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Genes, proteins and metabolites in the interaction of strawberry and fungal pathogen

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GENES, PROTEINS AND METABOLITES IN THE INTERACION OF STRAWBERRY AND FUNGAL PATHOGEN

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Dedikasyon

Para kina Benedicto at Asuncion, ang aking mga magulang,
ang mga unang guro na luminang sa aking kamalayan;

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"It always seems impossible until it's done."
-Nelson Mandela

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Preface

This manuscript is the final output of the author's doctoral research study conducted under the main supervision of Dr. Elena Baraldi. Most of the research activities were carried out at the Biotechnology Phytopathological Laboratory of the Department of Agricultural Science at School of Agriculture and Veterinary Medicine of the University of Bologna. A three-month period was also spent at the Department of Food Quality and Nutrition at the Fondazione Edmund Mach in San Michele All'Adige under the guidance of Dr. Urska Vrhovsek.

Shifting from her horticultural background, the author dealt on plant pathology topic for her PhD research. While conducting research on the interaction of strawberry and fungal pathogens, the author was able to enhance her skills on metabolomics. At the same time, the author was able to involve herself in molecular studies and learned several laboratory techniques and methods on data analysis.

In this manuscript, the results of the research activities carried out during the doctoral period is presented in three chapters (Chapters 2-4), following the format of a scientific publication. The study reported in Chapter 2 is for submission in a peer-reviewed journal, while Chapter 3 was already published in the Journal of Agricultural and Food Chemistry.

ABSTRACT

Colletrotrichum acutatum and *Botrytis cinerea* are among the major fungal pathogens of *Fragaria* spp. Both pathogens could infect strawberry fruits during the fruit's early developing stage and remain quiescent until ripening. In strawberry, a fruit ontogenic resistance to pathogen infection was described and correlated with fungal quiescence during the unripe stages of the fruit. Due to the period of fungal quiescence, the management of anthracnose and gray mould diseases becomes more complex as symptoms only manifest in ripe fruits. To identify the underlying component in the ontogenic resistance of strawberry fruits, transcriptomic and metabolomic approaches were used. White and red fruits of strawberry were artificially inoculated with *C. acutatum* and *B. cinerea.* Transcriptome profile of *B. cinerea* infected fruits exhibited a general up-regulation of defense-related genes in white fruits after 24 h of infection. Meanwhile, accumulation of phenolic compounds such as proanthocyanidins, catechins and the ellagitannin casuarictin was also observed in white fruits after 48 h of interacting with *C. acutatum* and *B. cinerea*. The acquisition of these findings could provide a benchmark to further investigate the interaction of strawberry against pathogens with latent infection. Hence, a strawberry transformation was performed to study the mechanism of a gene encoding for a mannose-binding lectin protein which was previously identified to be correlated with the resistance of white strawberry fruits to *C. acutatum*. The regeneration system utilized in the transformation is also discussed.

KEYWORDS: *Colletortichum acutatum, Botrytis cinerea,* fungal quiescence, latent infection, RNA-Seq, polyphenols, stable transformation

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Chapter 1

General Introduction

1.1. STRAWBERRY AS A PERISHABLE CROP

Strawberry (*Fragaria* spp.) fruits remain to be one of the most consumed fruits in the world due the nutritional benefits that could be derived, including minerals, vitamins, fatty acids, fibers and polyphenols[1]. To meet-up with the increasing demand, the world production of the crop increased from 6.5k to 7.7k metric tons in a span of five years (2010-2014)[2]. The increase in production, however, does not guarantee a higher supply of strawberry fruits since losses due to pathogen infection could be encountered anywhere along the postproduction and supply chain. Strawberry fruits are morphologically soft which makes the fruit highly perishable and susceptible to disease[3].

1.2. MAJOR DISEASE-CAUSING FUNGAL PATHOGENS OF *FRAGARIA* SPP. AND LATENT INFECTION

Two of the major diseases in strawberry are anthracnose caused by *Colletotrichum acutatum* and gray mould which is caused by *Botrytis cinerea*[4]. Both pathogens could infect their host during the early stages of development. In *C. acutatum,* conidial germination was reported at 24 h in infected white fruits of strawberry[5]. Meanwhile, floral organs were identified as important sites for *B. cinerea* infection[6], and were also known to infect strawberry inflorescence[7]. Upon penetration of the pathogens in the floral organs or unripe fruits of strawberry, the fungi enters a quiescent state and only resumes its activity once the fruit ripens[8,9,10].

The possibility of *C. acutatum* and *B. cinerea* to enter a quiescent state have been suggested to be influenced by: i) deficiency in the host nutritional resources required for pathogen development; ii) the presence of preformed or inducible fungistatic antifungal compounds in resistant unripe fruits; and iii) an unsuitable environment for the activation of fungal pathogenicity factors.[9] Due to latent infection posed by the two pathogens, the control and management of anthracnose and gray mould disease becomes more complex

as signs of infection do not appear early on and symptoms manifests only when the fruits have already reached their highest value.

1.3. ONTOGENIC RESISTANCE IN STRAWBERRY FRUITS

The latent infection in fruit crops is associated with the physico-chemical properties of the fruit during the time of pathogen infection. White fruits of the strawberry Alba inoculated with *C. acutatum* exhibited no symptoms after one week of incubation. On the other hand, *C. acutatum* inoculated red fruits manifested symptoms of the disease as early as three days post inoculation[5]. These responses exhibited the ontogenic resistance of strawberry fruits to fungal pathogens, with the unripe fruits exhibiting resistance and the red ones displaying susceptibility.

During ripening, strawberry fruits undergo a series of physico-chemical changes[11], which includes enhancement of color, sugars, acids, and volatiles that affect flavor and aroma, and textural modification via the alteration of cell turgor and cell wall structure[12]. These changes that occur during ripening also decrease the passive defense inherent in the fruit. For instance, modifications in cell wall polymers during ripening that causes fruit softening was reported to show a clear relationship susceptibility to pathogen infection[13]. Pre-formed biochemical barriers such as polyphenols, which are naturally concentrated in unripe fruit of strawberry, has been cited to have a proven toxic effect against pathogens and pests[14]. Recently, it was reported that aroma volatile compounds that are abundant in red strawberry fruits such as Furaneol, ethyl butanoate, and *cis*-3-hexenyl acetate stimulates the growth of *B. cinerea*[15]. This finding suggests that ontogenic resistance is not only dependent on the pre-formes structural and biochemical in immature fruits but also on ripeness-specific stimulating compounds.

1.4. AIMS OF THE STUDY

Several physico-chemical bases on the ontogenic resistance of strawberry fruits have been established, but clearly there are far more underlying factors that contributes to this phenomenon. The importance to unravel these factors could contribute to the improvement of the control and management strategies employed to pathogens that exhibits latent infection. Hence, the general objective of this study is to investigate on the interaction of unripe and ripe strawberry fruits to *C. acutatum* and *B. cinerea*, at the same time identify the properties that regulates the ontogenic resistance in strawberries. To achieve this aim, the –omics approach were utilized in the first two parts of the experiment: i). trancriptomic analysis of white and red fruits of woodland strawberry inoculated with *B. cinerea;* and, ii). metabolomic analysis of white and red strawberry fruits inoculated with *C. acutatum* and *B. cinerea*. In the third part of the study, an *Agrobacterium*-mediated transformation was performed in attempt to characterize the mechanism of a gene that encodes for a mannose-binding lectin protein. The said gene was first identified to be the most up-regulated in *C. acutatum* infected fruits in a previous transcriptomic study[5].

1.5 REFERENCES

1. Giampieri, F.; Tulipani, S.; Alvarez-Suarez, J. M.; Quiles, J. L.; Mezzetti, B.; Battino, M., The strawberry: Composition, nutritional quality, and impact on human health. *Nutrition* **2012,** *28* (1), 9-19.
2. Nations, F. a. A. O. o. t. U. FAOSTAT database http://faostat3.fao.org/home/E (accessed February 1).
3. Vicente, A. R.; Martínez, G. A.; Civello, P. M.; Chaves, A. R., Quality of heat-treated strawberry fruit during refrigerated storage. *Postharvest Biology and Technology* **2002,** *25* (1), 59-71.
4. Maas, J. L., *Compendium of strawberry diseases*. American Phytopathological Society (APS Press): 1998.
5. Guidarelli, M.; Carbone, F.; Mourgues, F.; Perrotta, G.; Rosati, C.; Bertolini, P.; Baraldi, E., Colletotrichum acutatum interactions with unripe and ripe strawberry fruits and differential responses at histological and transcriptional levels. *Plant Pathology* **2011,** *60* (4), 685-697.
6. Williamson, B.; Tudzynski, B.; Tudzynski, P.; van Kan, J. A. L., Botrytis cinerea: the cause of grey mould disease. *Molecular Plant Pathology* **2007,** *8* (5), 561-580.
7. Jarvis, W. R., The infection of strawberry and raspberry fruits by Botrytis cinerea Fr. *Annals of Applied Biology* **1962,** *50* (3), 569-575.
8. Pezet, R.; Viret, O.; Perret, C.; Tabacchi, R., Latency of Botrytis cinerea Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mould. *Journal of Phytopathology* **2003,** *151* (4), 208-214.
9. Prusky, D.; Lichter, A., Activation of quiescent infections by postharvest pathogens during transition from the biotrophic to the necrotrophic stage. *FEMS microbiology letters* **2007,** *268* (1), 1-8.
10. Alkan, N.; Friedlander, G.; Ment, D.; Prusky, D.; Fluhr, R., Simultaneous transcriptome analysis of Colletotrichum gloeosporioides and tomato fruit pathosystem reveals novel fungal pathogenicity and fruit defense strategies. *New Phytologist* **2015,** *205* (2), 801-815.
11. Symons, G. M.; Chua, Y. J.; Ross, J. J.; Quittenden, L. J.; Davies, N. W.; Reid, J. B., Hormonal changes during non-climacteric ripening in strawberry. *Journal of Experimental Botany* **2012,** *63* (13), 4741-4750.
12. Giovannoni, J. J., Genetic regulation of fruit development and ripening. *The plant cell* **2004,** *16* (suppl 1), S170-S180.
13. Barritt, B. H., Resistance of strawberry clones to Botrytis fruit rot. *Journal of the American Society for Horticultural Science* **1980**.
14. Dixon, R. A., Natural products and plant disease resistance. *Nature* **2001,** *411* (6839), 843-847.
15. Neri, F.; Cappellin, L.; Spadoni, A.; Cameldi, I.; Algarra Alarcon, A.; Aprea, E.; Romano, A.; Gasperi, F.; Biasioli, F., Role of strawberry volatile organic compounds in the development of Botrytis cinerea infection. *Plant Pathology* **2015,** *64* (3), 709-717.

Chapter 2

Comparative transcriptomic analysis of unripe and ripe strawberry fruits interacting with *Botrytis cinerea*

Comparative transcriptomic analysis of unripe and ripe strawberry fruits interacting with *Botrytis cinerea*

ABSTRACT

The fruit ontogenic resistance of *Fragaria vesca* to *Botrytis cinerea* has been described to vary depending on the ripening stage of the berry. White fruits were determined to be resistant to *B. cinerea* infection, while red fruits are easily susceptible. This phenomenon has been related to fungal quiescence where the pathogen remains dormant in the unripe stages of the fruit. In our study, a growth kinetic analysis showed an exponential growth of *B. cinerea* after 24 h in red fruits of woodland strawberry in contrast to the low activity of the pathogen in white fruits. To gain a better understanding on the varying response of the fruits interacting with *B. cinerea,* an RNA-Seq analysis was performed to highlight the transcriptome profile of unripe (white) and ripe (red) of fruits of strawberry infected with *B. cinerea.* Differential expression analysis was performed to investigate which genes responds to *B. cinerea* infection and how they differ between the unripe and ripe stages of strawberry fruits. The gene regulation in healthy and infected fruits of white and red woodland strawberry is presented in this study, with highlights on the defense-related genes.

KEYWORDS: *Fragaria vesca,* RNA-Seq, ripening stage, ontogenic resistance, fungal quiescence, latent infection

2.1. INTRODUCTION

Botrytis cinerea, the causal pathogen of gray mould, is one of the major causes of damage in strawberry (*Fragaria* spp.). Several strategies have been employed to reduce the losses caused by this nectotrophic fungus, but the ability of *B. cinerea* to remain quiescent after host infection makes the pathogen more difficult to manage[1].

The infection process of *B. cinerea* usually begins with the deposition of conidia to flower organs[2]. The pathogen then enters in a dormant state and remains inactive until fruit ripening is accomplished[3]. By the time *B. cinerea* resumes its activity, the fruits have already reached their marketable state and symptoms of the infection start to manifest which will render the fruits unfit for sale or consumption.

The latent infections that *B. cinerea* exhibits have been attributed to different factors such as the presence preformed fungistatic compounds in unripe fruits, the deficiency in the host nutritional resources required for pathogen development, and an unsuitable environment for the activation of fungal pathogenicity factors[4]. In strawberry fruits, the quiescence of *B. cinerea* has been demonstrated by artificially inoculating white and red fruits of a susceptible cultivar. Symptoms of the infection became evident on red fruits after seven days of incubation, while white fruits remained symptom-free. This phenotypic evidence is accompanied with an increase of polyphenols in white fruits such as proanthocyanidins and ellagitannins after 48 h of infection[5]. Indeed, the involvement of phenolic compounds in the ontogenic resistance of unripe strawberry fruits has been established as one of the earlier basis for the quiescence of *B. cinerea*[6]. Meanwhile, a recent study showed the involvement of strawberry volatile organic compounds (VOCs) in the growth of *B. cinerea,* demonstrating that ripening stage specific compound could either inhibit or stimulate the growth of the pathogen. Specifically, the volatile hexanal, which is more abundant in the unripe strawberry stages, inhibited mycelium growth of *B.*

cinerea, while VOCs that are abundant in red fruits such as Furaneol, ethyl-butanoate, and *cis*-3-hexenyl acetate, stimulated mycelium growth[7].

While biochemical basis on the resistance or susceptibility of strawberry has been established, several underlying factors that contribute to the ontogenic resistance of the fruit have yet to be unraveled. In the recent years, the application of RNA-Sequencing (RNA-Seq) allowed a more extensive transcriptome profiling and a precise elucidation of the gene expression alteration present within a particular sample[8]. In crop protection, the transcriptome-wide profiling has been applied to study the interactions of plant pathogens and their hosts and allowed to greatly increasing the knowledge on the mostly regulated transcripts upon pathogen infection[9,10,11].

In the present study, the trancriptome profile of the white and red fruits of woodland strawberry inoculated with *B. cinerea* were analyzed with an attempt to elucidate the fruit response of strawberry to pathogen attack. Taking advantage of the RNA-Seq technology, the variations in the gene expression of the susceptible and resistant stages of strawberry were highlighted. The growth kinetics of *Botrytis* on fruits of *F.vesca* was also determined to identify infection strategy of the pathogen through a qPCR-detection method.

2.2. MATERIALS AND METHODS

2.2.1. Pathogen inoculum and plant material

B05.10 strain of *Botrytis cinerea* was cultured on potato dextrose agar (Sigma) and grown at 21°C with a photoperiod of 12h (NUV light)[12]. Conidial suspension of 10^5 was prepared from 12 day-old cultures.

Meanwhile, potted plants of woodland strawberry (*Fragaria vesca*) were grown under controlled conditions at 22°C until fruits. Conventional management practices were observed and the plants were maintained pesticide-free.

2.2.2. Growth kinetics of *B. cinerea* and *F. vesca*

Inoculation and collection of samples. Three types of *F. vesca* tissue were inoculated with *B. cinerea* to investigate the infection strategy of the pathogen: flower, white fruit and red fruit. For each tissue, three biological replicates were used, where each replicate is composed of 10 open flowers (7 days after anthesis (DAA)) and five white (14 DAA) and red (21 DAA) fruits. The flowers were inoculated by dropping 10µL of the conidial suspension on the receptacle, while the fruits were inoculated on the fruit surface. The same procedure was done for the control treatment where distilled water was used in place of the conidial suspension. Each tissue was enclosed in a light plastic bag to maintain high relative humidity. The samples were frozen in LN_2 and homogenized with the use of mortar and pestle at 16h, 24h, 48h and 72 h.

DNA extraction. DNA of *B. cinerea* was extracted from homogenized strawberry tissue with fungal DNA kit (NucleoSpin Plant II, MN), following the manufacturer's instructions. On the other hand, DNA extraction from strawberry samples was carried out using a CTAB-method. In a 1.5 mL tube, a starting material of 100mg tissue was added with 1 mL extraction buffer (100mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4M NaCl, 2 % w/v CTAB) and 0.4ul of β-mercaptoethanol. The sample was vortexed for 1 min and incubated for 2 hours at 65 °C in a water-bath. One mL of chloroform/octanol (24:1) was added after incubation, followed by a thorough vortexing. The aqueous phase was separated by centrifuging the sample at 8,000 rpm for 5 min. RNA digestion was performed by adding 5ul of RNAse (1 mg in 0.1 ml-1) in the newly

separated aqueous phase and incubated at 37°C for 30min. An equivalent volume of chloroform/octanol was added and mixed, and eventually centrifuged for 5 min at 8,000rpm. The upper phase was transferred in a new tube and 0.8 volume of isopropanol was mixed by gently swirling the tube. The suspension was left in room temperature and upon the appearance of the DNA precipitate, the tube was centrifuged for 22 min at 14,000 rpm. The supernatant was withdrawn and the DNA pellets were washed with 500ul 70 % ethanol and centrifuged for 5 min at 12 000rpm. Finally the supernatant was air dried on the bench for 15-45 min. DNA pellets were re-suspended in 50-100ml of sterile water and the concentration read at 260 nm and 280 nm with an ND-1000 UV spectrophotometer. Purity was estimated by the OD260/OD280 ratio.

qPCR for DNA population assay. In order to amplify target regions for the growth kinetic study of *B. cinerea* and *F. vesca,* q-PCR assays were carried out with the MX3000 thermocycler (Stratagene, CA, USA). Specific primers targeting the ribosomal region between 28S and 18S genes (intergenic spacer) for *B. cinerea* (Suarez et al., 2005) were used, while the EFF and EFR were used to amplify a 100-bp fragment of Fragaria vesca (Supplemental Table 1).

A total of 12,5ul reaction was prepared, containing 2.5ul DNA with Maxima® SYBR Green/ROX qPCR Master Mix (2X; Fermentas) and 200nM of specific forward and reverse primers. In place of the DNA template, sterile water was used for the negative control. The cycling parameters are as follows: 5 min at 95°C, 40 cycles of 15s at 95°C, 25s at 61°C, 30s at 72°C. A melting curve was established from 55°C to 90°C by change 0.5°C every 10 s. The efficiency (E) of the PCR assay was calculated using the formula, E = (10 -1/slope -1) x 100, where the slope was extracted from the curve Ct = f(log Q0) and Q0 is the initial DNA or cell population in the assay. E was expressed as percentage.

Standard curve construction for growth analysis. Following the amplification the target genes, standard curves were constructed in order quantify *B. cinerea and F. vesca* along the time-points considered in the study. Genomic DNA of *B. cinerea* and *F. vesca* were extracted from 2-week old mycelium of B05.10 grown at 21°C and untreated red strawberry fruits respectively, following the protocol described above. A five-fold dilution series of genomic DNA of the pathogen and fruit on q-PCR was performed, which allowed the creation of a standard curve by plotting the starting concentration (ng) of log of DNA against the Ct value. Eventually, the Ct values of the target DNA from the first q-PCR experiment was used to quantify the initial amount of genomic DNA through extrapolation to its corresponding standard curve. The method was both applied for *B. cinerea* and *F. vesca.* The growth of the fungal pathogen in strawberry tissue was analyzed by normalizing the fungal DNA concentration to the ratio of the initial DNA concentration of *B. cinerea* and *F. vesca.* Fungal growth was expressed as pg of *B. cinerea*/ ng of *F. vesca*.

2.2.3. RNA sequencing and data processing

Sample inoculation. Samples used for the RNA-sequencing were white (14 DAA) and red (21 DAA) fruits of *F. vesca.* The fruits were treated as described above for both *B. cinerea* and mock-inoculated samples. Three replicates composed of five fruits each were used for the following experimental set-up: white mock-inoculated (WH), white *B. cinerea* inoculated (WI), red mock-inoculated (RH) and red *B. cinerea* inoculated (RI). The fruits were detached from the plants after 24h and frozen in liquid nitrogen for subsequent analysis.

RNA extraction. Total RNA of woodland strawberry was extracted from frozen samples with mortar and pestle following the protocol described by López-Gómez and M.A.

Gómez-Lim (1992), with few modifications. The integrity of the extracted RNA was visualized in agarose gel and quantified with an ND-1000 UV spectrophotometer.

Illumina sequencing. Next generation sequencing experiments, including sample quality control, were performed by Genomix4life S.R.L. (Baronissi, Salerno, Italy). Indexed libraries were prepared from 2 μg/ea purified RNA with TruSeq RNA Sample Prep Kit (Illumina) according to the manufacturer's instructions. Libraries were quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies) and pooled such that each index-tagged sample was present in equimolar amounts, with a final concentration of 2 nM for the pooled samples. The pooled samples were subject to cluster generation and sequencing using an Illumina HiSeq 2500 System (Illumina) in a 2x100 paired-end format at a final concentration of 8 pM.

The raw sequence files generated (.fastq files) underwent quality control analysis using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), and the Illumina paired-end reads were pre-processed for both quality and adapter trimming with fqtrim (https://ccb.jhu.edu/software/fqtrim/index.shtml). With the Subread aligner13, the pre-processed reads were aligned with the Fragaria vesca genome v1.1 assembly14. Raw read counts were extracted from the Subread alignments using the featureCount read summarization program15.

2.2.4. Differential expression analysis

The differential expression analysis was performed taking advantage of the voom method16, which estimates the mean-variance relationship of the log-counts, generating a precision weight for each observation that is fed into the limma empirical Bayes analysis pipeline17. The differential expression was conducted to analyze (i) which genes responds to ripening (RH/WH), which genes responds to pathogen inoculation on

the same fruit ripening stage (RI/WI, RI/RH, WI/WH). In the four conditions generated, differentially expressed genes (DEGs) of F. vesca were filtered according to their fold change (expressed in log2) and p-value (Volcano plot). DEGs were considered significant if the fold change in log2 (log2|FC|) ≥1 with a p-value <0.01.

2.2.5. Annotation and representation of DEGs

The annotation and functional categories of the significant DEGs for every condition were analyzed with MapMan 3.5.1R218. The available mapping system of MapMan for genes related with biotic stress was also utilized to determine DEGs associated with pathogen attack19. With the PageMan tool20, a GO enrichment was performed wherein data were subjected to Wilcoxon test. Heatmaps were drawn using Gene-E, while Venny 2.121 was used to determine the over-lapping of genes in the conditions studied.

2.2.6. Validation of RNAseq data with qRT-PCR

The expression level of selected genes was analyzed to validate RNAseq results (Supplemental Table 1). cDNA of *F. vesca* was generated from 1 µg of RNA using ImProm-II Reverse TranscriptaseTM (Promega, USA), following the provided protocol. Amplification of the cDNA was performed with MX3000 thermocycler (Stratagene, CA, USA), utilizing the same proportions of the mix and cycling parameters described in the growth kinetics trial. Quantification was carried out using the relative standard curve method[22]. Resulting expression of target genes was normalized with 30s housekeeping gene in all four conditions.

2.3. RESULTS AND DISCUSSION

2.3.1. Growth kinetics of *B. cinerea* as demonstrated in different tissues of *F. vesca*

The growth of *B. cinerea* on flower and unripe and ripe fruits of woodland strawberry was investigated by quantifying the genomic DNA of the fungal pathogen in the examined tissues by qPCR. Strawberry tissues were inoculated with 100 spores of *B. cinerea* and fungal DNA was quantified in in the inoculated open flowers at 0, 16, 24, and 48 HPI and in white and red fruits at 0, 24, 48 and 72 HPI. The concentration of *B. cinerea* and strawberry DNA was derived from the quantification standard curve previously generated on serial dilution of separated fungal or strawberry DNA. The concentration values of the fungal DNA were then normalized on the strawberry DNA quantities so to derive the final concentration of *B. cinerea* DNA (pg/ng).

