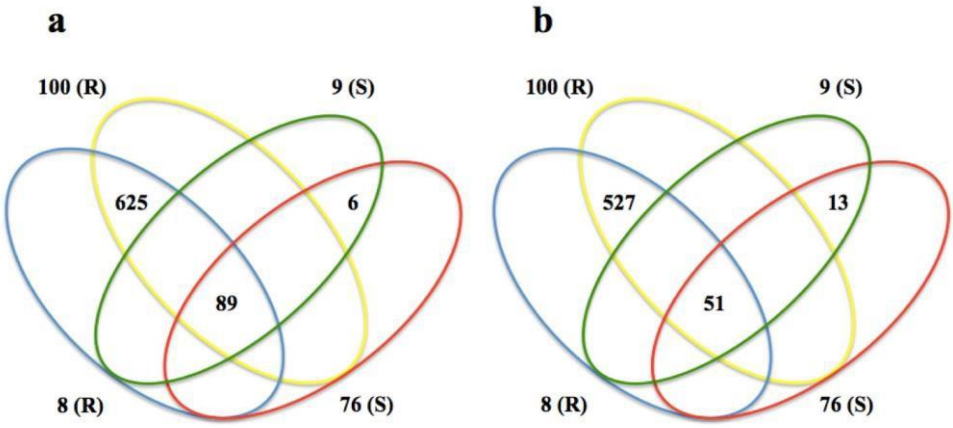

Figure 3. The Venny diagram of up-regulated (a) and down-regulated (b) genes in inoculation condition in the resistant (R) clones (100, 8) and susceptible (S) clones (76, 9).

3.2 Expression of *ABA8H* genes in response to *H. fraxinus*

Inoculation with *H. fraxinus* resulted in significantly longer necrotic lesions than wounding alone (Table 4). This could suggest that lesion was developed in the first week and then the progress was slowed down. There was also a substantial variation within replicates, which is illustrated by the lack of significance at the 5% level at 14 dpt despite the numerical difference between the two treatments.

Table 4. The effect of wounding or inoculation with *H. fraxinus* on lesion length (mm) in the European ash.

	Treatment			
Sampling time (day)	Wounding	Inoculation	SEM	P-value
1	3	3.6	0.9	0.64
7	1.2	7.8	1.2	0.004
14	0.8	6.8	1.9	0.06
21	0	2.6	0.8	0.04

The expression of the three *ABA8H* genes as measured with qRT-PCR indicates that the *FeABA8H3* and *FeABA8H2* genes showed the highest expression already at the first sampling, i.e. 1 dpt (Figure 4 b and c). These two genes had clearly a

lower expression at 7 dpt The regulation of *FeABA8H1* was not as strong but this gene too showed higher steady-state mRNA levels at 1 dpt (Figure 4a).

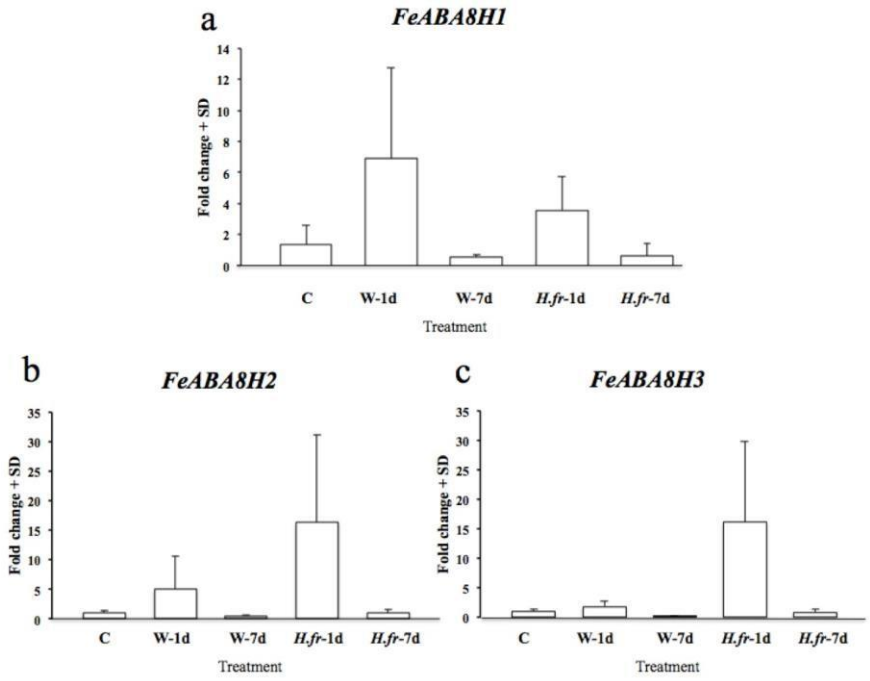

Figure 4.The relative expression of the three *ABA-8 hydroxylase* genes in the *Fraxinus excelsior* subjected to no-treatment (C), inoculation with *H. fraxineus* (*H.fr*) and wounding (W) at days 1 (1d), 7 (7d) and 21 (21d) post-treatment.

The relative expression of two *NAC* genes, *NAC72* and *NAC2*, potentially involved in controlling the ABA signaling pathway, was also quantified by qRT-PCR. *NAC72* appeared to be down-regulated in the wounding treatment at 7 dpt (Figure 5a). Both genes showed the highest steady-state mRNA levels at 1 dpt with *H. fraxineus* (Figure 5a and b) while, the expression levels reached to pre-treatment level (control) at 7 dpt.