Results showed the nil activity of *B. cinerea* on flower and white strawberry fruits throughout the time course (Figures 1a and 1b). In contrast, the growth of *B. cinerea* in red fruits started to increase at 24 HPI and continued with an exponential growth up to 72 HPI (Figure 1c). These findings suggest that while *B. cinerea* could infect inflorescence and unripe fruits of strawberry[23], the pathogen remains in a quiescent state and resumes growth once the fruit ripens as demonstrated by the increase of the fungal growth at 24 HPI in red fruits.

2.3.2. RNA-Seq of woodland strawberry inoculated with *B. cinerea*

The quantification of fungal DNA by qPCR indicated that on fruits the 24 HPI is a critical time point since, differently from white fruits, in red fruits the pathogen initiates its exponential growth. This time point was then selected for the RNA-Seq analysis of inoculated white and red fruits. This was performed to characterize the differences in gene expression profiles of *Fragaria vesca* unripe and ripe fruits interacting with

Botrytis cinerea. Only fruit tissues (white and red) were utilized as samples for the RNA-Seq analysis in order to have a parallel comparison in the defense responses of the two ripening stages of strawberry. White and red berries of woodland strawberry were mock-inoculated or artificially inoculated with *B. cinerea,* resulting to four experimental set-ups: WH (white mock-inoculated), WI (white inoculated), RH (red mock-inoculated) and RI (red inoculated). Three biological replicates composed of ten fruits each were used for every experimental set-up. A total of 166,693,718 quality-trimmed reads were generated with the Illumina HiSeq 2500 sequencer. For each biological replicate, 63 to 73% reads were uniquely mapped to the genome of woodland strawberry (Table 1).

2.3.3. Differential expression analysis

Differential expression among the four experimental set-ups (WH, WI, RH and RI) was performed with the goal to identify the genes that change in abundance level between two groups. The comparison in the expression level of genes in *B. cinerea* inoculated *F. vesca* fruits (RI/RH and WI/WH) were performed to investigate which genes respond to fungal infection and how they differ between the two ripening stages. Meanwhile, differential expression analysis of red and white fruits of strawberry (RI/WI) with *B. cinerea* inoculation was conducted to determine how the fungal infection influences the fruit ripening process. In order to discriminate between the gene expression differences induced by the pathogen and those which account for preformed fruit defense barriers during ripening, the difference in gene expression in white and red fruits in the absence of fungal pathogen was also considered (RH/WH). The results in the variation of the gene expression levels in the conditions mentioned are presented in detail in the succeeding sections.

A total of 17,169 differentially expressed genes (DEGs) resulted upon subjecting the raw read counts to analysis. From each condition, DEGs with $\log_2|FC|>1$ with a *p*-value <0.01 were filtered, and resulting genes were considered to have significant difference in expression. The resulting data set of significant DEGs was validated through a qRT-PCR of selected genes. A correlation analysis between the gene expression from RNA-Seq and relative expression from qRT-PCR gave a Pearson coefficient of r=0.93, suggesting a close linear relationship between the two data sets (Figure 2).

A Venn diagram of the conditions revealed high number of DEGs exclusive in RI/WI, RI/RH and WI/WH, which emphasizes the distinctiveness of each condition in response to *B. cinerea* (Figure 3). On another note, around 45% (106 genes) of the DEGs in RI/WI overlap with RH/WH, which could be attributed genes related with ripening, while the remaining 46% (107 genes) exclusive to RI/WI could be associated to plant defense. Contrary to the huge number of DEGs mutual between RI/WI and RH/WH, only 5% are similar between RI/RH and WI/WH. This suggests that the fruit response to pathogen attack is largely influenced by the ripening stage of strawberry.

The succeeding sections provide elaborated results of the variation in the expression levels of genes (expressed in $\log_2|FC|$) in each condition. The first case presents the ripening of *F. vesca* and how the infection of *B. cinerea* may have altered the ripening process. Individual profiles of gene expression in RI/WI and WI/WH, together with the variation of the two conditions are also presented.

2.3.4. Differential expression of genes during *F. vesca* ripening and upon *B. cinerea* infection

Differential expression during ripening. A total of 2,790 DEGs were found to be regulated during ripening of *F. vesca* (RH/WH). From the population, 859 genes were

up-regulated in mock-inoculated red fruits over the white ones, while 1,931 DEGs were down-regulated (Figure 4).

Based from the enrichment analysis of some selected functional class, most of up-regulated DEGs during ripening of strawberry are categorized under lipid metabolism, specifically fatty acid synthesis and elongation (Figure 5a). In strawberry, fatty acids serves as precursors to synthesis of VOCs responsible for the aroma of strawberry[24,25], which the fruits develop during ripening[7]. An enrichment in secondary metabolism (Figure 5b), specifically isoprenoids, phenylpropanoids, flavonoids, and in metabolism of the hormone auxin (Figure 5c) was also noted. Similar with fatty acids, terpenoids have been identified to contribute to the flavor profile of strawberry fruits[26,27], while phenolics are responsible for the red color that strawberry develops during ripening[28]. In addition, the involvement of auxin in ripening was first recognized as the main driver for the enlargement of the receptacle[29]. On another note, cell wall functions are mainly down-regulated in red fruits over white ones (Figure 5d) due to the berry softening that occur in the ripening process. Cell-wall polymers such as cellulose, hemicelluloses and pectin are hyrdrolyzed during ripening, thereby resulting to softening of the fruit[30].

Taking the DEGs individually, the most up-regulated gene in red fruits was the *CYP94B1* (gene08229) whose transcript is 6.1 fold higher than in white fruits (Table 2). Recently, the involvement of *CYP94B1*in the catabolism of jasmonates (JAs) in plants has been studied[31,32], and was reported to modulate several aspects of fruit ripening[33]. In line, the gene *OPR3* (gene12477) that encodes for 12-oxophytodienoate reductase 3 which are involved in the biosynthesis of JAs[33,34], was also up-regulated in red fruits (Table 2). Several other genes related to hormone metabolism are among the most up-regulated in RH/WH condition, including *DFL1* (gene30702) which encodes for an indole-3-acetic acid amido synthetase and a gene (gene04535) that encodes for an auxin-

independent growth promoter. The expression of genes involved in secondary metabolism was also higher in red fruits than in white such as *CAD8* (gene09243) and *TPS06* (gene09971) by 4.8 and 4.6- fold, respectively (Table 2).

While auxins are generally associated with the enlargement of the fruit receptacle during the development stage, we found that the most down-regulated gene during ripening is the *IAA4* with a fold change of -8.7 (Table 2). This gene encodes for an auxin-responsive protein IAA4, which are short-lived transcriptional factors that function as repressors of early auxin response genes at low auxin concentrations[35]. Other down-regulated genes in the RH/WH condition reflect biological processes that occur during ripening such as the two photosynthetic genes (gene00437 and gene24657), indicating that the photosynthetic activity decrease with the ripening of fruit [36]. The expression of several genes functionally classified under development was also down regulated in red fruits. These include a gene that encodes for dormancy-associated protein-like 1 (gene29730), *MYB domain 93* gene (gene30024), and a gene that encodes for the nodulin-like 21 protein (gene32625) (Table 2). It is also interesting to note that the transcript level of a gene that encodes for a probable disease resistance protein (gene28363) was down-regulated by -5.7 folds in red fruits over the white ones.

Differential expression in ripening strawberry with B. cinerea infection. The up- and down-regulated DEGs in *B. cinerea*-inoculated red versus white *F. vesca* (RI/WI) were 131 and 102, respectively (Figure 4). In the over-representation of the functional classes, the up-regulation of hormone metabolism, signaling, together with other miscellaneous functions were noted (Figure 5c, 5h, 5i), while lipid metabolism, secondary metabolism and protein are down-regulated (Figures 5a, 5b, 5g). It could be also noted that in general, stress is down-regulated in *B. cinerea*-inoculated red fruits over the white ones. However, a more specific discrimination revealed that the down-regulation is mostly related to the

function of abiotic stress; instead, stress caused by biotic factors such as the infection of *B. cinerea* is up-regulated (Figure 5).

To evaluate the DEGs that are directly involved in pathogen response of red and white fruits infected with *B. cinerea,* the subset of genes that are exclusively regulated only in RI/WI were filtered from the rest of the population (Table 3). A putative NADP-dependent oxidoreductase gene (gene10142) is the most up-regulated in ripening fruits of *F. vesca* infected with *B. cinerea* at a 5.3-fold change. The up-regulation of this gene could be associated with the rapid oxidative burst in plants as a hypersensitive response to pathogen attack. The NADP oxidoreductase functions as one of the co-factors in the release of reactive oxygen species (ROS) during rapid oxidative burst[37].

Two pathogenesis-related (PR) protein genes (gene30565 and gene27359) were also up-regulated by 3.6 and 3.2-folds respectively in infected red fruits, which are produced in the event of pathogen-attack (Table 3). Meanwhile, the infection of *B. cinerea* in red fruits cause an up-regulation to several receptor kinase genes such as the *BRL1* (gene01192), *LRR-RLK* (gene11302), and *PR5K* (gene19540 and gene20954), signaling the activation of plant defense pathway (Table 3).

Mirroring the down-regulation of secondary metabolism in the over-representation of DEGs (Figure 5b), the transcript of two laccase genes (gene18442 and gene24296) is lower by 6 and 5.1 folds, respectively in red fruits inoculated with *B. cinerea* than in white ones (Table 3). In higher plants, the laccase have been associated with the liginification of cell walls[38] and the down regulation of the genes that encodes for this enzyme may be caused by *B. cinerea* infection. A gene encoding for the terpene synthase 21 (gene12609) was also found to have lower transcript in red inoculated fruits (Table 3). Terpene synthase are precursors to the synthesis of terpenoids, which have been associated against herbivore attack through volatile emission synthesized in the

isprenoid/terpenoid pathway[39]. In line, a recent study reported that several strawberry VOCs are related to the latency of *B. cinerea* in infected white fruits[7]. The down-regulation of *terpene synthase 21* observed in RI/WI could be associated with the VOCs that are actively synthesized in the unripe stages that eventually decreased with ripening of strawberry which eventually lead to the activation of fungal activity in red fruits. The fatty acid desaturase 7 is one of the key enzymes that lead to the synthesis of JAs[40]. The gene that encodes for the said enzyme, *FAD7* (gene07920), was also down-regulated in *B. cinerea* inoculated red fruits (Table 4), consistent to the lower resistance of ripe berries as JAs are usually associated with defense against nectotrophic pathogens[41].

2.3.5. Variation of DEGs that are mutually regulated in the ripening of healthy fruits vs. ripening of infected fruits

The ripening of fruits naturally begins with the maturation of ovary to its edible form and eventually approach senescence with the advancement of ripening. The physiology of fruit ripening, however, changes with pathogen infection.

DEGs that are commonly regulated in healthy ripening fruits against fruits ripening inoculated with *B. cinerea* were identified to analyze how fungal infection affects ripening. There are 106 DEGs that are mutual between RH/WH and RI/WI only (Figure 3). From the subset, annotated DEGs whose that exhibits remarkable difference in fold change between the two (conditions is ≥0.6 are) the only ones presented (Figure 6).

The most notable variation among the common genes in RH/WH and RI/WI was noted from *CRK28* (gene17154) that encodes for the cysteine-rich receptor-like protein kinase 28 and involved in signal transduction. The transcript level of *CRK28* is up-regulated in RI/WI where *B. cinerea* infection is present during ripening and down-regulated in ripening healthy fruits (RH/WH) as CRKs have been suggested to play important roles in

the regulation of pathogen defense and programmed cell death. Transcript accumulation of several *CRK* genes in Arabidopsis, including *CRK28* was reported to be induced by ROS that could be produced during pathogen attack[42].

The rest of the genes common between RH/WH and RI/WI functions in photosynthesis, carbon metabolism, secondary metabolism, hormone metabolism, RNA, protein, signaling and several miscellaneous functions (Figure 6). In general, the DEGs from the mentioned functional classes displayed the same trend of regulation. However, a noticeable difference in the degree of up- or down- regulation was observed in some genes, which suggest the reconfiguration of primary and secondary metabolisms upon pathogen attack.

2.3.6. Differential expression of genes in pathogen- and mock- inoculated red fruits

A total of 242 significant DEGs were found in the differential expression of mock- and pathogen- inoculated red fruits of *F. vesca* (RI/RH; Figure 4). Based from the enrichment analysis, DEGs in RI/RH condition showed a general up-regulation in lipid metabolism, secondary metabolism, stress and signaling (Figures 5a, 5b, 5e and 5h). On the other hand, hormone metabolism and protein were down-regulated in pathogen inoculated red fruits (Figures 5c and 5g).

Mature, red strawberry fruits are highly susceptible to pathogen attack[7]. To characterize DEGs that are involved in *B. cinerea* infection in red fruits alone, only the genes that were exclusively regulated in RI/RH condition were considered. Several genes encoding for heat shock proteins (HSPs) (gene 20877, gene07767, gene20885) were among the most up-regulated in *B. cinerea* infected red fruits (Table 4). HSPs are usually induced with broad ranges of abiotic stress. However, several studies have reported on the

possible correlation of HSPs and ROS, suggesting that ROS also serves as signaling molecules to induce HSPs[43].

The *ACC1* (gene22077) functions in fatty-acid metabolism and are up-regulated in pathogen-inoculated red fruits by 2.7 (Table 4). As mentioned earlier, fatty acids are precursors to VOCs synthesis, which are generally up-regulated during ripening. In this condition, however, the up-regulation of *ACC1* indicates the activity of *B. cinerea* as the growth of the pathogen is stimulated by several strawberry VOCs that increase during ripening[7]. Meanwhile, due to the infection of *B. cinerea,* the transcript of protein kinase genes (gene12633, gene22229, gene01965) are higher in infected red fruits.

The role of abscisic acid (ABA) in plant defense is complex as it could either be positive or negative regulator of defense against pathogen attack[41]. In our study, one of the most down-regulated DEGs in *B. cinerea* infected fruits is the *HVA22I* (gene15316), which encodes for abscisic acid-responsive HVA22 family protein (Table 4). Typically, the concentration of ABA increases with strawberry ripening[44], and while studies have reported that the presence of ABA induces the susceptibility of fruits to pathogens[45], no reports have shown the possible relation between the down-regulation of ABA with *B. cinerea* infection. A gibberellin-regulated protein 10 (gene02539) was also down-regulated in red infected fruits by -2.4 fold (Table 4).

2.3.7. Differential expression of genes in pathogen- and mock- inoculated white fruits

Similar with the data presented in RI/RH, the DEGs characterized in this section are only the genes that were exclusively regulated in WI/WH. The number of significant DEGs in WI/WH totals to 331 genes, with the up- and down- regulated genes having almost the same share (167 and 163 respectively).

Most of the functional classes in the over-representation of DEGs exhibited up-regulation in white fruits infected with *B. cinerea* (Figure 5). Upon looking at the profile of individual DEGs, it was found that a glycosyltransferase family protein 2 gene (gene16131) was the most up-regulated in white strawberry fruits inoculated with *B. cinerea* which is 4.3-folds higher than healthy white fruits (Table 5). In addition, two UDP-glycosyltransferases (gene26265) and (gene04355) were also up-regulated in white infected fruits. Glycosyltransferases (GTs) constitute a large family of enzymes that are involved in the biosynthesis of oligosaccharides, polysaccharides, and glycoconjugates[46]. The GTs are involved in a range of functions from structure and storage to signaling[47], but no sufficient evidence have presented its involvement in defense response or its possible involvement in the resistance of white strawberry fruits.

The resistance of unripe (white or green) strawberry fruits against pathogen infection has been described in several studies[7,48,49]. In our study, several genes that encodes for secondary metabolites were identified to have higher transcript in white infected fruits (Table 5). In the phenylpropanoid pathway, the transferase family protein gene (gene 08635) and the *shikimate O-hydroxycinnamoyltransferase* are 3.5 and 2-folds higher in white infected fruits (Table 5). In the same way, genes that function in the flavonoid pathway, *naringenin-chalcone synthase* (gene26825) and *dihydroflavonol 4-reductase* (gene15174) were also found to have higher transcript level. The up-regulation of the said genes could be related to the resistance of white strawberry fruits to fungal pathogens, which was also reported in some studies[48, 49].

The up-regulation of two genes related to ethylene biosynthesis (gene08891 and gene07935; Table 5) could be related to defense against nectotrophic pathogens[41]. Meanwhile, two 9-cis-epoxycarotenoid dioxygenase genes (gene31335 and gene30616),

which are involved in ABA biosynthesis were also up-regulated in white fruits of *F. vesca* infected with *B. cinerea.*

Among the most down-regulated genes in WI/WH condition are involved in lipid degradation. The transcript of 1-phosphatidylinositol phosphodiesterase-related gene (gene22761) was -4.4 lower in white infected fruits. Similarly, the alanine:glyoxylate aminotransferase 3 gene is -1.6 lower (Table 5).

2.3.8. Defense response between unripe and ripe fruits of *F. vesca* interacting with *B. cinerea*

To further determine the difference in the response of white and red strawberry fruits to the infection caused by *B. cinerea*, DEGs in RI/RH and WI/WH were mapped using the built-in biotic stress pathway in MapMan. From each condition, 54 genes were mapped from RI/RH (Figure 7), while 77 were identified from WI/WH (Figure 8). Details of the mapped genes could be found in Supplemental Tables 2 and 3.

A general up-regulation of defense-related genes was observed in WI/WH than in RI/RH. The quiescence of *B. cinerea* in white fruits was reflected in the down-regulation genes encoding for PR-related proteins in the biotic stress pathway of WI/WH (Figure 8, gray box). This is contrary to the up-regulated PR-related protein gene in RI/RH (Figure 7, gray box). In line with the PR-related protein genes, signaling genes are up-regulated in RI/RH (Figure 7) while WI/WH exhibits down-regulation (Figure 8) due to the active infection of *B. cinerea* in red fruits. The same result was also observed when the mutual for signaling genes upon comparing the mutual genes between RI/RH and WI/WH (Figure 9).

An interesting difference between the two maps of defense related genes is the general up-regulation of secondary metabolites in WI/WH (Figure 8). As mentioned earlier, the secondary metabolites are involved in the ontogenic resistance of white strawberry

fruits[50], either as preformed or active defense. Aside from secondary metabolites, cell wall, beta glucanase, HSPs and hormones are more up-regulated in WI/WH (Figure 8).

2.4 . SUMMARY AND CONCLUSION

The difference in the defense response of unripe and ripe fruits of woodland strawberry to *B. cinerea* infection was analyzed in this study.

The fungal growth in the fruits of woodland strawberry was first analyzed through a qPCR method. A critical point in the difference of fungal activity between the unripe and ripe stages was identified at 24 h where *B. cinerea* exhibited exponential growth in red fruits in contrast to the white berries; this finding was in accordance with previous studies suggesting the fungal susceptibility of red fruits and resistance of white ones. From this, the RNA-Seq analysis was performed on white and red fruits of *F. vesca* after 24 HPI with *B. cinerea* to highlight the difference in the gene expression of the two ripening stages.

Based from the RNA-seq, the resistance of white fruits to *B. cinerea* infection could be attributed to the general up-regulation of defense-related genes in strawberry. Higher transcript level of genes encoding for key regulators of phenolic compounds production, cell wall functions and hormone signaling were noted over mock-inoculated white fruits. Meanwhile, the quiescence of the pathogen in white infected fruits could be further supported by the down-regulation of PR-related protein genes in infected white strawberry fruits.

The susceptibility of red fruits, on the other hand, was associated to the down-regulation of several defense-related genes upon *B. cinerea* infection. Several receptor kinases that

functions in signaling and PR-related proteins were up-regulated in infected fruits, suggesting an on-going infection in the fruit caused by the fungal pathogen.

The regulation of genes during ripening of *F. vesca* was also analyzed as possibly accounting for pre-formed defense associated elements. Regulation of DEGs between red and white healthy fruits of woodland strawberry was found to be in accordance with processes that the fruits undergo during ripening, such as the up-regulation of genes encoding for the synthesis of auxin and the down-regulation of a photosynthesis-related gene. Meanwhile, the up-regulation several defense-related genes in RI/WI suggests that fungal infection leads to an alteration in the normal ripening process of strawberry fruits.

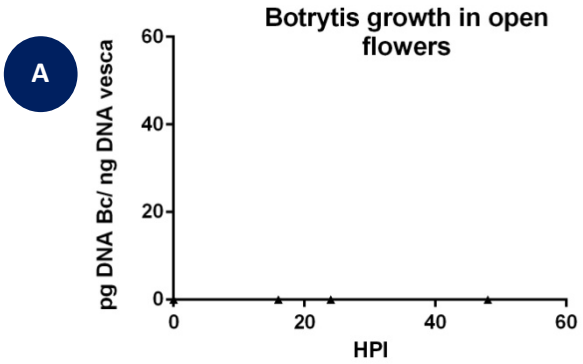
2.5. REFERENCES

1. Cadle-Davidson, L., Monitoring pathogenesis of natural Botrytis cinerea infections in developing grape berries. *American journal of enology and viticulture* **2008,** *59* (4), 387-395.
2. Williamson, B.; Tudzynski, B.; Tudzynski, P.; van Kan, J. A. L., Botrytis cinerea: the cause of grey mould disease. *Molecular Plant Pathology* **2007,** *8* (5), 561-580.
3. Jarvis, W. R., *Botryotinia and Botrytis species: taxonomy, physiology, and pathogenicity*. 1977.
4. Prusky, D., Pathogen quiescence in postharvest diseases. *Annual Review of Phytopathology* **1996,** *34*, 413-434.
5. Nagpala, E. G.; Guidarelli, M.; Gasperotti, M.; Masuero, D.; Bertolini, P.; Vrhovsek, U.; Baraldi, E., Polyphenols Variation in Fruits of the Susceptible Strawberry Cultivar Alba during Ripening and upon Fungal Pathogen Interaction and Possible Involvement in Unripe Fruit Tolerance. *Journal of Agricultural and Food Chemistry* **2016,** *64* (9), 1869-1878.
6. Jersch, S.; Scherer, C.; Huth, G.; Schlösser, E., Proanthocyanidins as basis for quiescence of Botrytis cinerea in immature strawberry fruits. *Zeitschrift fuer Pflanzenkrankheiten und Pflanzenschutz (Germany, FR)* **1989**.
7. Neri, F.; Cappellin, L.; Spadoni, A.; Cameldi, I.; Algarra Alarcon, A.; Aprea, E.; Romano, A.; Gasperi, F.; Biasioli, F., Role of strawberry volatile organic compounds in the development of Botrytis cinerea infection. *Plant Pathology* **2015,** *64* (3), 709-717.
8. Wang, Z.; Gerstein, M.; Snyder, M., RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet* **2009,** *10* (1), 57-63.
9. De Cremer, K.; Mathys, J.; Vos, C.; Froenicke, L.; Michelmore, R. W.; Cammue, B. P. A.; De Coninck, B., RNAseq-based transcriptome analysis of Lactuca sativa infected by the fungal necrotroph Botrytis cinerea. *Plant, Cell & Environment* **2013,** *36* (11), 1992-2007.
10. Gusberti, M.; Gessler, C.; Broggini, G. A. L., RNA-Seq Analysis Reveals Candidate Genes for Ontogenic Resistance in <italic>Malus-Venturia</italic> Pathosystem. *PLoS ONE* **2013,** *8* (11), e78457.
11. Alkan, N.; Friedlander, G.; Ment, D.; Prusky, D.; Fluhr, R., Simultaneous transcriptome analysis of Colletotrichum gloeosporioides and tomato fruit pathosystem reveals novel fungal pathogenicity and fruit defense strategies. *New Phytologist* **2015,** *205* (2), 801-815.
12. Gindro, K.; Pezet, R.; Viret, O.; Richter, H., Development of a rapid and highly sensitive direct-PCR assay to detect a single conidium of Botrytis cinerea Pers.:Fr in vitro and quiescent forms in planta. *Vitis - Journal of Grapevine Research* **2005,** *44* (3), 139-142.
13. Liao, Y.; Smyth, G. K.; Shi, W., The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Research* **2013,** *41* (10), e108-e108.
14. Shulaev, V.; Sargent, D. J.; Crowhurst, R. N.; Mockler, T. C.; Folkerts, O.; Delcher, A. L.; Jaiswal, P.; Mockaitis, K.; Liston, A.; Mane, S. P.; Burns, P.; Davis, T. M.; Slovin, J. P.; Bassil, N.; Hellens, R. P.; Evans, C.; Harkins, T.; Kodira, C.; Desany, B.; Crasta, O. R.; Jensen, R. V.; Allan, A. C.; Michael, T. P.; Setubal, J. C.; Celton, J.-M.; Rees, D. J. G.; Williams, K. P.; Holt, S. H.; Rojas, J. J. R.; Chatterjee, M.; Liu, B.; Silva, H.; Meisel, L.; Adato, A.; Filichkin, S. A.; Troggio, M.; Viola, R.; Ashman, T.-L.; Wang, H.; Dharmawardhana, P.; Elser, J.; Raja, R.; Priest, H. D.; Bryant, D. W.; Fox, S. E.; Givan, S. A.; Wilhelm, L. J.; Naithani, S.; Christoffels, A.; Salama, D. Y.; Carter, J.; Girona, E. L.; Zdepski, A.; Wang, W.; Kerstetter, R. A.; Schwab, W.; Korban, S. S.; Davik, J.; Monfort, A.; Denoyes-Rothan, B.; Arus, P.; Mittler, R.; Flinn, B.; Aharoni, A.; Bennetzen, J. L.; Salzberg, S. L.; Dickerman, A. W.; Velasco, R.; Borodovsky, M.; Veilleux, R. E.; Folta, K. M., The genome of woodland strawberry (Fragaria vesca). *Nat Genet* **2011,** *43* (2), 109-116.
15. Liao, Y.; Smyth, G. K.; Shi, W., featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **2014,** *30* (7), 923-930.
16. Law, C. W.; Chen, Y.; Shi, W.; Smyth, G. K., Voom: precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol* **2014,** *15* (2), R29.
17. Smyth, G. K., Limma: linear models for microarray data. In *Bioinformatics and computational biology solutions using R and Bioconductor*, Springer: 2005; pp 397-420.

18. Thimm, O.; Bläsing, O.; Gibon, Y.; Nagel, A.; Meyer, S.; Krüger, P.; Selbig, J.; Müller, L. A.; Rhee, S. Y.; Stitt, M., mapman: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal* **2004,** *37* (6), 914-939.
19. Rotter, A.; Camps, C.; Lohse, M.; Kappel, C.; Pilati, S.; Hren, M.; Stitt, M.; Coutos-Thévenot, P.; Moser, C.; Usadel, B.; Delrot, S.; Gruden, K., Gene expression profiling in susceptible interaction of grapevine with its fungal pathogen Eutypa lata: Extending MapMan ontology for grapevine. *BMC Plant Biology* **2009,** *9* (1), 1-14.
20. Usadel, B.; Poree, F.; Nagel, A.; Lohse, M.; Czedik-Eysenberg, A.; Stitt, M., A guide to using MapMan to visualize and compare Omics data in plants: a case study in the crop species, Maize. *Plant, Cell & Environment* **2009,** *32* (9), 1211-1229.
21. *Oliveros, J. C. Venny*. http://bioinfogp.cnb.csic.es/tools/venny/index.html.
22. Livak, K. J., ABI Prism 7700 sequence detection system. *User bulletin* **1997,** *2*, 929-34.
23. Viret, O.; Keller, M.; Jaudzems, V. G.; Cole, F. M., Botrytis cinerea infection of grape flowers: light and electron microscopical studies of infection sites. *Phytopathology* **2004,** *94* (8), 850-857.
24. Pérez, A. G.; Sanz, C.; Olías, R.; Olías, J. M., Lipoxygenase and hydroperoxide lyase activities in ripening strawberry fruits. *Journal of Agricultural and Food Chemistry* **1999,** *47* (1), 249-253.
25. Leone, A.; Bleve-Zacheo, T.; Gerardi, C.; Melillo, M. T.; Leo, L.; Zacheo, G., Lipoxygenase involvement in ripening strawberry. *Journal of Agricultural and Food chemistry* **2006,** *54* (18), 6835-6844.
26. Maarse, H., *Volatile compounds in foods and beverages*. CRC Press: 1991; Vol. 44.
27. Aharoni, A.; Giri, A. P.; Verstappen, F. W. A.; Bertea, C. M.; Sevenier, R.; Sun, Z.; Jongsma, M. A.; Schwab, W.; Bouwmeester, H. J., Gain and loss of fruit flavor compounds produced by wild and cultivated strawberry species. *The Plant Cell* **2004,** *16* (11), 3110-3131.
28. Halbwirth, H.; Puhl, I.; Haas, U.; Jezik, K.; Treutter, D.; Stich, K., Two-Phase Flavonoid Formation in Developing Strawberry (Fragaria × ananassa) Fruit. *Journal of Agricultural and Food Chemistry* **2006,** *54* (4), 1479-1485.
29. Nitsch, J. P., Growth and morphogenesis of the strawberry as related to auxin. *American Journal of Botany* **1950**, 211-215.
30. Payasi, A.; Mishra, N. N.; Chaves, A. L. S.; Singh, R., Biochemistry of fruit softening: an overview. *Physiology and Molecular Biology of Plants* **2009,** *15* (2), 103-113.
31. Koo, A. J. K.; Howe, G. A., Catabolism and Deactivation of the Lipid-derived Hormone Jasmonoyl-isoleucine. *Frontiers in Plant Science* **2012,** *3*.
32. Widemann, E.; Grausem, B.; Renault, H.; Pineau, E.; Heinrich, C.; Lugan, R.; Ullmann, P.; Miesch, L.; Aubert, Y.; Miesch, M.; Heitz, T.; Pinot, F., Sequential oxidation of Jasmonoyl-Phenylalanine and Jasmonoyl-Isoleucine by multiple cytochrome P450 of the CYP94 family through newly identified aldehyde intermediates. *Phytochemistry* **2015,** *117*, 388-399.
33. Creelman, R. A.; Mullet, J. E., Biosynthesis and action of jasmonates in plants. *Annual review of plant biology* **1997,** *48* (1), 355-381.
34. Wasternack, C., Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of botany* **2007,** *100* (4), 681-697.
35. Liscum, E.; Reed, J. W., Genetics of Aux/IAA and ARF action in plant growth and development. In *Auxin Molecular Biology*, Springer: 2002; pp 387-400.
36. Piechulla, B.; Glick, R. E.; Bahl, H.; Melis, A.; Gruissem, W., Changes in photosynthetic capacity and photosynthetic protein pattern during tomato fruit ripening. *Plant physiology* **1987,** *84* (3), 911-917.
37. Lamb, C.; Dixon, R. A., THE OXIDATIVE BURST IN PLANT DISEASE RESISTANCE. *Annual Review of Plant Physiology and Plant Molecular Biology* **1997,** *48* (1), 251-275.
38. Mayer, A. M.; Staples, R. C., Laccase: new functions for an old enzyme. *Phytochemistry* **2002,** *60* (6), 551-565.
39. Mithöfer, A.; Boland, W., Plant Defense Against Herbivores: Chemical Aspects. *Annual Review of Plant Biology* **2012,** *63* (1), 431-450.

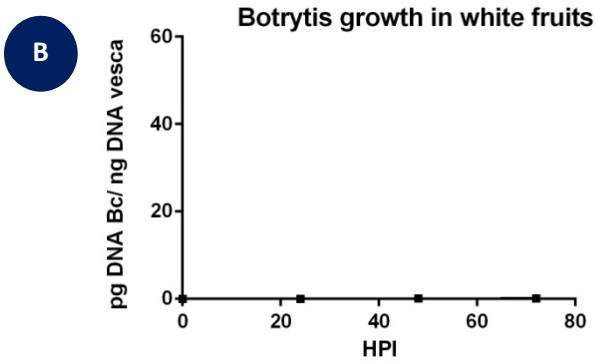
40. Stintzi, A.; Weber, H.; Reymond, P.; Browse, J.; Farmer, E. E., Plant defense in the absence of jasmonic acid: The role of cyclopentenones. *Proceedings of the National Academy of Sciences* **2001,** *98* (22), 12837-12842.
41. Bari, R.; Jones, J. D. G., Role of plant hormones in plant defence responses. *Plant molecular biology* **2009,** *69* (4), 473-488.
42. Wrzaczek, M.; Brosché, M.; Salojärvi, J.; Kangasjärvi, S.; Idänheimo, N.; Mersmann, S.; Robatzek, S.; Karpiński, S.; Karpińska, B.; Kangasjärvi, J., Transcriptional regulation of the CRK/DUF26 group of receptor-like protein kinases by ozone and plant hormones in Arabidopsis. *BMC plant biology* **2010,** *10* (1), 95.
43. Timperio, A. M.; Egidi, M. G.; Zolla, L., Proteomics applied on plant abiotic stresses: Role of heat shock proteins (HSP). *Journal of Proteomics* **2008,** *71* (4), 391-411.
44. Symons, G. M.; Chua, Y. J.; Ross, J. J.; Quittenden, L. J.; Davies, N. W.; Reid, J. B., Hormonal changes during non-climacteric ripening in strawberry. *Journal of Experimental Botany* **2012,** *63* (13), 4741-4750.
45. Mauch-Mani, B.; Mauch, F., The role of abscisic acid in plant–pathogen interactions. *Current Opinion in Plant Biology* **2005,** *8* (4), 409-414.
46. Taniguchi, N.; Honke, K.; Fukuda, M., *Handbook of glycosyltransferases and related genes*. Springer Science & Business Media: 2011.
47. Breton, C.; Šnajdrová, L.; Jeanneau, C.; Koča, J.; Imberty, A., Structures and mechanisms of glycosyltransferases. *Glycobiology* **2006,** *16* (2), 29R-37R.
48. Guidarelli, M.; Zoli, L.; Orlandini, A.; Bertolini, P.; Baraldi, E., The mannose-binding lectin gene FaMBL1 is involved in the resistance of unripe strawberry fruits to Colletotrichum acutatum. *Molecular plant pathology* **2014,** *15* (8), 832-840.
49. Weber, N.; Veberic, R.; Mikulic-Petkovsek, M.; Stampar, F.; Koron, D.; Munda, A.; Jakopic, J., Metabolite accumulation in strawberry (Fragaria × ananassa Duch.) fruits and runners in response to Colletotrichum nymphaeae infection. *Physiological and Molecular Plant Pathology* **2015,** *92*, 119-129.
50. Amil-Ruiz, F.; Blanco-Portales, R.; Muñoz-Blanco, J.; Caballero, J. L., The Strawberry Plant Defense Mechanism: A Molecular Review. *Plant and Cell Physiology* **2011,** *52* (11), 1873-1903.

FIGURES



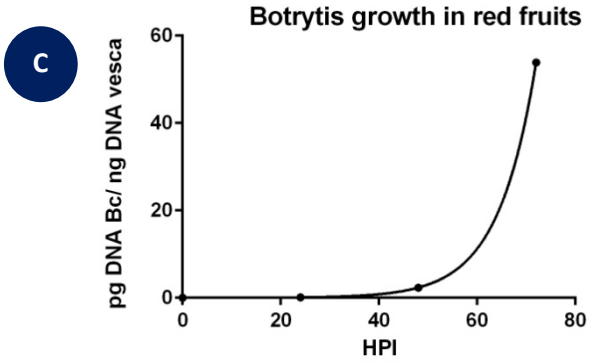
Samples	pg *Bc* /ng *Fv*
OFI-0h	0,0035
OFI- 16h	0,02844
OFI- 24h	0,03849
OFI - 48h	0,02167

$y = 0{,}0082e^{0{,}033x}$
$R^2 = 0{,}84$



Samples	pg *Bc* /ng *Fv*
WI-0h	0,0043
WI- 24h	0,0246
WI- 48h	0,11
WI- 72h	0,10

$y = 0{,}0064e^{0{,}0456x}$
$R^2 = 0{,}86$



Samples	pg *Bc* /ng *Fv*
RI-0h	0,0011
RI- 24h	0,114
RI- 48h	2,35
RI- 72h	53,9

$y = 0{,}0017e^{0{,}1476x}$
$R^2 = 0{,}99$

Figure 1. Growth kinetics of *B. cinerea* in three different tissues of *F. vesca*: A) flower; B)white fruits; C) red fruits.

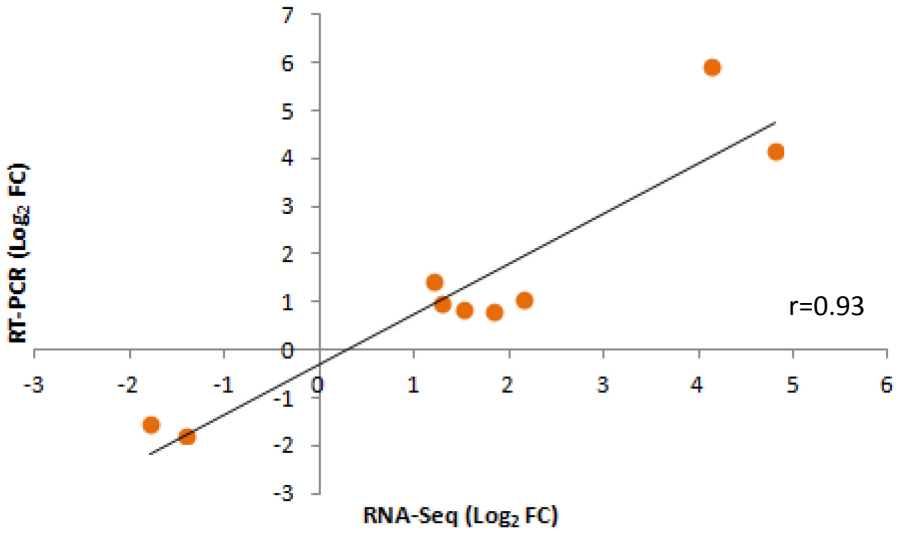


Figure 2. Correlation between the q-PCRand RNAseq with Pearson's correlation coefficient at $p<0.001$.

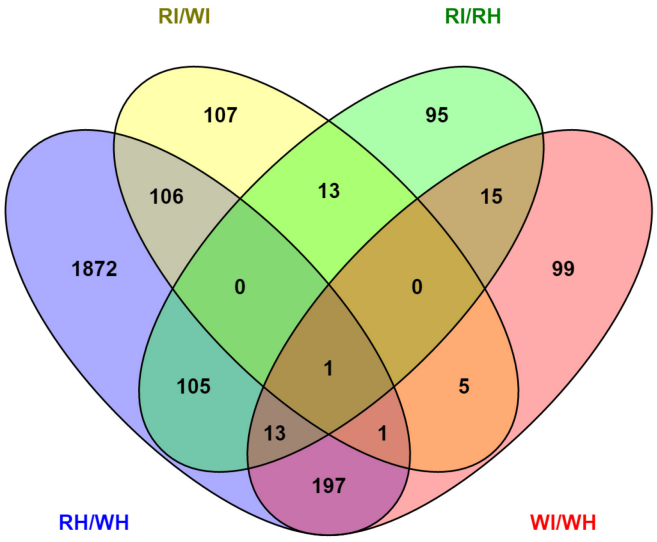


Figure 3. Exclusivity of significant DEGS (up and down-regulated) in four conditions studied: red and white healthy fruits (RI/RH), red infected and white infected fruits (RI/WI), red infected and healthy fruits (RI/RH), and white infected and healthy fruits (WI/WH).

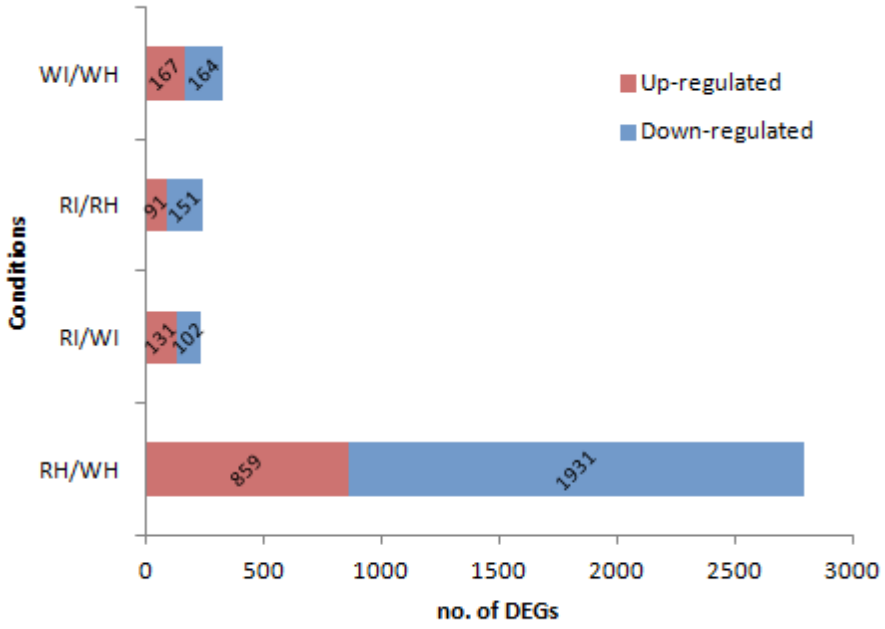


Figure 4. Number of differentially expressed genes (DEGS) and breakdown of significant DEGS. Genes are considered siginifcant if log2|FC|>1, with *p*-value <0.01.

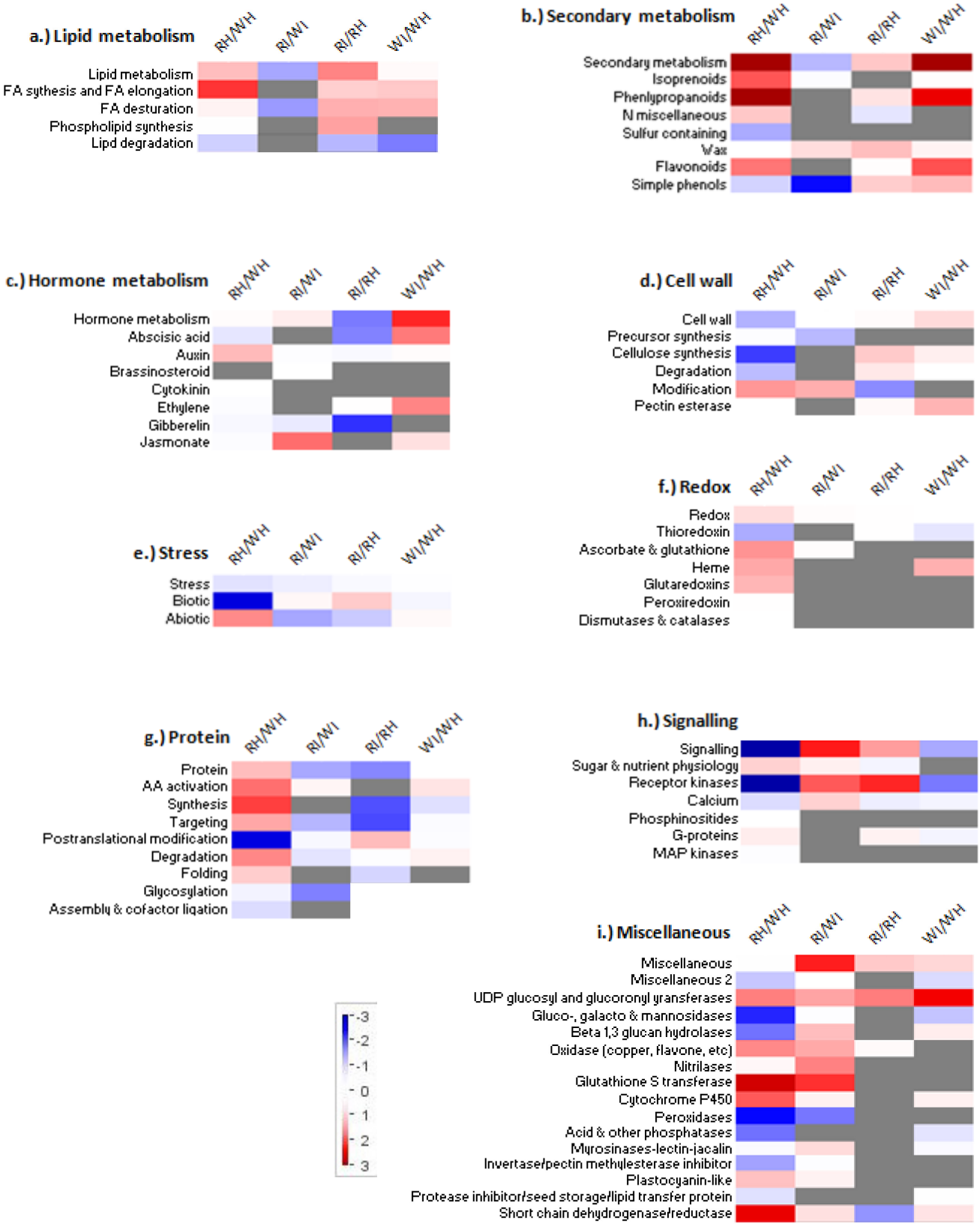
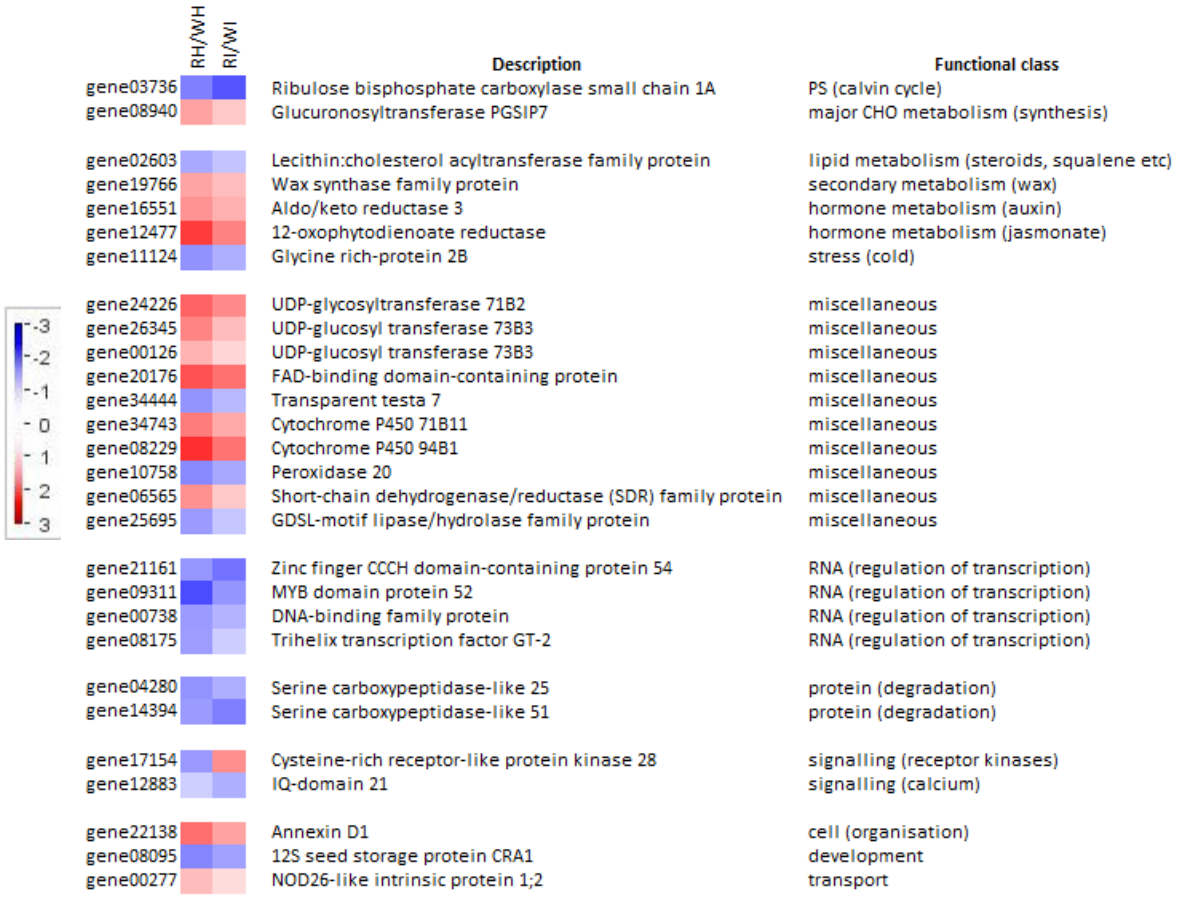


Figure 5. Enrichment of DEGs in selected functional classes as defined by PageMan. The first row of boxes represents the general representation of DEGs, while the succeeding rows denote more specific categories.