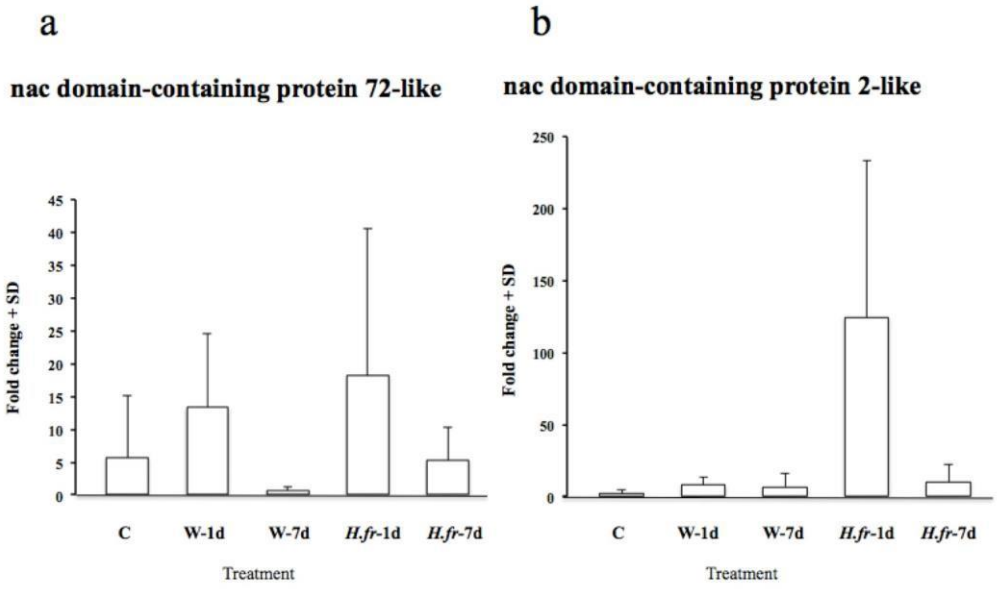

Figure 5. Expression of two *NAC* genes (NAC domain-containing protein 72-like (*NAC72*) (a) and 2-like (*NAC2*) (b)) in the European ash subjected to no-treatment (C), inoculation with *H. fraxineus* (*H.fr*) and wounding (W) at days 1 (1d), 7 (7d) and 21 (21d) post-treatment.

3.3 Validating the sequences of *ABA8H* genes

The *FeABA8H2* had the longest sequence with 451 amino acids. The *FeABA8H1* and *FeABA8H3* had a partial transcript with 239 and 336 amino acids, respectively. The amino acid sequences of *FeABA8H2* and *FeABA8H3* showed high degree of identity (96%) and similarity (97.3%) (Figure 6). *FeABA8H1* shared 239 and 184 amino acids with *FeABA8H2* and *FeABA8H3*, respectively. The *FeABA8H1* had 71% similarity and 55% identity with the *FeABA8H2* and 66% similarity and 51% identity with the *FeABA8H3* (Figure 6). The conserved sites among the three ABA8H amino acid sequences are presented in Figure 6. It appeared most parts of the sequences were similar.

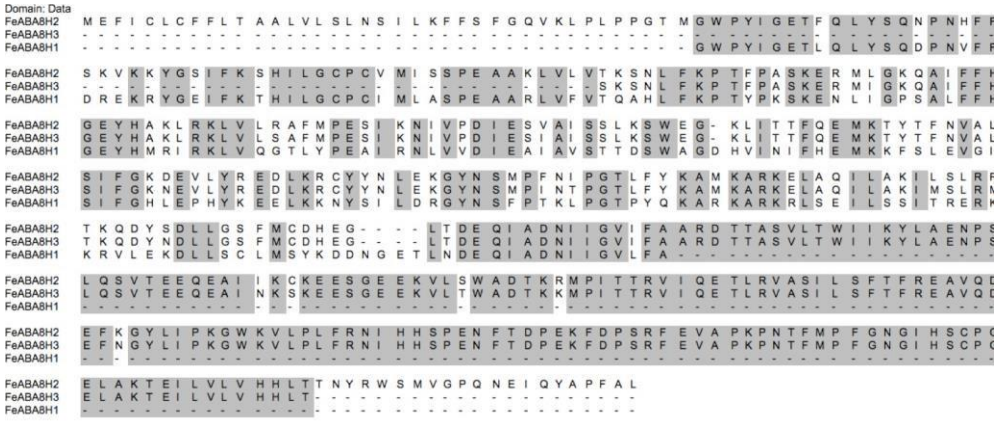

Figure 6. The alignment of three ABA8H amino acid sequences (FeABA8H1, FeABA8H2, FeABA8H3). The grey color shows the conserved sites.

3.3.1 The phylogenetic tree

The phylogenetic tree constructed from amino acid sequences of ABA8H in tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), *Erythrantha* (*Erythrantha guttatus)* and *Fraxinus excelsior* is presented in Figure 7. The scale number was 0.1 and the shortest branch was observed between ABA8H of tomato and potato. FeABA8H2 and FeABA8H3 were diverged with 100 % support from the other branches. Among the three ABA8H genes of the European ash, the shortest branch was also observed between FeABA8H2 and FeABA8H3.