	Description	Functional class
gene03736	Ribulose bisphosphate carboxylase small chain 1A	PS (calvin cycle)
gene08940	Glucuronosyltransferase PGSIP7	major CHO metabolism (synthesis)
gene02603	Lecithin:cholesterol acyltransferase family protein	lipid metabolism (steroids, squalene etc)
gene19766	Wax synthase family protein	secondary metabolism (wax)
gene16551	Aldo/keto reductase 3	hormone metabolism (auxin)
gene12477	12-oxophytodienoate reductase	hormone metabolism (jasmonate)
gene11124	Glycine rich-protein 2B	stress (cold)
gene24226	UDP-glycosyltransferase 71B2	miscellaneous
gene26345	UDP-glucosyl transferase 73B3	miscellaneous
gene00126	UDP-glucosyl transferase 73B3	miscellaneous
gene20176	FAD-binding domain-containing protein	miscellaneous
gene34444	Transparent testa 7	miscellaneous
gene34743	Cytochrome P450 71B11	miscellaneous
gene08229	Cytochrome P450 94B1	miscellaneous
gene10758	Peroxidase 20	miscellaneous
gene06565	Short-chain dehydrogenase/reductase (SDR) family protein	miscellaneous
gene25695	GDSL-motif lipase/hydrolase family protein	miscellaneous
gene21161	Zinc finger CCCH domain-containing protein 54	RNA (regulation of transcription)
gene09311	MYB domain protein 52	RNA (regulation of transcription)
gene00738	DNA-binding family protein	RNA (regulation of transcription)
gene08175	Trihelix transcription factor GT-2	RNA (regulation of transcription)
gene04280	Serine carboxypeptidase-like 25	protein (degradation)
gene14394	Serine carboxypeptidase-like 51	protein (degradation)
gene17154	Cysteine-rich receptor-like protein kinase 28	signalling (receptor kinases)
gene12883	IQ-domain 21	signalling (calcium)
gene22138	Annexin D1	cell (organisation)
gene08095	12S seed storage protein CRA1	development
gene00277	NOD26-like intrinsic protein 1;2	transport

Figure 6. Variation of DEGs between mock- and *B. cinerea* inoculated white and red strawberry fruits (RH/WH vs RI/WI). Boxes in red are up-regulated, while those in blue are down regulated.

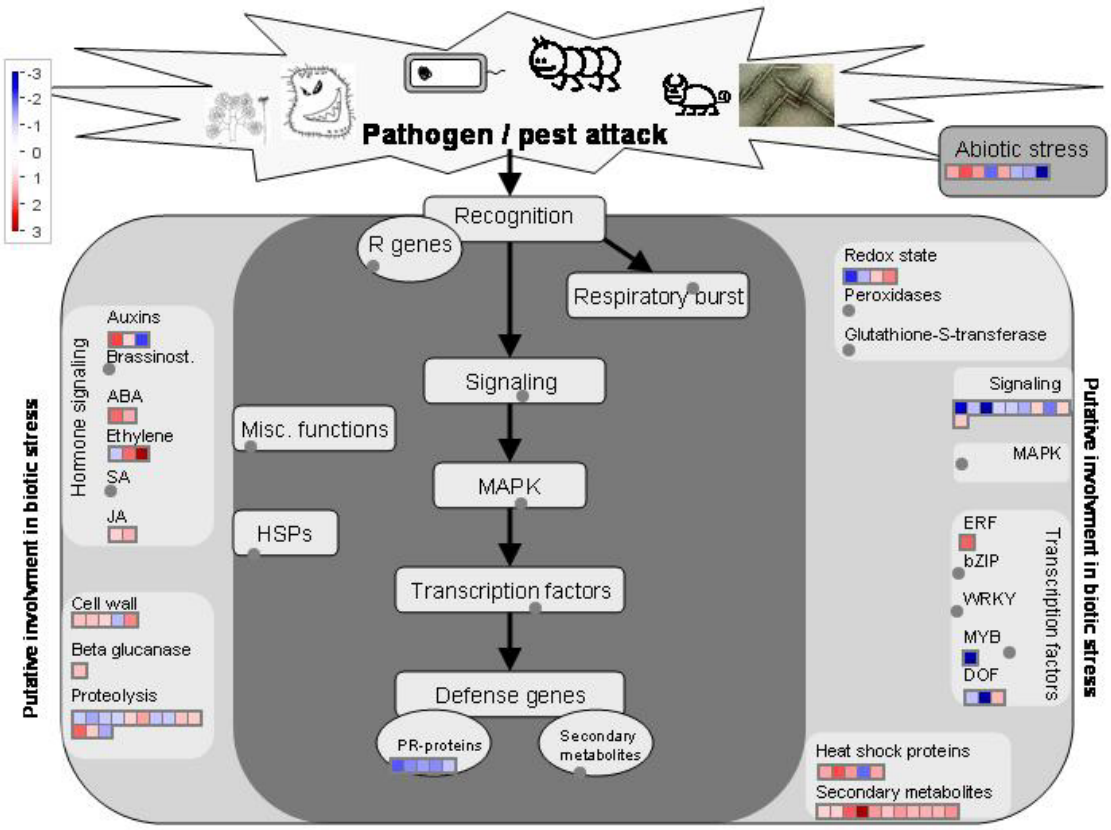


Figure 7. Mapped DEGS of RI/RH in the biotic stress pathway. The genes mapped in the dark gray box are involved in the biotic stress pathway, from recognition of the pathogen, up to the production of defense genes. Meanwhile, those that are outside the dark gray box are other genes putatively involved in biotic stress.

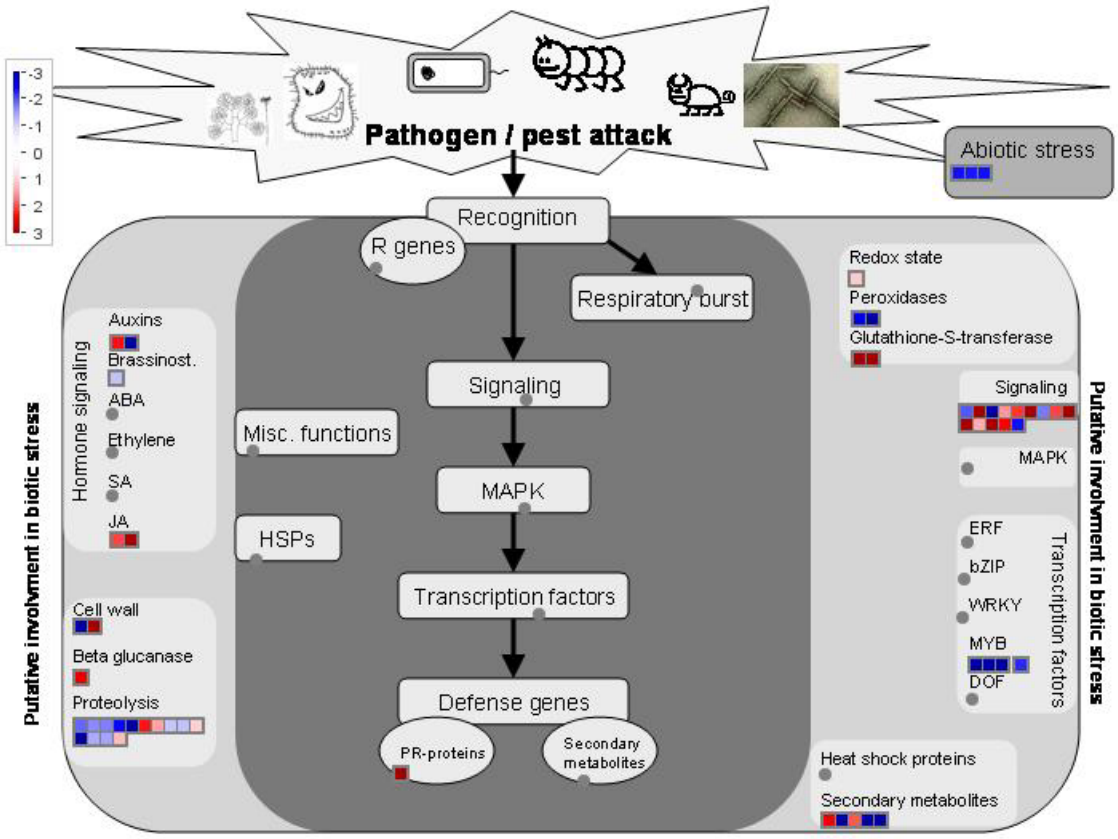


Figure 8. Mapped DEGS of WI/WH in the biotic stress pathway. The genes mapped in the dark gray box are involved in the biotic stress pathway, from recognition of the pathogen, up to the production of defense genes. Meanwhile, those that are outside the dark gray box are other genes putatively involved in biotic stress.

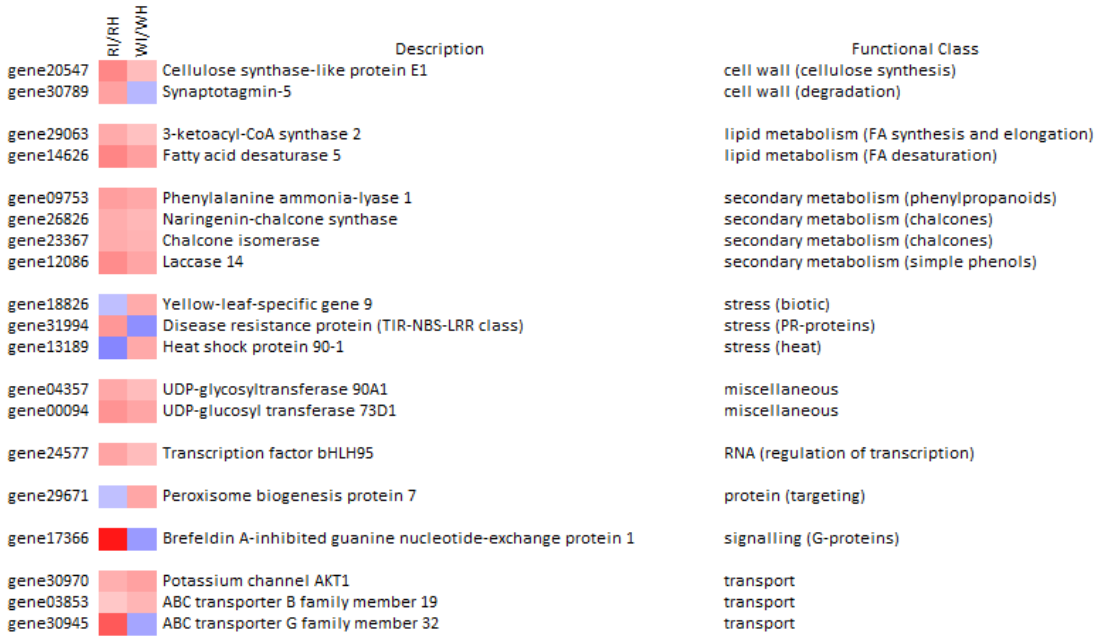


Figure 9. Defense-related DEGs similar between RI/RH and WI/WH. Boxes in red are up-regulated, while those in blue are down regulated.

TABLES

Table 1. Summary of trimmed and mapped and reads from F. vesca on all conditions studied (WH- white fruit, mock-inoculated; WI- white fruit, *B. cinerea* inoculated; RH- white fruit, mock-inoculated; RI- white fruit, *B. cinerea* inoculated).

Samples	Replicate	Quality-trimmed reads	Uniquely-mapped reads	Percentage (%)
WH	1	19,175,192	13,904,123	72.51
	2	12,193,360	8,714,291	71.47
	3	14,516,045	10,086,779	69.49
WI	1	14,706,293	9,219,560	62.69
	2	10,178,608	6,649,280	65.33
	3	13,947,656	9,977,591	71.54
RH	1	6,086,121	4,139,967	68.02
	2	13,854,723	9,877,082	71.29
	3	19,732,949	13,852,129	70.20
RI	1	14,447,738	10,259,351	71.01
	2	13,694,649	9,859,106	71.99
	3	14,160,384	9,498,992	67.08

Table 2. Subset of differentially expressed genes regulated during ripening of *F. vesca* (RH/WH).*

Gene ID	Description	Functional class	log2|FC|
UP-REGULATED			
gene08229	Cytochrome P450 94B1	miscellaneous	6.1
gene12477	12-oxophytodienoate reductase 3	hormone metabolism (jasmonate)	5.7
gene20699	Metalloendoproteinase 3-MMP	protein (degradation)	5.7
gene21848	26S proteasome regulatory complex subunit p42D, putative	protein (degradation)	5.6
gene23944	MATE efflux family protein	transport	5.4
gene30702	Indole-3-acetic acid amido synthetase	hormone metabolism (auxin)	5.3
gene08363	Ammonium transporter 1;1	transport	5.2
gene20176	FAD-binding domain-containing protein	miscellaneous (nitrilases)	5.1
gene21218	Mitochondrion-localized small heat shock protein 23.6	stress (abiotic)	5.0
gene22484	UDP-glycosyltransferase 92A1	miscellaneous (UDP glucosyl and glucoronyl transferases)	4.9
gene09243	Cinnamyl alcohol dehydrogenase 8	secondary metabolism (phenylpropanoids)	4.8
gene01310	Glycerol-3-phosphate acyltransferase 6	lipid metabolism (phospholipid synthesis)	4.7
gene19148	Putative ripening-related protein 2	development	4.7
gene09971	Terpenoid synthase 6	secondary metabolism (isoprenoids)	4.6
gene00899	Putative translation initiation factor IF-2	protein (synthesis)	4.6
gene05329	Beta-hydroxyisobutyryl-CoA hydrolase 1	amino acid metabolism (degradation)	4.6
gene09427	Glutathione S-transferase TAU 7	miscellaneous	4.6
gene30470	Alternative NADH dehydrogenase 1	mitochondrial electron transport / ATP synthesis	4.6
gene24226	UDP-glycosyltransferase 71B2	miscellaneous (UDP glucosyl and glucoronyl transferases)	4.6
gene04535	Auxin-independent growth promoter	hormone metabolism (auxin)	4.6

DOWN-REGULATED			
gene16569	Auxin-responsive protein IAA4	RNA (regulation of transcription)	-8.7
gene25908	Protein LYK2	protein (postranslational modification)	-6.7
gene00437	Cytochrome b6f complex subunit (petM), putative	PS (lightreaction)	-6.5
gene12486	Germin-like protein subfamily 3 member 3	stress (abiotic)	-6.2
gene32595	Indole-3-acetic acid inducible 14	RNA (regulation of transcription)	-6.2
gene25990	Glutathione S-transferase TAU 10	miscellaneous	-6.2
gene29730	Dormancy-associated protein-like 1	development	-6.2
gene04236	COBRA-like protein 4	cell wall (cellulose synthesis)	-6.1
gene27886	29 kDa ribonucleoprotein, chloroplast, putative	RNA (RNA binding)	-6.1
gene30024	MYB domain protein 93	development	-6.0
gene32625	Nodulin, putative	development	-5.9
gene28363	Probable disease resistance protein	stress (biotic)	-5.7
gene24657	Helicase domain-containing protein	PS (lightreaction photosystem II)	-5.5
gene03814	ATP-dependent RNA helicase DEAH13	protein (synthesis)	-5.5
gene31037	Peroxidase 25	miscellaneous (peroxidases)	-5.4
gene16711	GDSL esterase/lipase	miscellaneous (GDSL-motif lipase)	-5.4
gene22761	1-phosphatidylinositol phosphodiesterase-related protein	lipid metabolism (lipid degradation)	-5.4
gene04235	COBRA-like protein 4	cell wall (cellulose synthesis)	-5.4
gene00687	MLP-like protein 423	stress (abiotic)	-5.4
gene19713	Organic cation/carnitine transporter 3	transport (sugars)	-5.4

**Included in the list are the 20 most up- and down- regulated genes. Ranked genes with unknown annotation were excluded and replaced by genes next in rank.*

Table 3. Subset of differentially expressed genes exclusively regulated during ripening of *F. vesca* after 24 h of *B. cinerea* infection (RI/WI).*

Gene ID	Description	Functional class	log2|FC|
UP-REGULATED			
gene10142	NADP-dependent oxidoreductase, putative	miscellaneous (oxidases)	5.3
gene22897	Basic helix-loop-helix (bHLH) family protein	RNA (regulation of transcription)	4.1
gene30565	Pathogenesis-related 4	stress (biotic)	3.6
gene01192	Serine/threonine-protein kinase BRI1-like 1	signalling (receptor kinases)	3.5
gene19540	PR5-like receptor kinase	signalling (receptor kinases)	3.3
gene12671	Embryo sac development arrest 39	signalling (calcium)	3.3
gene28556	Glutamate receptor 2.9	signalling (sugar and nutrient physiology)	3.2
gene27359	Trypsin and protease inhibitor family protein	stress (PR-proteins)	3.2
gene13602	Cytochrome P450 714A1	miscellaneous	2.7
gene14817	Probable glucan endo-1,3-beta-glucosidase	miscellaneous	2.6
gene12609	Terepene synthase 21	secondary metabolism (terpenoids)	2.5
gene12773	AAA-type ATPase family protein	protein (degradation)	2.3
gene17719	Probable L-type lectin-domain containing receptor kinase	miscellaneous	2.0
gene04936	Cationic amino acid transporter 9	transport (amino acids)	2.0
gene22071	Sulfate transporter 1.3	transport (sulphate)	1.6
gene11302	Leucine-rich repeat family protein	signalling (receptor kinases)	1.6
gene21219	Glycerol-3-phosphate transporter, putative	transport	1.6
gene03185	Polyubiquitin 3	protein (degradation)	1.4
gene20954	PR5-like receptor kinase	signalling (receptor kinases)	1.4
gene29029	ATP Sulfurylase 1	S-assimilation	1.4

DOWN-REGULATED			
gene18442	Laccase 17	secondary metabolism (simple phenols)	-6.0
gene04896	Galactosyltransferase family protein	protein (glycosylation)	-5.7
gene24296	Laccase 14	secondary metabolism (simple phenols)	-5.1
gene01379	B3 domain-containing transcription factor FUS3	RNA (regulation of transcription)	-4.9
gene14551	Peroxidase 64	miscellaneous (peroxidases)	-4.8
gene07920	Fatty acid desaturase 7	lipid metabolism (FA desaturation)	-4.4
gene12094	Terepene synthase 21	secondary metabolism (terpenoids)	-4.2
gene29606	Pathogenesis-related protein, putative	stress (biotic)	-4.1
gene13577	Leucine-rich repeat transmembrane protein kinase, putative	signalling (receptor kinases)	-4.1
gene10847	Nudix hydrolase homolog 8	nucleotide metabolism	-4.0
gene01688	Auxin-responsive protein, putative	hormone metabolism (auxin)	-4.0
gene09222	Basic helix-loop-helix (bHLH) family protein	RNA (regulation of transcription)	-3.8
gene21825	Phosphatidylinositol/phosphatidylcholine transfer protein SFH3	protein (secretory pathway)	-3.5
gene13167	UDP-glycosyltransferase 88A1	miscellaneous (UDP glucosyl and glucoronyl transferases)	-3.5
gene06827	UDP-glucuronic acid decarboxylase 2	cell wall (precursor synthesis)	-3.4
gene26243	Probable acetyltransferase NATA1-like	miscellaneous (N-acetyltransferase)	-3.0
gene26226	Cytochrome P450, family 94	miscellaneous	-3.0
gene17408	Putative quinone-oxidoreductase homolog, chloroplastic	miscellaneous (oxidases)	-2.9
gene31056	Axial regulator YABBY 5	RNA (regulation of transcription)	-2.5
gene27854	Early-responsive to dehydration protein-related	stress (drought/salt)	-2.3

**Included in the list are the 20 most up- and down- regulated genes. Ranked genes with unknown annotation were excluded and replaced by genes next in rank.*

Table4. Subset of differentially expressed genes exclusively regulated in red fruits of *F. vesca* after 24 h of *B. cinerea* infection (RI/RH).*

Gene ID	Description	Functional class	log2|FC|
UP-REGULATED			
gene20877	17.6 kDa class I heat shock protein 2	stress (heat)	4.5
gene07736	CRINKLY 4-related kinase 1	protein (postranslational modification)	3.7
gene07767	17.6 kDa class I heat shock protein 2	stress (heat)	3.6
gene22871	U-box domain-containing protein 43	protein (degradation)	3.3
gene12633	Leucine-rich repeat family protein	signalling (receptor kinases)	3.2
gene21535	Flowering locus T	development	3.2
gene25287	Dead box RNA helicase 1	RNA (processing)	3.1
gene22229	Stubbelig-receptor family 2	signalling (receptor kinases)	2.9
gene28360	Bromo-adjacent homology (BAH) domain-containing protein	RNA (regulation of transcription)	2.8
gene22077	Acetyl-CoA carboxylase 1	lipid metabolism (FA synthesis and FA elongation)	2.7
gene23435	Nodulin family protein	development	2.7
gene14188	Disease resistance protein (TIR-NBS-LRR class), putative	stress (PR-proteins)	2.3
gene24479	WRKY transcription factor 6	RNA (regulation of transcription)	2.3
gene18829	Glycerol-3-phosphate acyltransferase 6	lipid metabolism (phospholipid synthesis)	2.3
gene03259	Ethylene-responsive protein -related	hormone metabolism (ethylene)	2.0
gene20885	Heat shock protein 18.2	stress (heat)	2.0
gene17000	Adagio protein 1	protein (degradation)	1.8
gene17430	Basic helix-loop-helix (bHLH) family protein	RNA (regulation of transcription)	1.8
gene01965	G-type lectin S-receptor-like serine/threonine-protein kinase B120	signalling (receptor kinases)	1.7
gene24713	C2 domain-containing protein	stress (cold)	1.7

DOWN-REGULATED			
gene22957	Proton-dependent oligopeptide transport (POT) family protein	transport (peptides and oligopeptides)	-3.5
gene15316	Abscisic acid-responsive HVA22 family protein	hormone metabolism (abscisic acid)	-2.9
gene24175	E3 ubiquitin-protein ligase RGLG1	protein (degradation)	-2.8
gene00661	Probable xyloglucan endotransglucosylase/hydrolase protein 33	cell wall (modification)	-2.4
gene02539	Gibberellin-regulated protein 10	hormone metabolism (gibberelin)	-2.4
gene01470	DNAJ heat shock N-terminal domain-containing protein	stress (heat)	-1.4
gene01771	Indole-3-butyric acid response 10	lipid metabolism (lipid degradation)	-1.4
gene05751	60S ribosomal protein L26-1	protein (synthesis)	-1.4
gene05358	60S ribosomal protein L26-2	protein (synthesis)	-1.4
gene16806	Zinc finger (C2H2 type) family protein	RNA (regulation of transcription)	-1.3
gene00230	VQ motif-containing protein	stress (drought/salt)	-1.3
gene24027	MYB domain protein 73	RNA (regulation of transcription)	-1.2
gene25489	Histone H3.2	DNA (synthesis/chromatin structure)	-1.2
gene03036	60S ribosomal protein L37a-1	protein (synthesis)	-1.2
gene12464	60s acidic ribosomal protein P1	protein (synthesis)	-1.1
gene14343	Annexin D8	cell (organization)	-1.1
gene09177	Alpha-crystallin domain of heat shock protein	stress (heat)	-1.1
gene25247	40S ribosomal protein S25-3	protein (synthesis)	-1.0

**Included in the list are the 20 most up- and down- regulated genes. Ranked genes with unknown annotation were excluded and replaced by genes next in rank.*

Table 5. Subset of differentially expressed genes in white fruits of *F. vesca* after 24 h of B. cinerea infection (WI/WH).*

Gene ID	Description	Functional class	log2|FC|
UP-REGULATED			
gene16131	Glycosyltransferase family protein 2	miscellaneous (UDP glucosyl and glucoronyl transferases)	4.3
gene08635	Transferase family protein	secondary metabolism (phenylpropanoids)	3.5
gene08891	2-oxoglutarate-dependent dioxygenase, putative	hormone metabolism (ethylene)	3.0
gene26265	UDP-glycosyltransferase 84A1	miscellaneous (UDP glucosyl and glucoronyl transferases)	2.4
gene01912	Oligopeptide transporter 7	transport (peptides and oligopeptides)	2.1
gene09721	Plasma membrabe intrinsic protein 2A	transport (major intrinsic proteins)	2.1
gene34068	Shikimate O-hydroxycinnamoyltransferase	secondary metabolism (phenylpropanoids)	2.0
gene31335	9-cis-epoxycarotenoid dioxygenase	hormone metabolism (abscisic acid)	1.9
gene07935	Gibberellin oxidase-like protein	hormone metabolism (ethylene)	1.9
gene04355	UDP-glycosyltransferase 73B1	miscellaneous (UDP glucosyl and glucoronyl transferases)	1.7
gene14626	Fatty acid desaturase 5	lipid metabolism (FA desaturation)	1.7
gene26825	Naringenin-chalcone synthase	secondary metabolism (chalcones)	1.5
gene00660	U-box domain-containing protein 19	protein (degradation)	1.5
gene30616	9-cis-epoxycarotenoid dioxygenase	hormone metabolism (abscisic acid)	1.4
gene23009	Zinc finger BED domain-containing protein	DNA (synthesis/chromatin structure)	1.3
gene15174	Dihydroflavonol 4-reductase	secondary metabolism (dihydroflavonols)	1.3
gene14601	Thaumatin	stress (biotic)	1.3
gene21537	Stearoyl-ACP desaturase, putative	lipid metabolism (FA synthesis and FA elongation)	1.3
gene21651	Thymidine kinase, putative	nucleotide metabolism	1.3
gene04304	Biotin carboxyl carrier protein 2	lipid metabolism (FA synthesis and FA elongation)	1.2

DOWN-REGULATED			
gene22761	1-phosphatidylinositol phosphodiesterase-related	lipid metabolism (lipid degradation)	-4.4
gene17365	Vacuolar protein sorting-associated protein 9b	protein (secretory pathway)	-4.2
gene01997	MYB domain protein 14 14	RNA (regulation of transcription)	-3.4
gene23280	Auxin response factor 16	RNA (regulation of transcription)	-3.2
gene29656	Copper-binding family protein	metal handling (binding)	-2.8
gene26498	NSP-interacting kinase 1	signalling (receptor kinases)	-2.8
gene01244	Ankyrin repeat family protein	cell (organisation)	-1.8
gene14171	Glycine-rich RNA-binding protein 2	RNA (RNA binding)	-1.8
gene21579	Amino acid transporter family protein	transport (amino acids)	-1.6
gene13811	Alanine:glyoxylate aminotransferase 3	lipid metabolism (lipid degradation)	-1.6
gene05732	Probable disease resistance protein	stress (PR-proteins)	-1.5
gene05181	Dehydration-induced 19 homolog 7	stress (drought/salt)	-1.5
gene15089	Metalloendopeptidase/ zinc ion binding	protein (degradation)	-1.4
gene18112	Pigment defective 149	development	-1.2
gene20593	Lectin protein kinase, putative	signalling (receptor kinases)	-1.2
gene16053	Disease resistance protein	stress (PR-proteins)	-1.2
gene00793	Riboflavin biosynthesis protein-related	co-factor and vitamin metabolism	-1.1
gene22573	Zinc finger (C3HC4-type RING finger) family protein	protein (degradation)	-1.1
gene14748	Anaphase-promoting complex, subunit 10 family	cell (division)	-1.1
gene15374	exonuclease family protein	DNA	-1.1

**Included in the list are the 20 most up- and down- regulated genes. Ranked genes with unknown annotation were excluded and replaced by genes next in rank.*

SUPPLEMENTAL INFORMATION

Supplemental Table 1. Primers used in qRT-PCR reactions.

Gene name		Primer sequences (5'-3')	Annealing temperature (°C)
GROWTH KINETIC STUDY			
Bc3F	F:	GCTGTAATTTCAATGTGCAGAATCC	61
Bc3R	R:	GGAGCAACAATTAATCGCATTTC	
EFF	F:	TGCTGTTGGAGTCATCAAGAATG	61
EFR	R:	TTGGCTGCAGACTTGGTCAC	
RNA-SEQ VALIDATION			
Cinnamyl alcohol dehydrogenase 6-related (gene09243)	F:	ATGGTGGTGTTGGGTTGTTG	60
	R:	TGAGCAACTGAACAACCGTG	
Cytochrome P450, 78A6-related (gene01896)	F:	TGACGTGAGCTTCTCTGTGT	61
	R:	CGACCCAGAAGCTCACCATA	
Myb-like DNA-binding protein MYB (gene09311)	F:	ATAACACAAGCGCCGATCAC	60
	R:	GATGACCTCGGCGTAGTAGT	
Fatty acid desaturase (gene14626)	F:	TTTCCAGCTTCCCAAATGGC	62
	R:	ATGGTACCTGTGTGTGCTCA	
ABC transporter (gene14806)	F:	CAATGAAATGGGCCTCCTCG	62
	R:	TATGCGAGGCCTTGTCAGAA	
Dirigent (gene13057)	F:	GAGGCACCGGTGATTTCAAG	60
	R:	GTTGCATAGGGAGGCCAGTA	
Glucan endo-1,3-beta-glucosidase 8-related (gene21072)	F:	ATGGTGGTGTTGGGTTGTTG	61
	R:	TGAGCAACTGAACAACCGTG	
NDH-dependent cyclic electron flow 5 (gene10423)	F:	TTGATGCTTCCTAGTGGCCT	62
	R:	GATGGTGTTGGCTTCTTGGG	
Nucleotide-diphospho-sugar transferases (gene09493)	F:	GCAGTTTTCCGCTTCCAGAA	61
	R:	CCCCAAACTGACTTGCTGAC	

Supplemetal Table 2. Mapped defense-related DEGs between *B. cinerea* and mock-inoculated red strawberry fruits (RI/RH).

Gene ID	Description	Functional Class	Log_2|FC|
PR PROTEIN			
gene27359	Trypsin and protease inhibitor family protein	stress (biotic)	3.2
HORMONE SIGNALING			
gene16551	Probable aldo-keto reductase 3	hormone metabolism (auxin)	2.3
gene01688	Auxin-responsive protein, putative	hormone metabolism (auxin)	-4.0
gene20646	DNA topoisomerase 6 subunit B	hormone metabolism (brassinosteroid)	-1.2
gene25085	12-oxophytodienoate reductase	hormone metabolism (jasmonate)	2.1
gene12477	12-oxophytodienoate reductase	hormone metabolism (jasmonate)	3.7
CELL WALL			
gene06827	UDP-glucuronic acid decarboxylase 2	cell wall (precursor synthesis)	-3.4
gene10527	Serine protease inhibitor, putative	cell wall (modification)	3.0
BETA GLUCANASE			
gene14817	Probable glucan endo-1,3-beta-glucosidase	miscellaneous (beta 1,3 glucan hydrolases)	2.6
PROTEOLYSIS			
gene23986	Subtilisin-like protease SBT1.7	protein (degradation)	-1.9
gene24861	Subtilisin-like protease SBT5.6	protein (degradation)	-1.6
gene01860	Subtilisin-like protease SBT3.8	protein (degradation)	-1.7
gene04280	Serine carboxypeptidase-like 25	protein (degradation)	-2.4
gene14394	Serine carboxypeptidase-like 51	protein (degradation)	-3.8
gene12773	AAA-type ATPase family protein	protein (degradation)	2.3

gene03185	Polyubiquitin 3	protein (degradation)	1.4
gene19865	C3H4 type zinc finger protein	protein (degradation)	-1.2
gene03647	E3 ubiquitin-protein ligase RHA2A	protein (degradation)	-1.2
gene27850	Protein binding / zinc ion binding	protein (degradation)	1.0
gene11227	Zinc finger (C3HC4-type RING finger) family protein	protein (degradation)	-3.2
gene30828	Zinc finger (C3HC4-type RING finger) family protein	protein (degradation)	-1.4
gene27352	F-box/LRR-repeat protein	protein (degradation)	-1.4
gene05741	F-box protein CPR30	protein (degradation)	1.2
REDOX STATE			
gene22311	Glutathione synthetase 2	redox (ascorbate and glutathione)	1.1
PEROXIDASES			
gene10758	Peroxidase 20	miscellaneous (peroxidases)	-2.6
gene14551	Peroxidase 64	miscellaneous (peroxidases)	-4.8
GLUTATHIONE-S-TRANSFERASE			
gene28763	Glutathione S-transferase PHI 9	miscellaneous (glutathione S transferases)	3.1
gene09427	Glutathione S-transferase TAU 7	miscellaneous (glutathione S transferases)	4.2
SIGNALING			
gene17332	Glutamate receptor 2	signalling (sugar and nutrient physiology)	-2.0
gene28556	Glutamate receptor 2.9	signalling (sugar and nutrient physiology)	3.2
gene13577	Leucine-rich repeat transmembrane protein kinase	signalling.receptor kinases.leucine rich repeat III	-4.1
gene11302	Leucine-rich repeat transmembrane protein kinase	signalling (receptor kinases)	1.6
gene00522	Polygalacturonase inhibiting protein 1	signalling (receptor kinases)	2.2
gene01192	Serine/threonine-protein kinase BRI1-like 1	signalling (receptor kinases)	3.5
gene19037	Receptor Like Protein 53	signalling (receptor kinases)	-1.8

gene30199	Leucine-rich repeat transmembrane protein kinase	signalling (receptor kinases)	2.1
gene17154	Cysteine-rich receptor-like protein kinase 29	signalling (receptor kinases)	3.3
gene19540	Cysteine-rich receptor-like protein kinase 25	signalling (receptor kinases)	3.3
gene20954	PR5-like receptor kinase	signalling (receptor kinases)	1.4
gene12671	Embryo sac development arrest 39	signalling (calcium)	3.3
gene17503	ARF-GAP domain 11	signalling (calcium)	2.5
gene12883	IQ-domain 21	signalling (calcium)	-2.4
TRANSCRIPTION FACTORS			
gene09311	MYB domain protein 52	RNA (regulation of transcription)	-3.1
gene29254	MYB family transcription factor 117	RNA (regulation of transcription)	-3.2
gene30024	MYB domain protein 93	RNA (regulation of transcription)	-5.5
gene00738	DNA-binding family protein	RNA (regulation of transcription)	-2.2
SECONDARY METABOLITES			
gene18442	Laccase 17	secondary metabolism (simple phenols)	-6.0
gene24296	Laccase 14	secondary metabolism (simple phenols)	-5.1
gene12609	Terpene synthase 21	secondary metabolism (isoprenoids)	2.5
gene12094	Terpene synthase 21	secondary metabolism (isoprenoids)	-4.2
gene19766	Wax synthase family protein	secondary metabolism (wax)	2.0
ABIOTIC STRESS			
gene11124	Glycine-rich protein 2B	stress (cold)	-2.3
gene27854	Early-responsive to dehydration protein-related	stress (drought/salt)	-2.3
gene29742	Response to water deprivation	stress (drought/salt)	-2.3

Supplemetal Table 3. Mapped defense-related DEGs between *B. cinerea* and mock-inoculated white strawberry fruits (WI/WH).

Gene ID	Description	Functional Class	Log_2|FC|
PR-PROTEINS			
gene31994	Disease resistance protein (TIR-NBS-LRR class), putative	stress (biotic)	-2.0
gene24446	Formin-like protein 13	stress (biotic)	-1.7
gene05732	Disease resistance protein (CC-NBS-LRR class), putative	stress (biotic)	-1.5
gene13372	Disease resistance protein (TIR-NBS-LRR class), putative	stress (biotic)	-1.6
gene16053	Disease resistance protein, putatuve	stress (biotic)	-1.2
HORMONE SIGNALLING			
gene31335	9-cis-epoxycarotenoid dioxygenase	hormone metabolism (ABA)	1.9
gene30616	9-cis-epoxycarotenoid dioxygenase	hormone metabolism (ABA)	1.4
gene17473	Cytochrome b561 and DOMON domain-containing protein	hormone metabolism (auxin)	2.1
gene03935	Auxin-responsive family protein	hormone metabolism (auxin)	1.0
gene31273	Unknown protein	hormone metabolism (auxin)	-2.1
gene15503	2-oxoglutarate-dependent dioxygenase, putative	hormone metabolism (ethylene)	-1.1
gene07935	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	hormone metabolism (ethylene)	1.9
gene08891	2-oxoglutarate-dependent dioxygenase, putative	hormone metabolism (ethylene)	3.0
gene03869	Allene-oxide cyclase 4	hormone metabolism (jasmonate)	1.0
gene25082	12-oxophytodienoate reductase	hormone metabolism (jasmonate)	1.3
CELL WALL			
gene20547	Cellulose synthase-like protein E1	cell wall (cellulose synthesis)	1.2
gene32087	Glycosyl hydrolase 9C2	cell wall (degradation)	1.2
gene05501	(1-4)-beta-mannan endohydrolase, putative	cell wall (degradation)	1.0
gene30789	Synaptotagmin-5	cell wall (degradation)	-1.3

gene05463	Pectinesterase 1	cell wall (pectin esterases)	1.7
BETA GLUCANASE			
gene21072	Glycosyl hydrolase family 17 protein	miscellaneous (beta 1,3 glucan hydrolases)	1.2
PROTEOLYSIS			
gene19235	CAAX prenyl protease 1 homolog	protein (degradation)	-1.1
gene15089	Metalloendopeptidase / zinc ion binding protein	protein (degradation)	-1.4
gene19372	no original description	protein (degradation)	-1.4
gene19269	RING-H2 finger B1A	protein (degradation)	1.0
gene00660	U-box domain-containing protein 19	protein (degradation)	1.5
gene09788	Zinc finger (C3HC4-type RING finger) family protein	protein (degradation)	-1.1
gene22573	Zinc finger (C3HC4-type RING finger) family protein	protein (degradation)	-1.1
gene08729	F-box protein AFR	protein (degradation)	1.2
gene03620	F-box family protein (FBL21)	protein (degradation)	1.1
gene15632	F-box family protein	protein (degradation)	1.9
gene32648	F-box family protein (FBX3)	protein (degradation)	1.1
gene31756	Autophagy-related protein 8f	protein (degradation)	-1.1
gene03347	Autophagy-related protein 8c	protein (degradation)	-1.0
REDOX STATE			
gene00630	Thioredoxin 2	redox (thioredoxin)	-2.2
gene26038	Atypical CYS HIS rich thioredoxin 4	redox (thioredoxin)	-1.3
gene12300	Thioredoxin X, chloroplastic	redox (thioredoxin)	1.1
gene18776	SOUL heme-binding-like protein	redox (heme)	1.7
SIGNALING			
gene10611	Photolyase/blue-light receptor 2	signalling (light)	1.0

gene29013	Early light-induced protein 1,	signalling (light)	1.1
gene20593	Lectin protein kinase, putative	signalling (receptor kinases)	-1.2
gene17154	Cysteine-rich receptor-like protein kinase 29	signalling (receptor kinases)	-4.4
gene02011	G-type lectin S-receptor-like serine/threonine-protein kinase	signalling (receptor kinases)	-1.0
gene26498	NSP-interacting kinase 1	signalling (receptor kinases)	-2.8
gene30694	Calmodulin-binding protein 60 B	signalling (calcium)	-1.1
gene12323	Calcium-transporting ATPase 8, plasma membrane-type	signalling (calcium)	-1.4
gene21284	Prenylated RAB acceptor 1.B1	signalling (G-proteins)	1.1
gene17366	Brefeldin A-inhibited guanine nucleotide-exchange protein 1	signalling (G-proteins)	-1.8
TRANSCRIPTION FACTORS			
gene32084	AP2 domain-containing transcription factor, putative	RNA (regulation of transcription)	1.9
gene01997	MYB domain protein 14	RNA (regulation of transcription)	-3.4
gene12818	Dof zinc finger protein DOF1.8	RNA (regulation of transcription)	-1.2
gene32187	Dof zinc finger protein DOF5.6	RNA (regulation of transcription)	-4.4
gene26135	Dof zinc finger protein DOF5.4	RNA (regulation of transcription)	1.3
HEAT SHOCK PROTEINS			
gene10105	Heat shock cognate 70 kDa protein 1	stress (heat)	1.4
gene07901	17.8 kDa class I heat shock protein (HSP17.8-CI)	stress (heat)	2.0
gene13189	Heat shock protein 90.1	stress (heat)	1.5
gene28462	DNAJ heat shock N-terminal domain-containing protein	stress (heat)	-1.9
gene03043	Early-responsive to dehydration 2	stress (heat)	1.4
SECONDARY METABOLITES			
gene12086	Laccase 14	secondary metabolism (simple phenols)	1.6
gene17114	Hydroxymethylglutaryl-CoA reductase 1	secondary metabolism (isoprenoids)	1.0
gene13195	Beta hydroxylase 1	secondary metabolism (isoprenoids)	1.0

gene09753	Phenylalanine ammonia-lyase 1	secondary metabolism (phenylpropanoids)	1.5
gene34068	Shikimate O-hydroxycinnamoyltransferase	secondary metabolism (phenylpropanoids)	2.0
gene08635	Acetyltranferase-like protein	secondary metabolism (phenylpropanoids)	3.5
gene26825	Naringenin-chalcone synthase	secondary metabolism (chalcones)	1.5
gene26826	Naringenin-chalcone synthase	secondary metabolism (chalcones)	1.3
gene23367	Chalcone isomerase	secondary metabolism (chalcones)	1.4
gene15174	Dihydrokaempferol 4-reductase	secondary metabolism (dihydroflavonols)	1.3
gene19765	Long-chain-alcohol O-fatty-acyltransferase family protein	secondary metabolism (wax)	1.2
ABIOTIC STRESS			
gene10105	Heat shock cognate protein 70-1	stress (heat)	1.4
gene07901	17.8 kDa class I heat shock protein (HSP17.8-CI)	stress (heat)	2.0
gene13189	Heat shock cognate protein 90.1	stress (heat)	1.5
gene28462	DNAJ heat shock N-terminal domain-containing protein	stress (heat)	-1.9
gene03043	Early responsive to dehydration 2	stress (heat)	1.4
gene18733	Universal stress protein (USP) family protein	stress (cold)	-1.3
gene05181	Hypersensitive to red and blue 1	stress (drought/salt)	-1.5
gene04560	Universal stress protein (USP) family protein	stress (unspecified)	-3.7

Chapter 3

Polyphenols Variation in Fruits of the Susceptible Strawberry cv. Alba during Ripening and upon Fungal Pathogen Interaction and Possible Involvement in the Unripe Fruit Tolerance

Polyphenols Variation in Fruits of the Susceptible Strawberry cv. Alba during Ripening and upon Fungal Pathogen Interaction and Possible Involvement in the Unripe Fruit Tolerance

ABSTRACT

Strawberry (*Fragaria* × *ananassa*) fruit contains high concentrations of health-promoting phenolic compounds, playing important roles for the fruit ontogenic tolerance to fungi. In the highly susceptible cultivar Alba, the two major strawberry fungal pathogens, Colletotrichum acutatum and Botrytis cinerea, displayed disease symptoms only at red ripe stages because immature fruits are tolerant to diseases. We analyzed and compared the variation of 47 polyphenols in the surface of unripe and ripe Alba fruits upon 24 and 48 h of C. acutatum and B. cinerea infection or mock inoculation. Significant alteration in phenolic content was detected only in white infected fruit, with differences specific for each pathogen. The expression analysis of phenylpropanoid, flavonoid, and shikimate pathway genes showed in only a few cases correlation with the relative metabolite abundance. The alteration in phenolic content and the lack of consistency with gene expression data are discussed in light of previously reported metabolome data of different susceptible and resistant strawberry genotypes.

KEYWORDS: *Fragaria* x *ananassa*, polyphenols, *Botrytis*, *Colletotrichum*, fungal quiescence, latent infection, ripening

3.1. INTRODUCTION

Besides its economic importance worldwide, strawberry *(Fragaria x ananassa)* crop is attracting much attention because of its nutritional benefits for human health since the fruit contains high levels of polyphenols with beneficial antioxidant, antibiotic and anti-inflammatory properties[1-3]. These phenolic compounds are present in strawberry fruits with concentration up to 40 mg per 100 g of fresh fruit[4]. Strawberry one of the most consumed fruit in the world. However, the soft and fleshy nature of strawberry fruit makes the crop highly perishable and susceptible to diseases[5], including a number of emerging threats affecting crop production in field[6- 8].

Two of the most important diseases affecting strawberry fruits are anthracnose caused by *Colletotrichum acutatum*[9] and grey mold caused by *Botrytis cinerea*[10]. These pathogens are particularly insidious because, although infection can occur in flowers or immature stages of fruits, the disease symptoms are manifested at mature red stages when the fruit has reached its highest value. This phenomenon is attributed to the physicochemical composition of immature fruits, which is not suitable for fungal growth; here pathogens can germinate and eventually develop early stages of colonization, but then they soon arrest their growth and survive as quiescent until the fruit is fully ripe. Once the fruit ripens, pathogens resume from the infection process and quickly invade the whole fruit and develop rot symptoms[11]. The infection strategy that *C. acutatum* displays on strawberry fruit has been studied on the susceptible cultivar Alba: at 24 h post-inoculation, pathogen growth is arrested in white fruits, whereas the pathogen already penetrated through intramural colonization in red fruits[12].

The involvement of secondary plant metabolites such as phenylpropanoids, flavonoids, benzoic acids and hydrolysable tannins in defense during fruit development is well established. These compounds, collectively known as polyphenols, are produced during

plant growth and development and are also induced when plants are under stress. For instance, fungal growth inhibition is linked to the accumulation of polyphenols during pathogen infection, and is cited as one of the possible determinants for the low susceptibility of unripe fruit to fungal rots[13]. In particular for fruits of *Fragaria* spp., flavonoids such as proanthocyanidins and flavan-3-ols are actively synthesized in unripe fruit stages and diminish as the fruit ripens causing an increased fruit susceptibility[14]. For instance, the concentration of proanthocyanidins is correlated with varying susceptibility of different strawberry cultivars to *B. cinerea*[14]. Furthermore, it was proven that catechin, a major flavan-3-ol in immature strawberry receptacles, plays a key role in determining the infection strategy of *B. cinerea* from flower infection to ripe fruit colonization[15]. Although no direct evidence of the involvement of phenolic compounds in the low susceptibility of immature strawberries to *C. acutatum* has been reported so far, the role of epicatechin in inhibiting the growth of *Colletorichum gloeosporoides* in avocado has been reported[16]. In addition, a recent study[17] demonstrated that both susceptible and resistant strawberry cultivars exhibited a significant increase of flavan-3-ols and ellagic acid conjugates upon infection with *Colletotrichum nymphaeae.* Accumulation of ellagitannins was reported also in strawberry leaves infected with *Colletotrichum fragariae*. The isolated compound, sprayed on plants induced resistance against *Colletotrichum acutatum* and *Xanthomonas citri*[18].

With this knowledge, the present study was performed in order to evaluate the involvement of phenolic compounds in the ontogenic resistance of immature strawberry fruits to *C. acutatum* and *B. cinerea* in a susceptible genetic background. For this, a UHPLC system coupled with triple quadruple mass spectrometer (UHPLC MS/MS) and a spectrophotometric assay were used to quantify polyphenols in white and red strawberry fruits at 24 and 48 h post-infection to highlight the early fruit response

determinants. Expression of the genes encoding for different enzymes involved in the synthesis of phenolic compounds was also monitored in order to study and correlate the transcriptional and metabolic responses. The results allowed us to identify the compounds mostly responsive to each pathogen.

3.2. MATERIALS AND METHODS

3.2.1. Pathogens and plant material

Isolate Maya-3 of *Colletotrichum acutatum*[12] was grown on potato dextrose agar (Sigma) at 20°C for ten days. Meanwhile, B05.10 strain of *Botrytis cinerea* was grown on same conditions and exposed under UV light to facilitate sporulation.