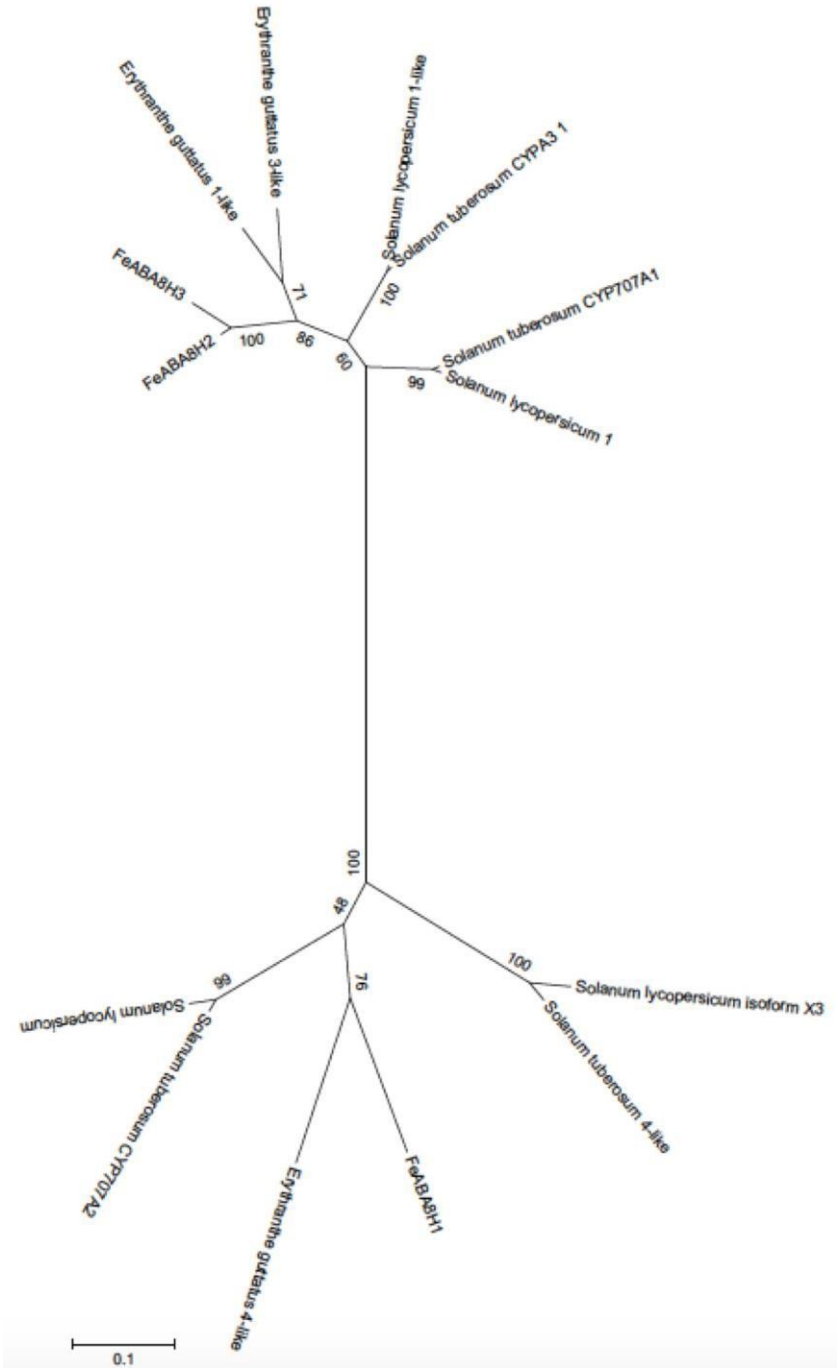

Figure 7. The maximum likelihood radial phylogenetic tree constructed based on amino acid sequences of three ABA8H proteins of the European ash (FeABA8H1, FeABA8H2, FeABA8H3) and four ABA8H proteins of tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), *Erythrantha* (*Erythrantha guttatus*). Bootstrap: 1000, partial deletion option: 95%.

4 Discussion

4.1 Inoculation on defined genotypes

The low mapping coverage of samples to *H. fraxineus* draft genome (0.1% to 6.6%) was most likely due to a low fungus activity in the sampling areas. This low activity of fungus was also observed in our pervious study (Eshghi Sahraei et al., 2015). The fungus activity might be to very low levels by the plant defense mechanism at the time of sampling, which was 9 months after inoculation. On the other hand, *H. fraxineus* is an invasive pathogen. Therefore, the low activity of fungus seems to be enough to induce disease (Gross et al., 2014).

The bioinformatic analysis revealed the presence of more than 87,000 transcripts in the libraries. An interesting observation is that the two resistant genotypes showed a higher number of regulated genes than the two susceptible genotypes. This can be partly explained by the deviating gene expression pattern of the clone 9, susceptible genotype, in response to *H. fraxineus* infection. However, the higher numbers of DEGs in the resistant compared to susceptible, clones is somehow expected. These results suggest that when a plant is encountered with a pathogen, some non-vital activities are switched off in the expense of initiating the defense mechanism and that the resistant genotypes possess a genetic component that allows them to activate a consistent defense.