Strawberry plants of cv. Alba, highly susceptible to several pathogens such as *Colletotrichum spp.*[12] and *Botrytis cinerea*, were tunnel-grown under conventional management practices in a local orchard (Cesena, Italy) and were maintained pesticide-free. The fruits were harvested 20 and 30 days after anthesis for the white and red berries, respectively and immediately brought to the laboratory for experiments. Fruits at white and red stages of ripening were used in order to compare fully tolerant and susceptible conditions.

3.2.2. Experimental set-up

For phenotypic assessment of the susceptibility of the strawberry Alba, three replicates of 10 fruits for each ripening stage were used and pathogen inoculated by dipping the fruits at a conidial suspension of 10^5/mL for 1 min. Another batch with the same number of fruits was dipped in water, serving as the control. Fruits were arranged in a lined-container and wrapped with polyethylene bags to maintain the relative humidity at level

of 70%. Incidence of *C. acutatum* and *B. cinerea* on the fruits were monitored daily for seven days (red fruits) and for 14 days (white fruits). Disease incidence was expressed as the percentage of infected fruits over the total number of samples in every treatment. Fruits were considered infected upon manifestation of symptoms.

For biochemical and molecular analyses of the phenolic content and gene expression profile, three replicates of 15 fruits for each ripening stage and for each type of pathogen infection were used. Fruits were inoculated as described above. After 24 and 48 h of inoculation, the fruit surface (3 mm thick) was excised and immediately frozen in LN_2. These time points were chosen based on previous histological and microarray analysis of the *C. acutatum* infection on strawberry fruits[12].

3.2.3. Metabolomic analysis

Extraction of polyphenols. Phenolic compounds were extracted by homogenizing 30 g of strawberry fruits with a 50 mL acetone/water mixture (70:30 v/v) for 90 seconds. This was done twice, after which the volume adjusted to 120 mL. The extracts were centrifuged and the supernatant were stored in -20°C for subsequent analysis.

Targeted analysis of polyphenols with UHPLC-MS/MS. Samples for fruit polyphenol analysis were prepared accordingly as described by Gasperotti et al.[4]. An aliquot of 1 mL of the extract was initially dried and re-suspended in a 1 mL methanol/water mixture (50:50 v/v) with 1 mg/L rosmarinic acid as the internal standard. The analysis were performed with an ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS) (Waters, Miliford, MA, USA) system coupled with triple quadruple (TQ) mass spectrometer[19]. The acquisition method was slightly modified, as two additional classes of compound were added to the analytical run: anthocyanins (eight compounds) and ellagitannins (four compounds). Ultra-performance liquid

chromatography was performed employing a Waters Acquity UPLC system (Milford, MA, USA) coupled to a Waters Xevo TQMS (Milford, MA, USA) working in ESI ionization mode. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column 1.8 μm, 100 mm × 2.1 mm (Milford, MA, USA), kept at 40°C, with two solvents: A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid). Calibration curves were prepared ranging from 5 x 10^{-3} to 20 mg/L with a proper relative standard reference for each analyzed compound. Information on MRM parameters are reported in Gasperotti et al.[4] and Vrhovsek et al.[19].

Bate Smith spectroscopy of high molecular weight proanthocyanidins (HMWP). HMWP were analyzed separately through the Bate-Smith assay following the method described by Rigo et al.[20].

3.2.4 RNA preparation and qRT-PCR Analysis

RNA was extracted from frozen fruits samples upon grinding with mortar and pestle as described by Lopez-Gomez and Gomez-Lim[21], with minor modifications. The extracted RNA was visualized in agarose gel to determine their integrity and quantified with an ND-1000 UV spectrophotometer. First-strand cDNA was synthesized from 1 μg of total RNA in a reaction of 20 μL with oligo-d(T) 17 as a primer using ImProm-II Reverse TranscriptaseTM (Promega, USA), following the provided protocol. The expression of genes belonging of the phenylpropanoid pathway, such as the *phenylalanine ammonia lyase* (*PAL*) and *cinnamate 4-hydroxylase* (*C4H*), and of the flavonoid pathway, such as *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*FHT*), *dihydroflavanol 4-reductase* (*DFR*) *anthocyanidin synthase* (*ANS*), *flavonoid-3-O-glucosyltransferase* (*FGT*), *leucoanthocyanidine reductase* (*LAR*), *anthocyanidin reductase* (*ANR*), and the expression level of a shikimate pathway gene, the *shikimate*

dehydrogenase (*SDH*) and *FaMYB1* transcription factor gene, a negative regulator of the flavonoid biosynthesis, were analyzed. These genes were amplified using strawberry primers specific to the most expressed gene isoforms[22]. The expression of target genes were normalized with elongation factor 1α (ef-1α) housekeeping gene. Amplifications were run in MX3000 thermocycler (Stratagene, CA, USA). Each reaction mixture contained: 1X of Platinum Sybr-Green Master mix (Invitrogen, Milan, Italy), 5 µM of each primer, 3.25 µl of nuclease-free water, and 2.5 µl of 1:12.5 dilution of cDNA, in a total volume of 12.5 µl. The following cycling conditions were used: an initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, annealing step at a specific temperature for 30 sec (Supplemental Table 1), and an extension step at 72°C for 30 sec. Melting curve analysis was performed by monitoring the fluorescence from 55°C to 95°C every. Data were analyzed using MXPro QPCR Software version 3.0 (Stratagene, USA). Quantification was carried out using the relative standard curve method[23]. For each sample, three independent biological replicates were made and each replicate was run three times.

3.2.5. Statistical Analysis

The data were processed using the statistical package STATISTICA (version 7, Statsoft Inc., Tulsa, OK, USA). All metabolomic data were initially analyzed with factorial ANOVA at $p<0.05$ to determine the significant variations between the ripening stage, pathogen and post-inoculation time. Subsequently, one-way ANOVA was performed on the resulting polyphenols from the earlier analysis. Expression levels of genes from qRT-PCR were also subjected to one-way ANOVA. Separation of means was performed with Duncan's Multiple Range Test (DMRT) at $p<0.05$. For the gradient correlation of transcript levels and metabolite concentration, Z scores were computed against the

average of each gene expression or compound in all the conditions studied. Meanwhile, the heatmap was drawn with R software (version 3.2.2), accompanied by a G-plot library.

3.3. RESULTS

3.3.1. Fruit susceptibility

No visible symptoms of infection by *Colletotrichum acutatum* and *Botrytis cinerea* were observed at 24 and 48 h post-inoculation (HPI) in both white and red strawberries (cv. Alba) compared to mock-inoculated (dipped in water) ones (Figure 1, Supplemental Figure 1). In red fruits, symptoms of dark-brown lesions appear at 3 days post-inoculation (DPI) of *B. cinerea* and at four DPI of *C. acutatum*, with recorded incidence of 27% and 20%, respectively (Figure 1). At 7 DPI, the lesions spread on most of the fruit surface in both types of infected red samples and, in fruits infected with *B. cinerea,* fungal egression occurs (Supplemental Figure 1). On the contrary, no symptoms were observed in inoculated white fruits at the same time points (Figure 1, Supplemental Figure 1) or later up to 14 days (not shown) despite the manifested red pigmentation.

3.3.2. Polyphenol profile in strawberry fruits during ripening and upon pathogen infection

A total of 47 compounds were detected from the surface of white and red strawberry fruits in inoculated or control conditions (Supplemental Table 2). Forty-six compounds were analyzed via targeted analysis with UHPLC MS/MS, while HMWP were quantified through Bates Smith spectrophotometric assay. The analyzed phenolic compounds belong to the following classes: benzoic acids and their derivatives, phenylpropanoids,

stilbenes, dihydrochalcones, flavones, flavonone, flavan-3-ols, flavonols, anthocyanins, ellagitannins, and proanthocyanidin (Supplemental Table 2).

All these classes were found to vary upon ripening and pathogen infection (Figure 2). Considering the metabolite classes, proanthocyanidins are the most concentrated group in both white and red fruits of Alba. In unripe strawberries, ellagitannins are the second most concentrated class, which is typical of this stage. On the other hand, anthocyanins are more concentrated in red fruits than ellagitannins due to ripening. Upon pathogen infection, an increase in polyphenol content is specifically noted in white fruits (Supplemental Table 2).

A more specific evaluation of individual polyphenols shows that the concentration of the compounds is affected by the ripening stage and pathogen infection (Figure 2). Upon ranking the abundance of compounds in each condition, it is revealed that HMWP are the most abundant polyphenol in strawberry fruits regardless of the ripening stage, presence and type of pathogen or the time after inoculation. Meanwhile, pelargonidin-3-glucoside is the second most concentrated polyphenol in all conditions of red fruits and in mock-inoculated white fruits at 48 HPI. This compound is the main anthocyanin in strawberry. Moreover, the increase of the ellagitannin casuarictin over pelargonidin-3-glucoside in infected white fruits of Alba suggests that this compound is involved in infection related response (Table 1).

On the basis of other ranked compounds, it is apparent that the majority of polyphenols in red fruits do not exhibit differences upon pathogen infection. On the contrary, the abundance of the compounds in unripe Alba is greatly influenced by the presence of *C. acutatum* and *B. cinerea*. For instance, the flavan-3-ol catechin shows higher abundance in pathogen-inoculated white fruits than in control.

3.3.3. Variation of different classes of polyphenols in unripe and ripe strawberries upon fungal infection

To analyze the influence of the fruit ripening stage (R), of the fungal pathogen species (P) and of the post-inoculation time (T) on the variation of each polyphenol, a factorial ANOVA was performed on all analyzed compound (Supplemental Table 3). Ten of 47 (21%) phenolic compounds were found to significantly vary between the pathogens, whereas 37 (79%) and 22 (47%) are significantly different between the ripening stage and the infection time, respectively (Table 2). Upon consideration of all three variables, 12 (26%) of the phenolic compounds were found to be significantly influenced (Table 2, PxRxT). These belong to the groups of benzoic acids and derivatives, ellagitannins, flavonols, flavan-3-ols, and proanthocyanidin.

Flavonols. Considering the total concentration, no significant variation of flavonol compounds was detected in strawberry fruits among different ripening stages or pathogen inoculation (Figure 2 and Supplemental Table 2). On the other hand, significant interactions between the pathogen, ripening stage and time were highlighted upon analysis of individual flavonol compounds (Table 2). In particular, the concentration of kaempferol-3-rutinoside is 73% higher in red fruits than in white. However, both *C. acutatum* and *B. cinerea* caused a decrease of this compound at 24 HPI infected red fruits, which could possibly be related to the susceptibility of red berries. Meanwhile, a significant accumulation of isorhamnetin-3-rutinoside is measured in white fruits inoculated with *B. cinerea* at 48 HPI (Figure 3).

Flavan-3-ols. Consistent with previous reports, control strawberry fruits exhibited a decrease in flavan-3-ols during ripening (Figure 2 and Supplemental Table 2). A decrease in flavan-3-ols is also detected in control white fruits from 24 to 48 HPI, possibly as a postharvest effect on phenolic metabolisms. The infection with *C. acutatum*

and *B. cinerea* influences the concentration of these polyphenols only in white fruits: *C. acutatum* infection does not lead to the flavan-3-ol decrease from 24 to 48 HPI, whereas *B. cinerea* first exhibited a decrease (24 HPI) and then an accumulation at 48 HPI. On the contrary, no variation is detected in pathogen inoculated red fruits compared to control.

Taken individually, catechin, procyanidin B1 and procyanidin B3 vary similarly to the total flavan-3-ols (Figure 3). The accumulation of flavan-3-ols exclusively in white infected fruits suggests that these polyphenols could be determinant for the low susceptibility of white fruit to pathogens.

Proanthocyanidins. The levels of strawberry proanthocyanidin found in our study decrease with ripening, similar to previous results[14]. Within white fruits, the concentration of HMWP does not significantly vary upon 24 HPI with both pathogens. A significant increase of HMWP is detectable only in white fruit as late response (48 HPI) with both pathogen species. No significant differences were found in red fruits (Figure 2 and 3).

Benzoic acids and derivatives. Although present in relatively smaller concentrations, benzoic acids and their derivatives were found to have significant differences between fruit ripening stage, type of pathogen and time (Table 2 and Supplemental Table 2). In general, the concentration of total benzoic acids is significantly higher in white fruits than the red ones. Interestingly, this variation is independent from the pathogen infection in all the condition tested, except for *B. cinerea* inoculated white fruits at 24 HPI, where a significant decrease from control is measured. In red fruits, on the other hand, the concentration of total benzoic acids and derivatives does not show any significant variation among the condition tested except for 48 HPI *B. cinerea,* where these compounds are found significantly increased (Supplemental Table 2).

Of the eight analyzed compound from the class, *p*-hydroxybenzoic acid and 2,6-dihydroxy benzoic, methyl gallate, and catechol acid were found to be the only ones significantly contributing to the benzoic acids variation described above (Figure 3). It is noteworthy, that methyl gallate concentration increases in *B. cinerea* infection in white fruits at 24 HPI, which is in contrast to the general trend (Figure 3).

Ellagitannins. Total ellagitannins significantly decreases from 24 to 48 HPI, in both white and red control fruits of about 61 and 54%, respectively (Figure 2 and Supplemental Table 2). Infection with both fungal pathogens differently influences fruit ellagitannin concentration, depending on the ripening stage. In white fruits, ellagitannins remain stable from 24 to 48 HPI and do not decrease as in control. In contrast, a significant decrease in these compounds are detected in 24 HPI *B. cinerea* infected red fruits, but not in *C. acutatum* ones.

In the present study, casuarictin appears as the major ellagitannin compound, representing more than half of the total ellagitannin concentration in all the treatment conditions (Figure 2). Both casuarictin and agrimoniin change their concentration mirroring the total ellagitannins variations described above (Figure 3 and Supplemental Table 2).

3.3.4. Expression of genes of the polyphenol pathway during ripening and upon pathogen infection

The expression of genes encoding for enzymes involved in polyphenols biosynthesis was analyzed by RT-qPCR. Genes regulating the synthesis of flavan-3-ols and proanthocyanidins, such *ANS* and *LAR* showed higher expression at unripe stages, both infected and not (Figure 4), whereas the *FHT* gene, serving in the synthesis of early flavonol precursors, increases its expression in red control fruits. Interestingly, the

expression of *FaMYB1* does not differ from white to red control fruits. In red fruits, the presence of pathogen infection does not seem to significantly alter the abundance profile of the transcript level of most of the genes. Particular decrease in the expression of few genes is exhibited only at 24 HPI. Upon *C. acutatum* infection, the transcript levels of *DFR*, which is involved in flavan-3-ols synthesis, and *FHT,* decrease, while *FGT* genes regulating anthocyanin production, together with *ANS,* decrease upon infection of *B. cinerea*. Meanwhile, in white infected fruits, only *ANS* exhibited a decrease in expression at 24 HPI in response to both types of pathogens. Contrary to this, major differences in gene expression are detected at 48 HPI: phenylpropanoid gene *C4H,* and the flavonoid genes *CHI* and *CHS* (Figure 4) significantly increase their transcript level in infected fruits independent of the type of pathogen. Similarly, *ANS* and *LAR* show a clear activation in transcription upon infection with both pathogens (Figure 4).

No significant differences in the expression of *PAL,* the gene encoding for the first enzyme in the phenylpropanoid pathway, were detected during ripening or upon infection. Similarly, no major differences are detected in the expression of *SDH,* the gene that regulates benzoic acid and ellagitannin biosynthesis[24] (Figure 4).

3.3.5. Metabolite and transcript profiles correlation

With the purpose of highlighting the possible correlations, the Z scores of the phenolic compound concentration and of the expression level of the genes involved in their synthesis were calculated. It should be noted that the Z scores, represented as color changes, are standardized on the average value of each condition and do not take into account statistical significance (Figure 5). The phenolic compounds showing the most important variation along with the treatment condition or those with high concentration were considered (Supplemental Table 2).

Considering that the differences detected in the expression of *PAL* gene is not significant in any of the condition tested, and *C4H* only varies significantly only in white 48 HPI inoculated fruits, weak correspondence between gene expression and metabolite concentration is apparent for the phenylpropanoid pathway (Figure 5). With respect to the flavonoid pathway, the expression of *FHT* gene does not seem to influence the concentration of these metabolites. The expression of *FGT* gene, regulating the synthesis of anthocyanins, is not correlated likewise with any of the examined pelargonidin and cyanidin compounds. On the other hand, the expression of *LAR, ANS,* and *ANR*, but not *DFR*, reflects fairly close the different concentrations of catechin, procyanidins and HMWP (Figure 5).

Finally for to the shikimate pathway, the higher expression of *SDH* gene in white fruits correlates with the higher concentration of most of the benzoic acid metabolites and ellagitannins (Figure 5).

3.4. DISCUSSION

The evolutionary role of fruit during ripening is dual because, initially, it protects the embryo until seed becomes lignified, and later promotes seed dispersal into the surrounding environment. For this, unripe fruits have very efficient physical and chemical defense mechanisms so that the majority of fungal pathogens attacking these stages stop their growth and become quiescent. In contrast, attractive colors and aromas develop in ripe fruits and defense barriers diminish, allowing animal dispersion of seeds and resumption of fungal pathogen growth

The impact of fruit ontogeny on the infection strategy of fungal pathogens has been studied and widely documented for several fruit species[11]. In particular for strawberry,

the tolerance of unripe fruits to the two major fruit pathogens, *Colletotrichum acutatum* and *Botrytis cinerea* is well known: *C. acutatum* is found quiescent as appressorium in white immature fruits[12], whereas for *B. cinerea* infection can occur at flowering stage but symptoms develop only at red fruit stages[25]. Furthermore, aroma volatile compounds typical of ripe strawberry, such as Furaneol, ethyl butanoate and *cis*-3-hexenyl acetate, clearly stimulate the growth of both these fungi, suggesting that fungal quiescence is not only dependent on inhibitory factors in the immature fruits, but also on ripeness-specific stimulating compounds[26].

In our study we confirm that the unripe fruit stages of a susceptible strawberry cultivar is tolerant to anthracnose and grey mold disease (Figure 1, Supplemental Figure 1). Contrary to unripe strawberry fruits that mature on the plant, the fruits harvested at unripe stages and inoculated with the *C. acutatum* or *B. cinerea* never developed symptoms of any fungal disease even as the fruits turned red. This could be due either to fungal death occurring at late time points or also to the nonclimacteric nature of strawberry. Unripe strawberry fruits are not able to satisfy all the physiological requirements needed to accomplish maturation, and these are probably required to restore fruit susceptibility and fungal growth.

Both pre-formed and induced factors have been addressed as involved in the tolerance of immature fruits[11], and phenolic compounds fall in both these categories, playing roles as pre-formed (phytoanticipin) or induced (phytoalexin) defenses[13, 27]. In 1989, the hypothesis that *B. cinerea* quiescence in strawberry green fruits was due to proanthocyanidins, which are particularly abundant at these stages[28]. This hypothesis was later supported by several studies addressing both the phenolic compounds antimicrobial properties[29] and their accumulation in different immature fruit species[30, 31].

The results presented here support a major role of polyphenols as pre-formed contribution to the disease tolerance of immature fruits (Figure 2). Indeed, according to previous reports on polyphenol variation during ripening[32] and their role in plant defense[33], we find that compounds such as flavan-3-ols, proanthocyanidins, benzoic acids and ellagitannins strongly decrease with ripening in the absence of pathogens. Consistently, genes such as *ANS* and *LAR*, regulating the synthesis of catechins and proanthocyanidins are down-regulated in red fruits (Figure 4). Conversely, the expression of the *FaMYB1* that negatively regulate the production of anthocyanins in *F.* x *ananassa*[34] does not vary during ripening. However, other *MYB* genes have been recognized in strawberry[35, 36] that could be involved in the regulation of flavonoid gene expression. Interestingly, a recent study addressing the profile of phenolic compounds in strawberry fruits of different cultivars indicated that the level of flavan-3-ols in healthy fruits of a tolerant cultivar is much higher than in a susceptible one and that the differences between tolerant and susceptible cultivars laid more in the pre-existing phenolic profiles than in the pathogen induced ones[17]. Although supporting a key role for these compounds in fruit resistance, our data suggest that the mechanisms involved fruit ontogenic resistance is similar to those conferring genotype resistance.

Considering the ripening stages, pathogen, and post-infection time, most significant differences in the profile of individual polyphenols were detected at 48 HPI in white fruits, suggesting that fruit response to pathogens intensifies at this time (Figure 2 and Table 1). In particular, HMWP, the most concentrated polyphenol in strawberry fruits[4, 37] are also the most responsive to pathogen infections, increasing their level up to 44% with respect to control. The fact that the level of flavan-3-ols, proanthocyanidins and ellagitannins is maintained in 48 HPI white fruits infected with both pathogens suggests that the fruit response to pathogens inhibits the normal postharvest metabolisms to

maintain high the concentration of antimicrobial compounds. These alterations are probably related to the temporary tolerance of white fruits, independent of the susceptible genetic background. Accumulation of ellagitannins and ellagic acid conjugates as defense response have been reported in strawberry leaves, where these compounds can elicit hypersensitive response and salicylic acid mediated gene expression[18, 38], and in ripe fruits inoculated with *Colletotrichum nymphaeae*[17] or with *Colletotrichum simmondsii*[39].

Differently from *Colletotrichum*, *Botrytis* induces a decrease in benzoic acids (except methyl gallate), phenylpropanoid and flavan-3-ols exclusively associated to the early stage of the interaction (24 HPI white fruits) (Figure 2). Since these compounds have all been recognized as active in immature defense response, their decrease at early stage of infection could be associated to a different infection strategy of the two fungi: *C. acutatum* has a general hemibiotroph *habitus*, while *B. cinerea* is a typical necrotroph. Therefore, it is reasonable that at early interaction steps they communicate differently with the host.

It is notable in red fruits that neither of the two pathogens provokes important variation in metabolite profiles compared to control, except for few individual compounds that undergo a significant decrease (Supplemental Table 2). These ripe fruits contain lower levels of polyphenols, are fully susceptible to these pathogens and develop disease symptoms in few days (Figure 1). Thus, the decrease or lack of response in the concentration of phenolic compounds in this type of fruit is consistent with the establishment of a fully compatible interaction between the pathogen and its host. Furthermore, contrary to our findings, a significant increase in ellagic acid derivatives, flavan-3-ols and flavonols was recently reported not only in unripe but also in ripe strawberry fruits of the cultivar ‘Asia’ after infection with *C. nymphaeae* [40]. Provided

that these results were obtained from a different strawberry cultivar, which already accounts for strong metabolic differences, it must be taken into consideration that only the external layers of the fruit were used to extract the phenolic compounds in our study and not the whole fruit; this was carried out with the purpose to examine only the tissues directly interacting with the pathogen during 24 and 48 HPI. Indeed, it is known that the spatial distribution of phenolic compounds inside the strawberry fruit tissue is discontinuous through the fruit section, with flavan-3-ols being much more abundant in the fruit core than into the external receptacle tissues[22]. This could explain the strong differences in the ellagitannins concentration found in our analysis with the data previously reported for the same cultivar utilizing the same method[41], where casuarictin, agrimoniin and ellagic acid were found 5-10-fold less concentrated. A different predetermined spatial organization of the phenolic compounds could meet a different functional requirement of these metabolites in the various parts of the fruit during ripening. However, defense-related compounds, such as benzoic acids, ellagitannins, flavan-3-ols and HMWPs, could also be induced to mobilize across the fruit layers at the site of infection upon pathogen perception. This hypothesis could also explain the disassociation between transcriptional activation of some of the regulatory genes and corresponding polyphenol accumulation that we have found in this study.

In conclusion, our results support a key role for phenolic compounds in the ontogenic fruit disease tolerance to two major postharvest strawberry diseases. However, further studies using cultivars with different level of disease susceptibility are needed to fully uncover the molecular mechanisms involved in unripe fruit tolerance. These can provide new important elements for the development of new cultivars less susceptible to *Colletotrichum* and *Botrytis*.