The two-way hierarchical clustering (Figure 2) indicates that gene expression was divided into two categories as expected, highlighting differences in gene expression between healthy and inoculation conditions.

The up-regulation of *ERFs* and *ACC oxidase* which are involved in ET pathway and the up-regulation of WRKY-TFs in inoculated resistant clones could be viewed as signs of activation of defense to *H. fraxineus*, and an indication of molecular signaling components of this defense responses. Both *ACC Oxidase* and *ERFs* act in the ET signaling pathway (Van Loon et al., 2006). ET is produced in

response to abiotic and biotic stress (Wang et al., 2002) The transcriptional responses to *H. fraxineus* therefore suggest that ET signaling might be important to activate resistance to *H. fraxineus*. However, it is obvious that the regulation of the members of the *F. excelsior ERFs* in response to *H. fraxineus* is complex as other members of the gene family were down-regulated in *H. fraxineus* inoculated resistant and susceptible clones. The regulation of the ET pathway is complex and still not fully understood (Guo and Ecker, 2004). Detailed studies on the regulation of *F. excelsior ERFs* in response to *H. fraxineus* infection or other stresses are needed to improve our understanding.

Members of the WRKY-TFs gene family are known to control responses to several types of stress and the members of this gene family have a conserved DNA binding site namely the W-box (Eulgem et al., 2000; Dong et al., 2003). The importance of WRKY-TFs in the regulation of genes involved in the defense mechanism has been established in *Arabidopsis* (Eulgem et al., 2000; Dong et al., 2003). Interestingly enough, a previous transcriptomics study on *Fraxinus* phloem aiming to profile the responses to emerald ash borer (Bai et al., 2011) identified a WRKY-TF, which accumulated to higher levels in *F. mandschurica* than in other *Fraxinus* taxa. It remains to be investigated whether the WRKY-TF that we identified, is identical to this WRKY-TF or not.

The HID2 enzyme synthesizes isoflavonoids, a group of secondary metabolites produced by plants in response to biotic and abiotic stress (Akashi et al., 2005; Nascimento and Fett-Neto, 2010). The *HID2* gene was not expressed in the resistant clones, but it was expressed in the inoculated susceptible clones suggesting that the expression of this gene is associated with the development of disease. The concomitant induction of a *COMT* and a *farnesyl diphosphate synthase* in the susceptible genotypes in response to *H. fraxinus* further supports the possibility that particular metabolites accumulate in association with disease symptoms (Cleary et al., 2014). The up-regulation of *HID2* gene in the inoculated susceptible clones would have possibly resulted in an increased level of isoflavonoids. Our previous study showed that the flavonoids kaempferol and quercetin are major components of the *F. excelsior* metabolome and that both components were repressed after viridiol treatment in resistant genotypes but not in susceptible genotypes that developed disease symptoms (Cleary et al., 2014).

The over expression of *flavonol synthase flavanone 3-hydroxylase-like*, *phenylalanine ammonia-lyase*, *shikimate chloroplastic-like* were observed in both susceptible and resistant clones in response to inoculation with *H. fraxineus.* The two first components, *flavonol synthase flavanone 3-hydroxylase-like* and *phenylalanine ammonia-lyase*, are part of secondary and flavonoid metabolic pathway (Hahlbrock and Grisebach, 1979). The *phenylalanine ammonia-lyase* produced phenilalanyne which is also a precursor for many secondary metabolites

(Hahlbrock and Grisebach, 1979). It appears that a potential link between secondary metabolites, such as isoflavonoids, and development of disease symptoms should further be studied. The up-regulation of *secoisolariciresinol dehydrogenase-like* was also detected in both resistant and susceptible clones after inoculation with *H. fraxineus*. Secoisolariciresinol dehydrogenase is an enzyme that catalyzes secoisolariciresinol into matairesinol and has a role in some intermediate steps, which produce components of defense mechanism in vascular plant (Moinuddin et al., 2006). The xanthoxin dehydrogenase catalyzes Xanthoxin to abscisic aldehyde and is a sub group of secoisolariciresinol dehydrogenase-like (http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=187587). Secoisolariciresinol dehydrogenase-like is tightly associated with the Xanthoxin and abscisic aldehyde, which are intermediate components in ABA biosynthesise. The Xanthoxin and abscisic aldehyde were detected after application of viridiol, produced from *H. fraxineus*, on the *Fraxinus excelsior* (Cleary et al., 2014). This observation and the ABA8H genes down regulation detected after inoculation with *H. fraxinus* suggest that ABA plays important roles in defense mechanism (Eshghi Sahraei et al., 2015).

The defense mechanism in plants is a complex process, which is regulated by the cross-talk between ET, SA and JA pathways (Almagro et al., 2008). Secondary metabolites and reactive oxygen species (ROS) are also produced in plant in response to invasion by a pathogen, resulting in HR in the infection site (Almagro et al., 2008). Peroxidases are involved in the production of ROS (Almagro et al., 2008). The expression of peroxidase and PR protein in both susceptible and resistant clones could be indicative of activation of the SA, JA and ET pathways (Almagro et al., 2008).

Overall, up-regulation of proteins involved in the defense mechanism was observed in both susceptible and resistant clones, with the resistant clones showing a higher up-regulation. The down-regulation of PS I, with a function in transporting light-driven electrons, could be due to a reduced activity of pathways responsible for the plant growth at the time of defense mechanism activation (Scheller et al., 2001).