3.5. REFERENCES

1. Giampieri, F.; Alvarez-Suarez, J. M.; Battino, M., Strawberry and Human Health: Effects beyond Antioxidant Activity. *Journal of Agricultural and Food Chemistry* **2014,** *62* (18), 3867-3876.
2. Forbes-Hernandez, T. Y.; Gasparrini, M.; Afrin, S.; Bompadre, S.; Mezzetti, B.; Quiles, J. L.; Giampieri, F.; Battino, M., The healthy effects of strawberry polyphenols: which strategy behind antioxidant capacity? *Critical Reviews in Food Science and Nutrition* **2015,** (just-accepted), 00-00.
3. Giampieri, F.; Forbes-Hernandez, T. Y.; Gasparrini, M.; Alvarez-Suarez, J. M.; Afrin, S.; Bompadre, S.; Quiles, J. L.; Mezzetti, B.; Battino, M., Strawberry as a health promoter: an evidence based review. *Food & Function* **2015,** *6* (5), 1386-1398.
4. Gasperotti, M.; Masuero, D.; Mattivi, F.; Vrhovsek, U., Overall dietary polyphenol intake in a bowl of strawberries: The influence of Fragaria spp. in nutritional studies. *Journal of Functional Foods* **2015,** *18*, 1057-1069.
5. Vincente, A.R.; Martinez, G. A.; Civello, P. M.; Chaves, A. R., Quality of heat-treated strawberry fruit during refrigerated storage. *Postharvest Biology and Technology* **2002,** *25* (1), 59-71.
6. Pope, T.; Gundalai, E.; Elliott, L.; Blackshaw, R.; Hough, G.; Wood, A.; Bennison, J.; Prince, G.; Chandler, D., Recording the movement of adult vine weevil within strawberry crops using radio frequency identification tags. *Journal of Berry Research* (Preprint), 1-10.
7. De Ros, G.; Conci, S.; Pantezzi, T.; Savini, G., The economic impact of invasive pest Drosophila suzukii on berry production in the Province of Trento, Italy. *Journal of Berry Research* **2015,** (Preprint), 1-8.
8. Wenneker, M.; Bergsma-Vlami, M., Erwinia pyrifoliae, a new pathogen on strawberry in the Netherlands. *Journal of Berry Research* **2015,** *5* (1), 17-22.
9. Buddie, A. G.; Martínez-Culebras, P.; Bridge, P. D.; García, M. D.; Querol, A.; Cannon, P. F.; Monte, E., Molecular characterization of Colletotrichum strains derived from strawberry. *Mycological Research* **1999,** *103* (4), 385-394.
10. Maas, J. L., *Compendium of strawberry diseases*. American Phytopathological Society (APS Press): 1998.
11. Prusky, D., Pathogen quiescence in postharvest diseases. *Annual Review of Phytopathology* **1996,** *34*, 413-434.
12. Guidarelli, M.; Carbone, F.; Mourgues, F.; Perrotta, G.; Rosati, C.; Bertolini, P.; Baraldi, E., Colletotrichum acutatum interactions with unripe and ripe strawberry fruits and differential responses at histological and transcriptional levels. *Plant Pathology* **2011,** *60* (4), 685-697.
13. Amil-Ruiz, F.; Blanco-Portales, R.; Muñoz-Blanco, J.; Caballero, J. L., The Strawberry Plant Defense Mechanism: A Molecular Review. *Plant and Cell Physiology* **2011,** *52* (11), 1873-1903.
14. Di Venere, D.; Linsalata, V.; Ippolito, A.; Nigro, F.; Arcuti, P.; Lattanzio, V., Endogenous phenolics, ripening and susceptibility of strawberry fruits (Fragaria ananassa Duch.) to post-harvest diseases. *Polyphenols Communication* **1998,** *98*, 459-460.
15. Puhl, I.; Treutter, D., Ontogenetic variation of catechin biosynthesis as basis for infection and quiescence of Botrytis cinerea in developing strawberry fruits. *J. Plant Dis. Prot* **2008,** *115*, 247-251.
16. Guetsky, R.; Kobiler, I.; Wang, X.; Perlman, N.; Gollop, N.; Avila-Quezada, G.; Hadar, I.; Prusky, D., Metabolism of the Flavonoid Epicatechin by Laccase of Colletotrichum

gloeosporioides and Its Effect on Pathogenicity on Avocado Fruits. *Phytopathology* **2005,** *95* (11), 1341-1348.
17. Mikulic-Petkovsek, M.; Schmitzer, V.; Slatnar, A.; Weber, N.; Veberic, R.; Stampar, F.; Munda, A.; Koron, D., Alteration of the content of primary and secondary metabolites in strawberry fruit by Colletotrichum nymphaeae infection. *Journal of Agricultural and Food Chemistry* **2013,** *61* (25), 5987-5995.
18. Mamani, A.; Filippone, M.; Grellet, C.; Welin, B.; Castagnaro, A.; Ricci, J., Pathogen-Induced Accumulation of an Ellagitannin Elicits Plant Defense Response. *Molecular Plant-Microbe Interactions* **2012,** *25* (11), 1430-1439.
19. Vrhovsek, U.; Masuero, D.; Gasperotti, M.; Franceschi, P.; Caputi, L.; Viola, R.; Mattivi, F., A Versatile Targeted Metabolomics Method for the Rapid Quantification of Multiple Classes of Phenolics in Fruits and Beverages. *Journal of Agricultural and Food Chemistry* **2012,** *60* (36), 8831-8840.
20. Rigo, A.; Vianello, F.; Clementi, G.; Rossetto, M.; Scarpa, M.; Vrhovšek, U.; Mattivi, F., Contribution of proanthocyanidins to the peroxy radical scavenging capacity of some Italian red wines. *Journal of agricultural and food chemistry* **2000,** *48* (6), 1996-2002.
21. López-Gómez, R.; Gómez-Lim, M. A., A Method for Extracting Intact RNA from Fruits Rich in Polysaccharides using Ripe Mango Mesocarp. *HortScience* **1992,** *27* (5), 440-442.
22. Almeida, J. R. M.; D'Amico, E.; Preuss, A.; Carbone, F.; de Vos, C. H. R.; Deiml, B.; Mourgues, F.; Perrotta, G.; Fischer, T. C.; Bovy, A. G.; Martens, S.; Rosati, C., Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (Fragaria × ananassa). *Archives of Biochemistry and Biophysics* **2007,** *465* (1), 61-71.
23. Livak, K. J., ABI Prism 7700 sequence detection system. *User bulletin* **1997,** *2*, 929-34.
24. Widhalm, Joshua R.; Dudareva, N., A Familiar Ring to It: Biosynthesis of Plant Benzoic Acids. *Molecular Plant* **2015,** *8* (1), 83-97.
25. Williamson, B.; Tudzynski, B.; Tudzynski, P.; van Kan, J. A. L., Botrytis cinerea: the cause of grey mold disease. *Molecular Plant Pathology* **2007,** *8* (5), 561-580.
26. Neri, F.; Cappellin, L.; Spadoni, A.; Cameldi, I.; Algarra Alarcon, A.; Aprea, E.; Romano, A.; Gasperi, F.; Biasioli, F., Role of strawberry volatile organic compounds in the development of Botrytis cinerea infection. *Plant Pathology* **2015,** *64* (3), 709-717.
27. VanEtten, H. D.; Mansfield, J. W.; Bailey, J. A.; Farmer, E. E., Two Classes of Plant Antibiotics: Phytoalexins versus "Phytoanticipins". *The Plant Cell* **1994,** *6* (9), 1191-1192.
28. Jersch, S.; Scherer, C.; Huth, G.; Schlösser, E., Proanthocyanidins as basis for quiescence of Botrytis cinerea in immature strawberry fruits. *Zeitschrift fuer Pflanzenkrankheiten und Pflanzenschutz (Germany, FR)* **1989**.
29. Feucht, W.; Treutter, D., The role of flavan-3-ols and proanthocyanidins in plant defense. CRC Press: Boca Raton, FL, USA: 1999; pp 307-338.
30. Keller, M.; Viret, O.; Cole, F. M., Botrytis cinerea Infection in Grape Flowers: Defense Reaction, Latency, and Disease Expression. *Phytopathology* **2003,** *93* (3), 316-322.
31. Pezet, R.; Viret, O.; Perret, C.; Tabacchi, R., Latency of Botrytis cinerea Pers.: Fr. and biochemical studies during growth and ripening of two grape berry cultivars, respectively susceptible and resistant to grey mold. *Journal of Phytopathology* **2003,** *151* (4), 208-214.
32. Aaby, K.; Mazur, S.; Nes, A.; Skrede, G., Phenolic compounds in strawberry (Fragaria x ananassa Duch.) fruits: Composition in 27 cultivars and changes during ripening. *Food Chemistry* **2012,** *132* (1), 86-97.

33. Halbwirth, H.; Puhl, I.; Haas, U.; Jezik, K.; Treutter, D.; Stich, K., Two-Phase Flavonoid Formation in Developing Strawberry (Fragaria × ananassa) Fruit. *Journal of Agricultural and Food Chemistry* **2006,** *54* (4), 1479-1485.
34. Aharoni, A.; De Vos, C. H.; Wein, M.; Sun, Z.; Greco, R.; Kroon, A.; Mol, J. N. M.; O'Connell, A. P., The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. *The Plant Journal* **2001,** *28* (3), 319-332.
35. Medina-Puche, L.; Cumplido-Laso, G.; Amil-Ruiz, F.; Hoffmann, T.; Ring, L.; Rodríguez-Franco, A.; Caballero, J. L.; Schwab, W.; Muñoz-Blanco, J.; Blanco-Portales, R., MYB10 plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during ripening of Fragaria× ananassa fruits. *Journal of experimental botany* **2014,** *65* (2), 401-417.
36. Medina-Puche, L.; Molina-Hidalgo, F. J.; Boersma, M. R.; Schuurink, R. C.; López-Vidriero, I.; Solano, R.; Franco-Zorrilla, J.-M.; Caballero, J. L.; Blanco-Portales, R.; Muñoz-Blanco, J., A R2R3-MYB transcription factor (FaEOBII) regulates eugenol production in ripe strawberry (Fragaria× ananassa) fruit receptacles. *Plant physiology* **2015**, pp-114.
37. Buendía, B.; Gil, M. I.; Tudela, J. A.; Gady, A. L.; Medina, J. J.; Soria, C.; López, J. M.; Tomás-Barberán, F. A., HPLC-MS Analysis of Proanthocyanidin Oligomers and Other Phenolics in 15 Strawberry Cultivars. *Journal of Agricultural and Food Chemistry* **2010,** *58* (7), 3916-3926.
38. Hukkanen, A. T.; Kokko, H. I.; Buchala, A. J.; McDougall, G. J.; Stewart, D.; Kärenlampi, S. O.; Karjalainen, R. O., Benzothiadiazole Induces the Accumulation of Phenolics and Improves Resistance to Powdery Mildew in Strawberries. *Journal of Agricultural and Food Chemistry* **2007,** *55* (5), 1862-1870.
39. Weber, N.; Schmitzer, V.; Jakopic, J.; Mikulic-Petkovsek, M.; Stampar, F.; Koron, D.; Veberic, R., Influence of Colletotrichum simmondsii R. G. Shives & Y. P. Tan infection on selected primary and secondary metabolites in strawberry (Fragaria x ananassa Duch.) fruit and runners. *European Journal of Plant Pathology* **2013,** *136* (2), 281-290.
40. Weber, N.; Veberic, R.; Mikulic-Petkovsek, M.; Stampar, F.; Koron, D.; Munda, A.; Jakopic, J., Metabolite accumulation in strawberry (Fragaria × ananassa Duch.) fruits and runners in response to Colletotrichum nymphaeae infection. *Physiological and Molecular Plant Pathology* **2015,** *92*, 119-129.
41. Gasperotti, M.; Masuero, D.; Guella, G.; Palmieri, L.; Martinatti, P.; Pojer, E.; Mattivi, F.; Vrhovsek, U., Evolution of Ellagitannin Content and Profile during Fruit Ripening in Fragaria spp. *Journal of Agricultural and Food Chemistry* **2013,** *61* (36), 8597-8607.

3.6 FIGURES

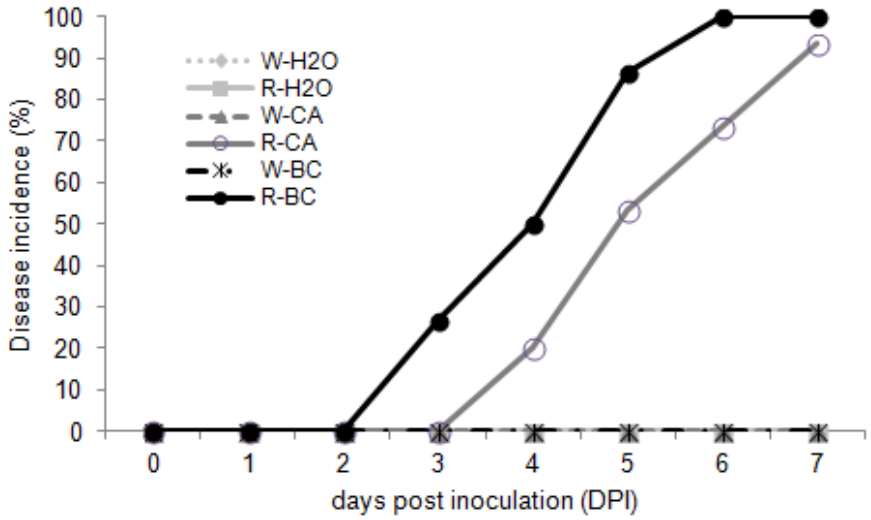


Figure 1. Incidence of *C. acutatum* and *B. cinerea* in white and red fruits of Alba up to 7 DPI. For consistency, results are all presented in DPI, including disease incidence recorded at 24 and 48 HPI. W and R: white and red fruits, respectively; H_2O, CA and BC: mock-, *C. acutatum*-, and *B. cinerea*- inoculated fruits, respectively.

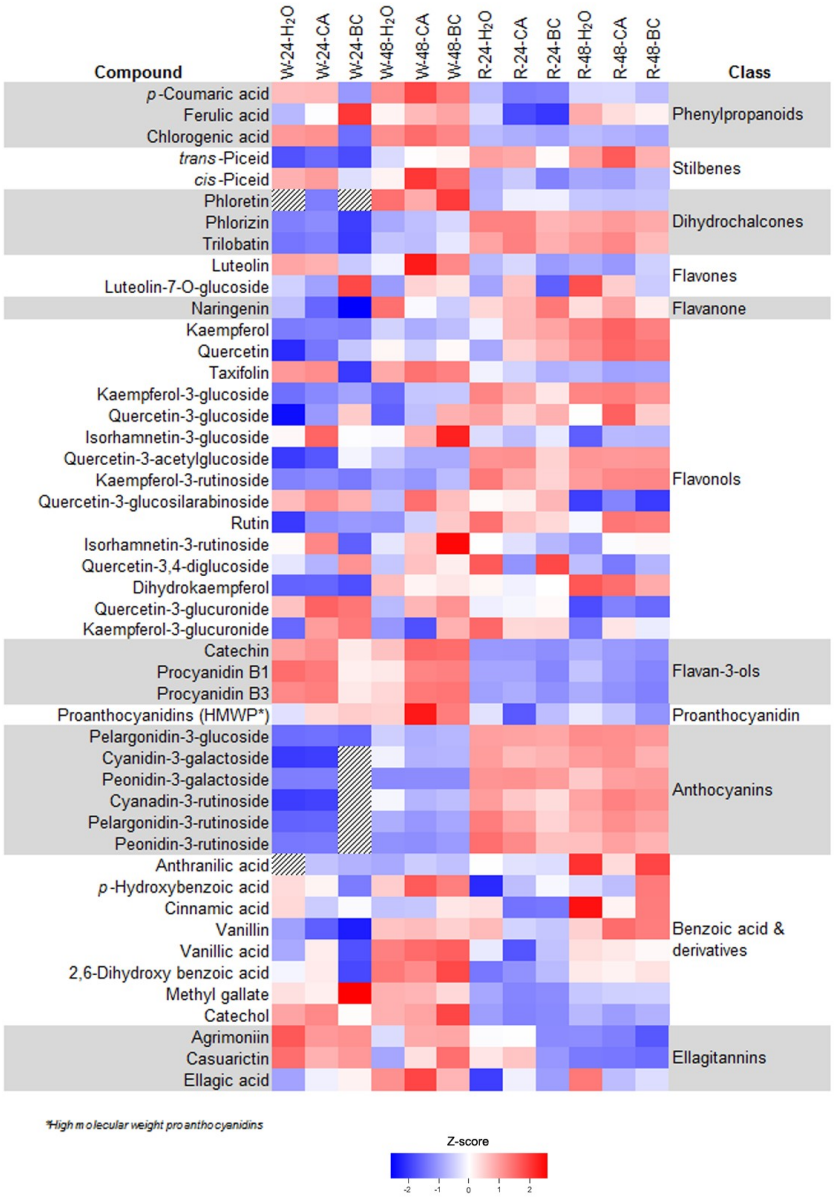


Figure 2. Concentrations of polyphenols expressed as Z-scores (bottom colored bar) in white and red strawberry fruits as affected by *C. acutatum* and *B. cinerea* infection visualized as a heat map. W and R: white and red fruits, respectively; 24 and 48: post-inoculation hours, respectively; H_2O, CA and BC: mock-, *C. acutatum*- and *B. cinerea*- inoculated fruits, respectively.

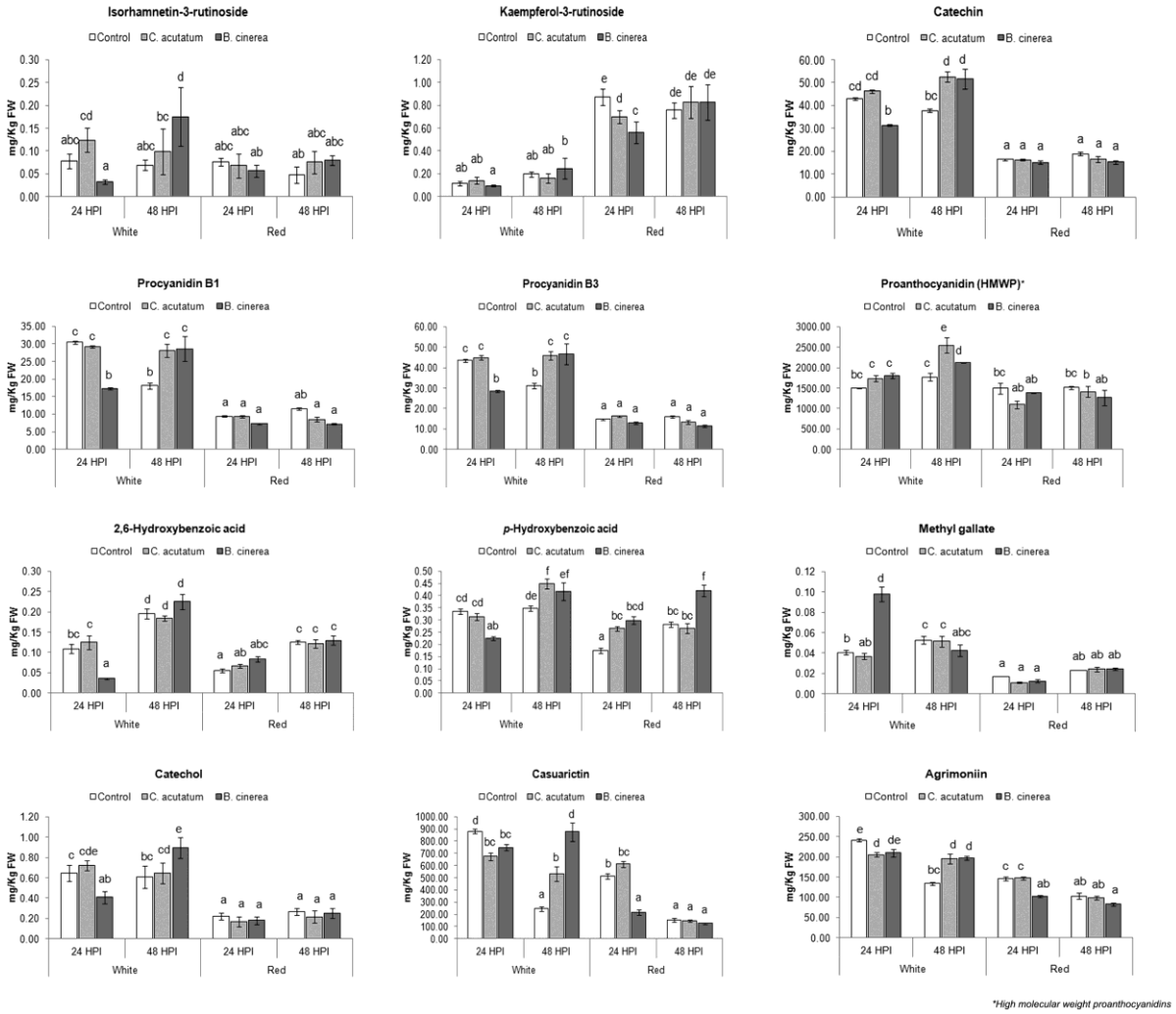


Figure 3. Variation of fruit polyphenols in white and red Alba strawberry upon infection of *C. acutatum* and *B. cinerea*. The 12 compounds found to be significantly influenced by fungal pathogens species (P), ripening stage (R) and post-inoculation time (T) are reported. Quantities are expressed as mg/Kg fresh weight (FW). Each data is an average of three biological replicates with its standard error. Means with the same letter are not significantly different at $p<0.05$ (DMRT).

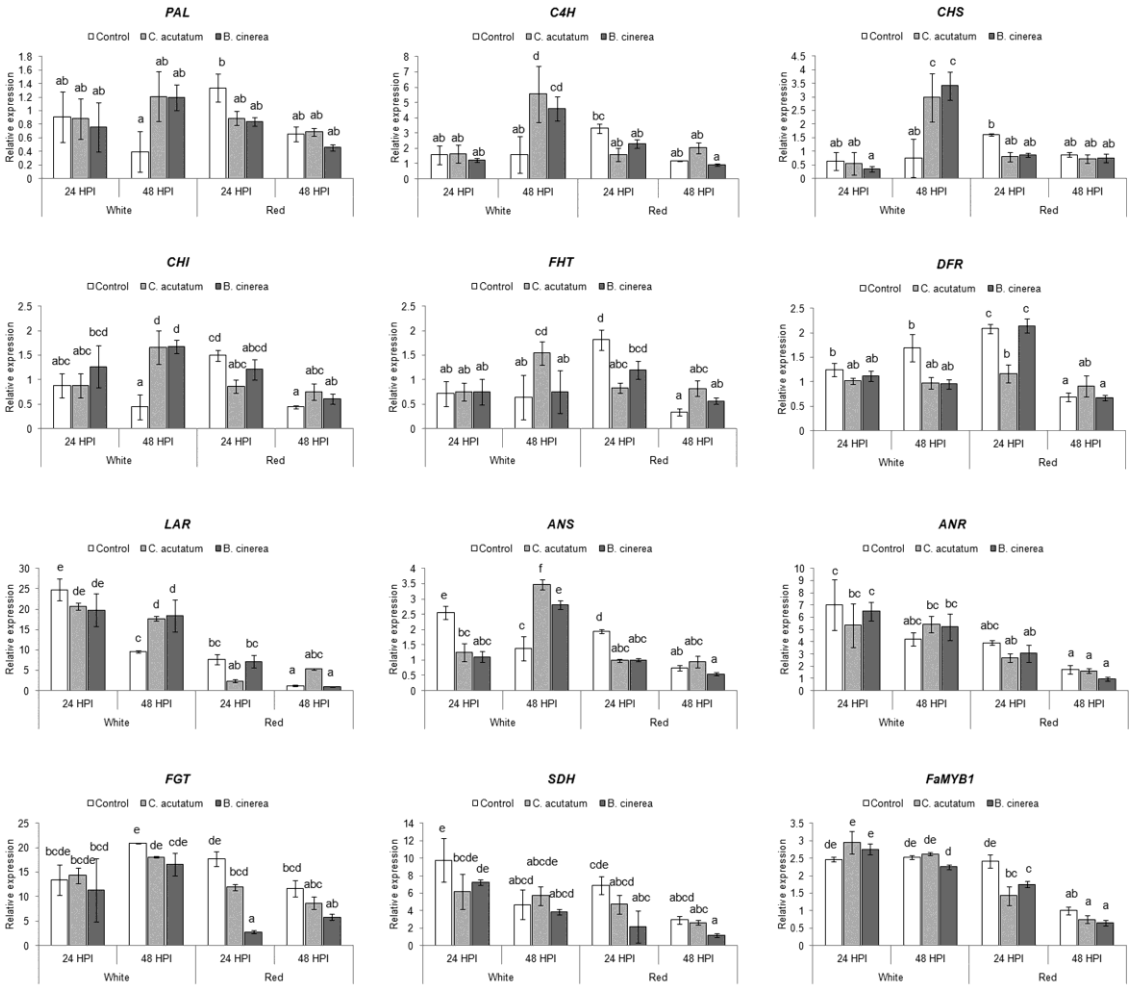


Figure 4. Relative expression levels of genes in white and red strawberry fruits as affected by *C. acutatum* and *B. cinerea* inoculation. All values were normalized to the expression level of the *elongation factor 1α* housekeeping gene. Each data is an average of three biological replicates with its standard error. Means with the same letter are not significantly different at $p<0.05$ (DMRT).