4.2 Expression of *ABA8H* genes in response to biotic stress

The down-regulation of the *ABA8H* genes in response to inoculation with *H. fraxinus* is in accordance with our previous observations (Cleary et al., 2014; Eshghi Sahraei et al., 2015). As the concentration of ABA is negatively correlated to *ABA8H* gene expression (Saito et al., 2004), we could expect that the level of ABA was increased in the inoculation treatment but, unfortunately, we did not measure the ABA level in the inoculated tissues.

The similar fold change of *FeABA8H2* and *FeABA8H3* in response to *H. fraxineus* could be due to the high similarity of these two genes. A high similarity was also observed after aligning the amino acid sequences of these genes (Figure 6) suggesting that these genes have a same function in *F.excelsior*.

Based on our previous observation (Eshghi Sahraei et al., 2015) and the results from qRT-PCR study, it seems that *ABA8H* genes are first up-regulated and then down-regulated in response to inoculation with *H. fraxinus*. This was also supported from the transcriptomics study on samples obtained 9 month after inoculation. In the green-house experiment, it seems that there was a correlation between lesion length and the activity of *ABA8H* genes. The increase in the lesion length in the inoculated samples in day 7 of the treatment was coincided with a down-regulation of *ABA8H* genes, indicating a possible increase in ABA level in the tissue. However, the large variation found in the results makes it difficult to draw firm conclusions. A possible explanation for this variability is that the plants used in this experiment had an infection to *H. fraxineus* prior to the study, which became evident at the end of the experiment. Possibly, the individual plants were at different infection stages and as a result, response to treatment differed among biological replicates.

A similar expression pattern was observed in the qRT-PCR study between *ABA8H3* and *NAC2*. A same expression pattern was also found between *NAC72* and *ABA8H1* and *ABA8H2*.The increased expression of *NAC72* and *NAC2* in response to inoculation and wounding could be expected because it is known that this particular group of *NAC* gene family members often are involved in mitigating the biotic and abiotic stress (Puranik et al., 2012; Nuruzzaman et al., 2013). These genes are responsible for the regulation of genes encoding precursor enzymes needed for the ABA formation (Puranik et al., 2012). In addition, the *NAC* genes regulate positively the JA/ET pathway and negatively the ABA signaling pathway in stress (Puranik et al., 2012).

4.3 Validating the sequences of *ABA8H* genes

In the current experiment and our earlier study (Eshghi Sahraei et al., 2015), we found that *FeABA8H1*, *FeABA8H2* and *FeABA8H3* are down-regulated after inoculation with *H. fraxineus*. The initial qRT-PCR primers were based on predicted gene models obtained from the ash draft genome (Harper et al., 2016). The genes were re-sequenced to validate the predicted sequences of *ABA8H*. The results from data similarity and identity as well as the phylogenetic tree (Figure 6 & 7) suggest that *FeABA8H2* and *FeABA8H3* are paralogous in *F.excelsior* and that they share a common evolutionary ancestor with their ortholog, which is *Erythrantha* (Figure 7). The length of branch in phylogenetic tree indicates extent of genetic changes

over time (http://epidemic.bio.ed.ac.uk/how_to_read_a_phylogeny). Therefore, a short length of branch between ABA8H of tomato and potato suggests a high amino acid similarity (Figure 7). The high similarity and identity between *FeABA8H2* and *FeABA8H3* (96% and 97.3% respectively) suggest that may be the result of a gene duplication. Sub-functionalization and neo-functionalization are the main reasons for keeping duplicate genes in biological systems (Rastogi and Liberles, 2005). In neo-functionalization, one of the copies becomes responsible for a different function but in sub-functionalization, these duplicated genes become partially involved in a same function (Rastogi and Liberles, 2005). It is also possible that several genes become responsible for the production of a same enzyme due to the need of plant (personal communication- Magnus Karlsson)

In conclusions, genes were differentially expressed in resistant and susceptible clones in response to inoculation with *H. fraxinus.* Genes responsible for the defense mechanism were up-regulated in both resistant and susceptible clones. Most genes with a role in the defense mechanism such as *ACC*, ER-TFs and WRKY-TFs were presented in the inoculated resistant clones. The number of DEGs in the resistant clones was higher than in the susceptible clones. The longest lesions were observed in the inoculated barks and at 7 dpt and 14 dpt. The *ABA8H* had the highest expression at day 1 dpt.

Due to the high similarity between *FeABA8H2* and *FeABA8H3*, we believe gene duplication has occurred in the genus of *Fraxinus*. The phylogeny analysis revealed *ABA8H* in the *F.excelsior* has a high similarity with the *ABA8H* in *Erythrantha*.

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Appendix

Appendix 1. Primers used for the sequencing and the real time quantitative polymerase chain reaction (qRT-PCR).

Primers	Sequence	Annealing temperature (°C)	Product size (bp)	Aim of design
20478-F	CAATGGGGTGGCCTTATA	56.78	722	Sequencing
20478-R	TGCAAAAAGTACTCCGATGA	56.94		Sequencing
40775 F	CTCTCAGTCTTTCTGTACTGTCC	54.96	1392	Sequencing
40775 R	GAAGAGCAAAGGGTGCATAC	57.42		Sequencing
64553-F	CAGCAAATCAAATCTCTTCA	54.01	1013	Sequencing
64553 R	TGGTCAGGTGATGTACAAGG	56.90		Sequencing
40775F2_1501	CATGAATGCGCGCAGAACTA	59.35		qRT-PCR
40775R2_1501	TCCCTGCAAGCAAAGAGAGA	58.94	101	qRT-PCR

Appendix 1. (cont.)

Primers	Sequence	Annealing temperature (°C)	Product size (bp)	Aim of design
64552F2_1501	GCATGATACTCGCCCTGATGA	60.00	80	qRT-PCR
64552R2_1501	TTCAAGCCCACATTCCCTGC	60.90		qRT-PCR
20478_F	TTGCAGCACAAGACACGACT	60.66	100	qRT-PCR
20478_R	CCTTCTGTTCTGCCCTTGAC	59.84		qRT-PCR
48802-F	GCGCCTTACCAGCATAAAAC	59.75	102	qRT-PCR
48802-R	CTGGGAGTGGGTACTGGAAG	59.57		qRT-PCR
39046-F	TGAAGAGCTCATGGTGCAAT	59.40	108	qRT-PCR
39046-R	GGAAGTTCCCATGGATCAAA	59.73		qRT-PCR
α-Tubulin-F	CACCTCCTCCAACGGTCTTA	60.10	104	qRT-PCR
α-Tubulin-R	GGCTGGTATTCAGGTTGGAA	59.93		qRT-PCR

Appendix 2. The genes with highest fold change were up regulated in inoculation condition in resistant clones

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_006133_TCONS_00010496	-inf	cysteine-rich repeat secretory protein 39-like	238.424	1.63E-73
XLOC_007763_TCONS_00013361	-inf	non-symbiotic hemoglobin class 1	291.197	2.34E-96
XLOC_009072_TCONS_00015657	-inf	eugenol synthase 1-like	452.595	9.91E-156
XLOC_009510_TCONS_00016410	-inf	stem-specific protein tsjt1-like	154.451	1.34E-55
XLOC_012110_TCONS_00020893	-inf	ethylene-responsive transcription factor erf096-like	145.976	2.56E-40
XLOC_014574_TCONS_00025151	-inf	7-deoxyloganetin glucosyltransferase-like	239.195	1.20E-101
XLOC_014593_TCONS_00025184	-inf	7-deoxyloganetin glucosyltransferase-like	239.195	1.32E-106
XLOC_014962_TCONS_00025829	-inf	viridiflorene synthase	93.2041	5.21E-31
XLOC_015003_TCONS_00025898	-inf	ethylene-responsive transcription factor erf098-like	125.176	4.64E-32
XLOC_015502_TCONS_00026749	-inf	glyoxylate hydroxypyruvate reductase hpr3-like	192.586	1.69E-56
XLOC_018570_TCONS_00032009	-inf	germacrene a	241.121	2.07E-73
XLOC_019287_TCONS_00033230	-inf	cytochrome p450 71d95-like	81.2629	3.36E-16
XLOC_019288_TCONS_00033231	-inf	premnaspirodiene oxygenase-like	98.5969	3.37E-22
XLOC_022097_TCONS_00038102	-inf	hypothetical protein VITISV_014759	142.124	3.34E-35
XLOC_022764_TCONS_00039332	-inf	nac domain-containing protein 21 22-like	97.0561	1.41E-19
XLOC_022884_TCONS_00039543	-inf	hypothetical protein JCGZ_00710	62.003	9.08E-10
XLOC_026611_TCONS_00046198	-inf	copal-8-ol diphosphate chloroplastic-like	880.937	0
XLOC_026680_TCONS_00046306	-inf	dirigent protein 21-like	214.542	1.69E-66
XLOC_026927_TCONS_00046765	-inf	respiratory burst oxidase homolog protein a	138.272	2.63E-33
XLOC_028981_TCONS_00050332	-inf	bon1-associated protein 2-like	172.94	1.00E-48

Appendix 2. (cont.)

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_031266_TCONS_00054438	-inf	PREDICTED: uncharacterized protein LOC105176650	78.1814	2.21E-14
XLOC_031711_TCONS_00055210	-inf	e3 ubiquitin-protein ligase rha2a-like	125.946	4.87E-32
XLOC_032640_TCONS_00056866	-inf	beta-amyrin synthase	219.55	1.33E-70
XLOC_033068_TCONS_00057646	-inf	probable galactinol--sucrose galactosyltransferase 1	205.682	1.59E-59
XLOC_033809_TCONS_00058977	-inf	cannabidiolic acid synthase-like	821.617	0
XLOC_034414_TCONS_00060035	-inf	nac domain-containing protein 100-like	413.69	6.03E-139
XLOC_045813_TCONS_00079964	-inf	ethylene-responsive transcription factor 1b	220.32	3.54E-68
XLOC_049524_TCONS_00086395	-inf	wall-associated receptor kinase 2-like	174.481	3.46E-47
XLOC_057734_TCONS_00100443	-inf	receptor-like protein kinase hsl1	1300.8	0
XLOC_072968_TCONS_00126361	-inf	probable wrky transcription factor 75	218.009	6.03E-68
XLOC_035858_TCONS_00062570	6.52261	1-aminocyclopropane-1-carboxylate oxidase	415.616	2.12E-140

Appendix 3. The genes with highest fold change were down regulated in resistant clones.

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_000087_TCONS_00000152	Inf	photosystem i reaction center subunit chloroplastic-like	216.468	8.24E-66
XLOC_002414_TCONS_00004125	Inf	probable lrr receptor-like serine threonine-protein kinase at3g47570	233.417	3.49E-67
XLOC_004766_TCONS_00008183	Inf	ring-h2 finger protein atl67-like	228.024	1.15E-71
XLOC_006139_TCONS_00010504	Inf	protein walls are thin 1-like	652.514	0
XLOC_013490_TCONS_00023262	Inf	della protein rgl1-like	492.271	2.00E-163
XLOC_020304_TCONS_00034972	Inf	shoot meristemless-like protein	119.398	5.55E-32
XLOC_029697_TCONS_00051588	Inf	nuclear transcription factor y subunit a-3-like isoform x1	118.627	3.97E-29
XLOC_032542_TCONS_00056679	Inf	sugar transport protein 8-like	219.55	5.23E-66
XLOC_039428_TCONS_00068741	Inf	vetispiradiene synthase 3-like	831.247	0
XLOC_040858_TCONS_00071349	Inf	ty1-copia-like retrotransposon	111.309	1.52E-27
XLOC_042092_TCONS_00073400	Inf	probable cellulose synthase a catalytic subunit 5	287.73	1.42E-128
XLOC_043220_TCONS_00075464	Inf	cytochrome p450 94c1-like	259.225	7.81E-81
XLOC_044773_TCONS_00078180	Inf	spx domain-containing membrane protein at4g22990-like	790.801	0
XLOC_052052_TCONS_00090680	Inf	ethylene-responsive transcription factor erf034	244.202	9.88E-75
XLOC_054117_TCONS_00094197	Inf	udp-sugar transporter sqv-7-like	141.739	4.37E-37
XLOC_055643_TCONS_00096803	Inf	glucan endo- -beta-glucosidase 12	398.667	7.59E-131
XLOC_058674_TCONS_00102098	Inf	stress responsive a b barrel domain family protein	149.828	1.57E-42
XLOC_062742_TCONS_00109015	Inf	dna binding isoform 1	200.29	1.24E-57
XLOC_062744_TCONS_00109019	Inf	heat stress transcription factor a-6b-like	169.474	2.14E-46
XLOC_063003_TCONS_00109446	Inf	ap2-like ethylene-responsive transcription factor ant	352.443	1.29E-114
XLOC_063565_TCONS_00110420	Inf	sulfate thiosulfate import atp-binding protein	359.762	9.41E-117

Appendix 3. (cont.)

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_063954_TCONS_00111099	Inf	copia partial	106.301	6.16E-40
XLOC_064483_TCONS_00112018	Inf	PREDICTED: uncharacterized protein LOC103456039	60.4622	2.10E-07
XLOC_065070_TCONS_00113053	Inf	sericin 1-like	281.952	6.92E-86
XLOC_065631_TCONS_00114028	Inf	protein iq-domain 14	357.066	4.10E-117
XLOC_066274_TCONS_00115081	Inf	protein scarecrow-like	345.895	8.64E-110
XLOC_066736_TCONS_00115850	Inf	odontogenic ameloblast-associated	124.02	3.82E-32
XLOC_067775_TCONS_00117643	Inf	PREDICTED: uncharacterized protein LOC104596423	58.151	7.82E-08
XLOC_067780_TCONS_00117651	Inf	PREDICTED: uncharacterized protein LOC104596423	58.151	5.28E-08

Appendix 4. The genes with highest fold change were up regulated in both susceptible and resistant clones in response to *H. fraxineus* infection

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_017222_TCONS_00029640	6.05402	polyphenol oxidase chloroplastic-like	841.647	0
XLOC_066018_TCONS_00114639	5.69566	peroxidase 12-like	457.603	1.88E-157
XLOC_032284_TCONS_00056219	5.08033	hypothetical protein L484_011462	106.686	1.37E-25
XLOC_045728_TCONS_00079815	5.07811	tetraketide alpha-pyrone reductase 1-like	503.827	7.41E-175
XLOC_024935_TCONS_00043210	4.8516	pathogen-related protein	392.889	2.70E-134
XLOC_001413_TCONS_00002492	4.27279	pectate lyase-like	688.337	0
XLOC_009763_TCONS_00016851	4.08676	septum-promoting gtp-binding protein 1-like	392.504	1.11E-130
XLOC_083556_TCONS_00144060	3.99429	secoisolariciresinol dehydrogenase-like	253.062	1.17E-81
XLOC_023212_TCONS_00040115	3.95319	beta- -galactosyltransferase 7 isoform x1	461.84	2.94E-159
XLOC_008342_TCONS_00014342	3.91484	glycosyltransferase 7	335.109	4.18E-171
XLOC_027430_TCONS_00047630	3.90662	ole e 5 olive pollen allergen	289.656	1.13E-95
XLOC_032374_TCONS_00056375	3.79042	geraniol dehydrogenase	504.982	6.41E-175
XLOC_075513_TCONS_00130734	3.7396	flavonol synthase flavanone 3-hydroxylase-like	532.332	0
XLOC_035381_TCONS_00061730	3.69402	phenylalanine ammonia-lyase	236.113	8.66E-69
XLOC_086929_TCONS_00149810	3.59717	probable protein s-acyltransferase 14	382.874	4.18E-126
XLOC_025513_TCONS_00044155	3.59273	abc transporter g family member 35-like	1447.57	0
XLOC_017247_TCONS_00029668	3.55523	aldolase-type tim barrel family protein isoform 1	664.07	0
XLOC_001565_TCONS_00002743	3.51909	udp-glycosyltransferase 87a1-like	248.825	2.69E-75
XLOC_042820_TCONS_00074747	3.45658	shikimate chloroplastic-like	270.011	5.24E-86
XLOC_041759_TCONS_00072836	3.44375	PREDICTED: uncharacterized protein LOC104221096	289.271	6.28E-94

Appendix 5**.** The genes with highest fold change up regulated in susceptible clones in response to *H. fraxineus* infection

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_029892_TCONS_00051933	4.22221	2-hydroxyisoflavanone dehydratase-like	462.225	6.80E-158
XLOC_081268_TCONS_00140275	3.43026	caffeoyl- o-methyltransferase	481.871	3.40E-169
XLOC_013177_TCONS_00022721	2.84388	methylesterase chloroplastic	228.024	6.35E-69
XLOC_075548_TCONS_00130778	2.74928	probable pectate lyase 8	721.465	0
XLOC_037171_TCONS_00064912	2.43327	farnesyl diphosphate synthase	546.969	0

Appendix 6. The genes with highest fold change were down regulated in susceptible clones in response to *H. fraxineus* infection

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_077476_TCONS_00134037	3.90252	laccase-14-like	714.916	0
XLOC_021054_TCONS_00036307	3.12166	ethylene-responsive transcription factor 5-like	197.593	2.48E-55
XLOC_085606_TCONS_00147634	3.05935	low affinity sulfate transporter 3	265.003	8.53E-80
XLOC_009899_TCONS_00017075	2.97609	vacuolar-processing enzyme	574.704	0
XLOC_046270_TCONS_00080773	2.76108	protein tify 10b-like	227.254	1.79E-65
XLOC_055641_TCONS_00096801	2.65917	calcium-transporting atpase plasma membrane-type-like	324.709	6.55E-98
XLOC_030407_TCONS_00052827	2.40128	plastid-targeted protein 2	154.836	9.92E-42
XLOC_018383_TCONS_00031669	2.20534	mitogen-activated protein kinase kinase 5-like isoform x4	190.66	2.37E-50
XLOC_033558_TCONS_00058578	2.17488	calmodulin-binding family protein	746.503	0
XLOC_031518_TCONS_00054910	2.10841	probable e3 ubiquitin-protein ligase rha2b	173.711	9.58E-50

Appendix 7**.** The genes with highest fold change were down regulated in both susceptible and resistant clones in response to *H. fraxineus* infection

Locus	Log FC	best blastX hit	Bit-Score	E-Value
XLOC_022125_TCONS_00038138	3.97762	phospholipase a1- chloroplastic-like	860.907	0
XLOC_062857_TCONS_00109218	3.56667	protein tify 10b-like	223.016	7.21E-65
XLOC_030086_TCONS_00052237	3.38428	fk506-binding protein 4-like	107.842	3.61E-25
XLOC_060449_TCONS_00105135	3.24324	poly polymerase	168.318	1.53E-47
XLOC_068050_TCONS_00118132	2.97728	transcription factor bhlh36-like	178.718	5.57E-50
XLOC_000577_TCONS_00001017	2.93593	btb poz domain-containing protein at5g41330	433.335	4.65E-146
XLOC_054121_TCONS_00094214	2.73374	PREDICTED: uncharacterized protein LOC105161463	650.203	0
XLOC_012088_TCONS_00020856	2.69476	protein tify 10b-like	241.121	2.72E-71
XLOC_016588_TCONS_00028600	2.59843	polyketide cyclase dehydrase and lipid transport superfamily protein	631.713	0
XLOC_085093_TCONS_00146775	2.58072	PREDICTED: uncharacterized protein LOC105156865 isoform X1	415.616	1.24E-141
XLOC_031339_TCONS_00054571	2.23917	major latex-like protein	201.445	2.70E-60
XLOC_079277_TCONS_00136993	2.23082	ethylene-responsive transcription factor erf017-like	204.527	1.61E-60
XLOC_061562_TCONS_00107016	2.21894	protein spiral1-like 5	88.1965	1.94E-18
XLOC_064856_TCONS_00112687	2.20384	probable sucrose-phosphate synthase 4	478.404	1.08E-153
XLOC_047146_TCONS_00082312	2.13957	auxin-responsive protein iaa26-like	350.517	1.63E-113
XLOC_058264_TCONS_00101364	2.12713	probable alpha-amylase 2	364.385	3.74E-118
XLOC_056992_TCONS_00099101	2.12224	probable lrr receptor-like serine threonine-protein kinase at5g63710	855.899	0
XLOC_014848_TCONS_00025635	2.12198	protein sym-1	270.396	4.68E-84
XLOC_007459_TCONS_00012839	2.01788	transcription factor bhlh35-like	321.242	1.81E-104
XLOC_050921_TCONS_00088776	2.00463	dof zinc finger	129.413	1.41E-34