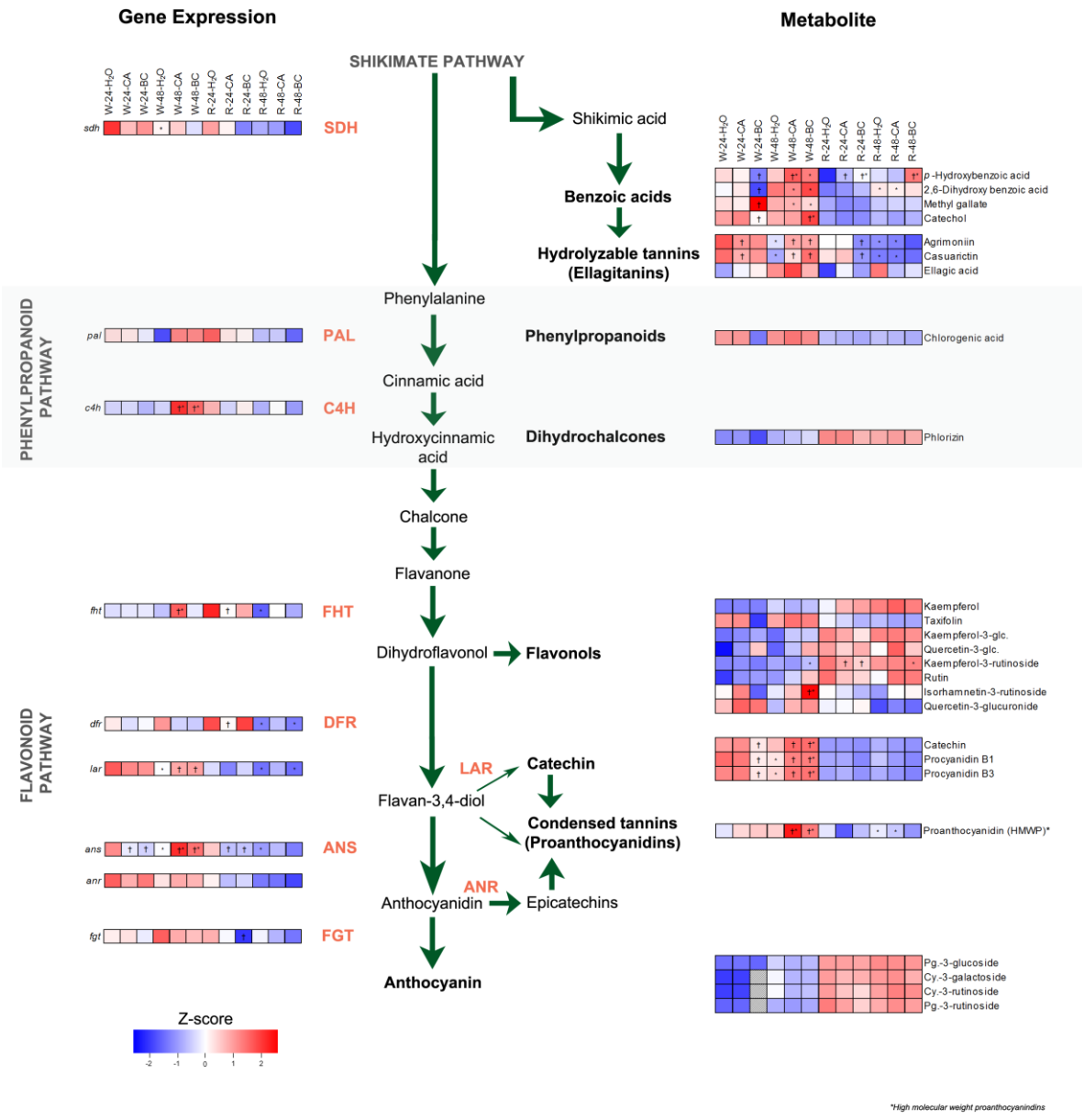


Figure 5. Correlation of gene expression and metabolite concentration expressed as Z-scores in coloured boxes. Genes or compound that significantly vary based on the one-way ANOVA are designated in the boxes with an * (significance between 24 and 48 HPI), and/or a † (significance with respect to control). W and R: white and red fruits, respectively; 24 and 48: post-inoculation hours, respectively; H_2O, CA and BC: mock-, *C. acutatum*- and *B. cinerea*- inoculated fruits, respectively.

Table 1. Top ten most abundant polyphenols in the different conditions tested[a]

Compounds	Conditions											
	W-24-H_2O	W-24-CA	W-24-BC	W-48-H_2O	W-48-CA	W-48-BC	R-24-H_2O	R-24-CA	R-24-BC	R-48-H_2O	R-48-CA	R-48-BC
HMWP[b]	1	1	1	1	1	1	1	1	1	1	1	1
casuarictin	2	2	2	3	2	2	3	3	3	3	3	3
agrimoniin	3	3	3	4	4	4	4	4	4	4	4	4
ellagic acid	4	4	4	5	5	5	5	5	5	5	5	5
procyanidin B3	5	8	7	6	7	8	10	10	10	9	10	10
quercetin-3-glucuronide	6	5	5	7	8	7	8	7	7	10	7	8
catechin	7	6	6	8	6	6	9	9	9	8	9	9
pelargonidin-3-glucoside	8	7	10	2	3	3	2	2	2	2	2	2
procyanidin B1	9	9	8	9	9	9	—	—	—	—	—	—
kaempferol-3-glucuronide	10	10	9	—	—	—	—	—	—	—	—	—
cyanidin-3-galactoside	—	—	—	10	10	10	6	6	6	6	6	6
pelargonidin-3-rutinoside	—	—	—	—	—	—	7	8	8	7	8	7

[a]W and R: white and red fruits, respectively; 24 and 48: post-inoculation hours, respectively; H_2O, CA and BC: mock-, *C. acutatum*- and *B. cinerea*-inoculated fruits, respectively. Numbers in column represents the ranking position of each compound within the conditions, with 1 being the most abundant.
[b]High molecular weight proanthocyanidin

Table 2. Number of statistically significant compounds in each condition as influenced by of pathogen (P), ripening stage of strawberry fruit (R), and post-inoculation time (T), as tested with factorial ANOVA at $p<0.05$ and $p<0.01$.

Class of polyphenol	P	R	T	PxR	PxT	RxT	PxRxT
benzoic acid and derivatives	3	6	4	3	4	5	4
phenylpropanoids	1	3	2	2	0	2	0
stilbenes	0	2	1	0	0	0	0
dihydrochalcones	2	2	2	0	1	2	0
flavones	0	1	0	1	0	0	0
flavonone	0	1	1	1	0	1	0
flavan-3-ols	0	3	1	0	3	0	3
flavonols	3	13	5	2	4	2	2
anthocyanins	0	2	2	0	0	2	0
ellagitannins	0	3	3	2	3	0	2
proanthocyanidins	1	1	1	1	1	0	1
TOTAL	10	37	22	12	16	14	12

3.8. SUPPORTING INFORMATION

Supplemental Table 1. Primers used in qRT-PCR reactions.

Gene name	Primer sequences (5'-3')	Annealing temperature (°C)
Phenylalanine ammonia lyase (pal)	F: TTGAAGCTCATGTCTTCCAC R: CAAGTTCTCCTCCAAATG	56
Cinnamate 4-hydroxylase (c4h)	F: TGCCCTTGGCTTCATGACT R: GCTTGACACTACGGAGAAAGGT	56
Chalcone synthase (chs)	F: GGCTCACCGTCGAGACCG R: GGAGAAGATCACTCGAATCA	56
Chalcone isomerase (chi)	F: GCCGGAAATGGGAAAGTG R: GCTCAGTTTCATGCCTTGAC	56
Flavnonoid 3-hydroxylase (fht)	F: ATCACCGTTCAACCTGTGGAAG R: TCTGGAATGTGGCTATGGACAAC	56
Dihydroflavonol 4-reductase (dfr)	F: GGGCCGCTACATCTGTTC R: GGAACATTATACTTGGGGTAT	56
Leucoanthocyanidin reductase (lar)	F: CAACTTCCCCATTGAAGG R: ATTGAAGCACTCATCCAAAG	56
Anthocyanidin reductase (anr)	F: CCTGAATACAAAGTCCCGACTGAG R: GTACTTGAAAGTGAACCCCTCCTTC	56
Anthocyanidin synthase (ans)	F: GACTTGTCCATTTGGCCTC R: CCCCCTCAGTTCCTTAGCATACTC	56
Flavonoid glycosyltransferase (fgt)	F: CAAGCAGTCCAACAGCTCAATC R: GAAAACATACCCCTCCGGCAC	56
Shikimate dehydrogenase (sdh)	F: TTGGTGCTGACCTTGTGGAGAT R: TAAGTGAAAAGAGTTGGCA	58
FaMYB1 (myb)	F: GCAACTTGAGGATCAGCC R: GGTGCCTGAGTTGAATCTC	56
1α (elongation factor)	F: TGCTGTTGGAGTCATCAAGAATG R: TTGGCTGCAGACTTGGTCAC	56

Supplemental Table 2. Mean concentration of polyphenols in white and red strawberry fruits inoculated with *C. acutatum* and *B. cinerea.* Standard errors of the mean are in subscript. Each value is the average of three biological replicates. Means with the same letter in each row are not significantly different at $p<0.05$ (DMRT).

Compound (mg/kg fresh weight)	White fruits						Red fruits					
	24 HPI			48 HPI			24 HPI			48 HPI		
	Control	*C. acutatum*	*B. cinerea*	Control	*C. acutatum*	*B. cinerea*	Control	*C. acutatum*	*B. cinerea*	Control	*C. acutatum*	*B. cinerea*
p -Coumaric acid	0.17 0.01	0.17 0.01	0.08 0.01	0.20 0.01	0.24 0.02	0.21 0.02	0.09 0.00	0.07 0.01	0.07 0.00	0.11 0.01	0.11 0.01	0.10 0.01
Ferulic acid	0.00 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.00 0.00	0.00 0.00	0.00 0.00	0.01 0.00	0.01 0.00	0.01 0.00
Chlorogenic acid	1.59 0.11	1.65 0.05	0.10 0.01	1.67 0.12	1.92 0.12	1.73 0.22	0.50 0.02	0.43 0.03	0.37 0.02	0.50 0.01	0.45 0.05	0.39 0.03
TOTAL PHENYLPROPANOIDS	**1.76** 0.11	**1.82** 0.05	**0.19** 0.02	**1.87** 0.13	**2.17** 0.13	**1.95** 0.24	**0.60** 0.02	**0.50** 0.02	**0.44** 0.02	**0.61** 0.02	**0.56** 0.05	**0.49** 0.03
trans -Piceid	0.01 0.00	0.02 0.00	0.01 0.00	0.03 0.00	0.03 0.00	0.03 0.00	0.04 0.00	0.04 0.00	0.03 0.00	0.04 0.00	0.05 0.00	0.04 0.00
cis -Piceid	0.08 0.00	0.08 0.00	0.06 0.00	0.07 0.00	0.10 0.01	0.09 0.01	0.06 0.00	0.06 0.00	0.05 0.00	0.05 0.00	0.05 0.01	0.06 0.00
TOTAL STILBENES	**0.09** 0.01	**0.10** 0.00	**0.07** 0.00	**0.10** 0.01	**0.13** 0.01	**0.13** 0.01	**0.10** 0.00	**0.10** 0.00	**0.07** 0.01	**0.10** 0.00	**0.11** 0.01	**0.10** 0.00
Phloretin		0.00 0.00		0.01 0.00	0.01 0.00	0.01 0.00	0.00 0.00	0.01 0.00	0.01 0.00	0.00 0.00	0.00 0.00	0.00 0.00
Phlorizin	0.59 0.01	0.66 0.03	0.17 0.01	0.85 0.03	0.98 0.05	1.13 0.09	2.52 0.06	2.52 0.07	2.06 0.08	2.16 0.05	2.31 0.12	2.16 0.08
Trilobatin	0.03 0.00	0.03 0.00	0.02 0.00	0.04 0.00	0.04 0.00	0.05 0.00	0.08 0.00	0.09 0.00	0.08 0.00	0.08 0.00	0.09 0.00	0.07 0.00
TOTAL DIHYDROCHALCONES	**0.62** 0.01	**0.69** 0.03	**0.19** 0.01	**0.90** 0.03	**1.03** 0.05	**1.19** 0.09	**2.61** 0.06	**2.62** 0.07	**2.14** 0.09	**2.25** 0.05	**2.40** 0.12	**2.24** 0.08
Luteolin	0.23 0.01	0.22 0.03	0.11 0.01	0.14 0.01	0.35 0.07	0.25 0.03	0.10 0.00	0.12 0.01	0.08 0.01	0.09 0.02	0.08 0.01	0.12 0.01
Luteolin-7-O-glucoside	0.02 0.00	0.02 0.00	0.03 0.00	0.02 0.00	0.02 0.00	0.02 0.00	0.02 0.00	0.02 0.00	0.01 0.00	0.03 0.00	0.02 0.00	0.02 0.00
TOTAL FLAVONES	**0.25** 0.01	**0.23** 0.03	**0.14** 0.01	**0.15** 0.01	**0.38** 0.07	**0.27** 0.03	**0.12** 0.00	**0.14** 0.01	**0.10** 0.01	**0.12** 0.03	**0.10** 0.01	**0.13** 0.01
Naringenin	0.01 0.00	0.00 0.00	0.00 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00	0.01 0.00
TOTAL FLAVANONE	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00	**0.01** 0.00
Kaempferol	0.01 0.00	0.01 0.00	0.01 0.00	0.08 0.00	0.05 0.01	0.06 0.01	0.10 0.00	0.19 0.01	0.21 0.01	0.25 0.00	0.29 0.02	0.25 0.01
Quercetin	0.02 0.00	0.03 0.01	0.05 0.01	0.06 0.00	0.05 0.01	0.06 0.01	0.04 0.01	0.07 0.00	0.08 0.01	0.09 0.01	0.10 0.01	0.10 0.01
Taxifolin	0.58 0.02	0.61 0.01	0.00 0.00	0.54 0.03	0.67 0.05	0.64 0.09	0.31 0.01	0.26 0.01	0.20 0.01	0.22 0.02	0.18 0.02	0.18 0.02
Kaempferol-3-glucoside	0.92 0.04	0.99 0.11	1.07 0.04	0.90 0.04	1.16 0.18	1.17 0.06	1.84 0.09	1.67 0.03	1.44 0.08	1.84 0.07	1.86 0.09	1.78 0.08
Quercetin-3-glucoside	1.15 0.13	1.62 0.16	2.22 0.11	1.43 0.10	1.75 0.19	2.35 0.51	2.43 0.21	2.18 0.10	2.35 0.17	1.96 0.15	2.74 0.13	2.21 0.10
Isorhamnetin-3-glucoside	0.12 0.01	0.19 0.02	0.12 0.01	0.12 0.01	0.16 0.02	0.22 0.04	0.11 0.01	0.10 0.01	0.11 0.01	0.07 0.01	0.10 0.01	0.10 0.00
Quercetin-3-acetylglucoside	0.11 0.01	0.17 0.01	0.50 0.04	0.41 0.02	0.34 0.06	0.34 0.06	0.85 0.03	0.85 0.07	0.66 0.05	0.84 0.07	0.83 0.07	0.84 0.07
Kaempferol-3-rutinoside	0.11^{ab} 0.01	0.14^{ab} 0.01	0.09^{a} 0.00	0.20^{ab} 0.01	0.16^{ab} 0.01	0.25^{b} 0.03	0.87^{e} 0.02	0.70^{d} 0.02	0.56^{c} 0.03	0.76^{de} 0.02	0.83^{de} 0.05	0.83^{de} 0.05
Quercetin-3-glucosilarabinoside	0.13 0.02	0.16 0.02	0.14 0.01	0.07 0.01	0.18 0.04	0.13 0.04	0.10 0.02	0.10 0.01	0.14 0.02	0.02 0.00	0.04 0.01	0.02 0.00
Rutin	0.23 0.01	0.32 0.02	0.32 0.02	0.32 0.01	0.38 0.03	0.50 0.07	0.62 0.04	0.50 0.04	0.48 0.03	0.41 0.05	0.61 0.04	0.60 0.05
Isorhamnetin-3-rutinoside	0.08^{abc} 0.01	0.12^{cd} 0.01	0.03^{a} 0.00	0.07^{abc} 0.00	0.10^{cd} 0.02	0.18^{d} 0.02	0.08^{abc} 0.00	0.07^{abc} 0.01	0.06^{ab} 0.00	0.05^{ab} 0.01	0.08^{abc} 0.01	0.08^{abc} 0.00
Quercetin-3,4-diglucoside	0.02 0.00	0.02 0.00	0.03 0.00	0.02 0.00	0.03 0.00	0.03 0.01	0.03 0.00	0.02 0.00	0.03 0.01	0.02 0.00	0.02 0.00	0.02 0.00
Dihydrokaempferol	0.05 0.01	0.05 0.01	0.01 0.00	0.42 0.03	0.30 0.08	0.33 0.06	0.30 0.02	0.26 0.02	0.28 0.03	0.64 0.05	0.59 0.06	0.46 0.06
Quercetin-3-glucuronide	43.26 2.39	58.51 4.15	55.36 1.37	25.78 1.37	45.22 7.23	50.67 6.36	31.67 1.76	32.50 1.13	34.45 2.31	13.72 1.39	19.66 1.93	16.99 1.81
Kaempferol-3-glucuronide	7.75 0.65	10.88 0.52	11.41 0.19	8.23 0.42	7.48 1.18	10.55 1.00	11.68 1.05	9.95 0.34	9.98 0.54	7.90 0.40	9.75 0.53	9.14 0.62
TOTAL FLAVONOLS	**54.54** 3.14	**73.82** 4.97	**71.36** 1.73	**38.64** 1.78	**58.01** 8.69	**67.47** 8.09	**51.01** 5.14	**49.44** 1.38	**51.03** 3.21	**28.79** 2.13	**37.68** 2.62	**33.60** 2.21
Catechin	43.08^{cd} 0.55	46.24^{cd} 0.61	31.38^{b} 0.38	37.80^{bc} 0.75	52.60^{d} 2.40	51.78^{d} 4.24	16.32^{a} 0.30	16.22^{a} 0.37	15.08^{a} 0.83	18.85^{a} 0.69	16.45^{a} 1.23	15.28^{a} 0.70
Procyanidin B1	30.46^{c} 0.45	29.20^{c} 0.33	17.37^{b} 0.33	18.06^{b} 0.89	28.10^{c} 1.84	28.58^{c} 3.55	9.41^{a} 0.22	9.411^{a} 0.33	7.27^{a} 0.24	11.51^{ab} 0.28	8.54^{a} 0.63	7.19^{a} 0.25
Procyanidin B3	43.53^{c} 0.86	44.94^{c} 1.27	28.59^{b} 0.64	31.30^{b} 1.29	45.92^{c} 2.19	46.59^{c} 5.05	14.83^{a} 0.41	16.22^{a} 0.38	13.10^{a} 0.61	16.05^{a} 0.60	13.33^{a} 1.04	11.41^{a} 0.46
TOTAL FLAVAN-3-OLS	**117.07^{c}** 1.21	**120.38^{c}** 1.87	**77.34^{b}** 1.22	**87.16^{b}** 2.86	**126.62^{c}** 6.36	**126.95^{c}** 12.82	**40.55^{a}** 0.86	**41.85^{a}** 0.94	**35.45^{a}** 1.65	**46.41^{a}** 1.54	**38.32^{a}** 2.85	**33.87^{a}** 1.32
Proanthocyanidins (HMWP)*	1493.22^{cde} 5.44	1736.27^{de} 81.97	1796.99^{de} 65.95	1767.59^{e} 93.98	2550.78^{f} 191.60	2128.30^{f} 13.02	864.66^{a} 135.46	1097.40^{ab} 91.67	1386.67^{bc} 11.65	1512.15^{cde} 38.52	1412.78^{cd} 130.44	1267.19^{bc} 189.96
TOTAL PROANTHOCYANIDINS	**1493.22^{cde}** 5.44	**1736.27^{de}** 81.97	**1796.99^{de}** 65.95	**1767.59^{e}** 93.98	**2550.78^{f}** 191.60	**2128.30^{f}** 13.02	**864.66^{a}** 135.46	**1097.40^{ab}** 91.67	**1386.67^{bc}** 11.65	**1512.15^{cde}** 38.52	**1412.78^{cd}** 130.44	**1267.19^{bc}** 189.96
Pelargonidin-3-glucoside	34.81 1.05	45.01 4.05	10.96 3.41	301.93 3.07	213.16 48.09	236.77 51.38	813.30 7.24	800.81 11.46	781.13 12.05	885.88 29.38	873.11 19.71	837.75 37.34
Cyanidin-3-galactoside	1.16 0.07	1.72 0.26	0.49 0.06	26.25 0.47	17.54 4.55	17.85 4.70	47.36 1.75	41.72 1.73	43.25 1.48	47.76 3.55	49.92 1.46	43.52 1.15
Peonidin-3-galactoside	0.06 0.00	0.07 0.02		0.13 0.01	0.14 0.03	0.14 0.03	1.67 0.10	1.69 0.15	1.60 0.11	1.24 0.12	1.58 0.20	1.62 0.13
Cyanadin-3-rutinoside	0.04 0.00	0.06 0.01		1.05 0.02	0.69 0.18	0.75 0.21	1.89 0.03	1.55 0.08	1.38 0.08	1.83 0.12	2.10 0.08	1.99 0.04

Pelargonidin-3-rutinoside	1.45	0.03	1.64	0.14			8.31	0.24	6.29	1.46	7.73	1.67	32.77	0.64	27.77	0.90	21.47	0.93	26.46	0.95	30.53	1.06	28.16	1.04
Peonidin-3-rutinoside	0.00	0.00	0.00	0.00			0.01	0.00	0.01	0.00	0.02	0.00	0.12	0.01	0.11	0.01	0.08	0.01	0.08	0.01	0.10	0.01	0.09	0.01
TOTAL ANTHOCYANINS	37.52	1.12	48.51	4.47	11.45	3.46	337.69	3.52	237.83	54.30	263.25	57.86	897.10	8.75	873.64	14.26	848.91	14.49	963.25	34.07	957.34	22.29	913.12	39.41
Anthranilic acid			0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.01	0.00
p -Hydroxybenzoic acid	0.33^{cd}	0.01	0.31^{cd}	0.01	0.22^{ab}	0.01	0.35^{de}	0.01	0.45^{f}	0.02	0.42^{ef}	0.04	0.18^{a}	0.01	0.26^{bc}	0.01	0.30^{bcd}	0.01	0.28^{bc}	0.01	0.26^{bc}	0.02	0.42^{f}	0.02
Cinnamic acid	0.09	0.00	0.06	0.01	0.08	0.00	0.06	0.00	0.06	0.01	0.09	0.01	0.09	0.00	0.04	0.01	0.04	0.00	0.17	0.03	0.08	0.02	0.13	0.04
Vanillin	0.03	0.00	0.03	0.00	0.02	0.00	0.04	0.00	0.04	0.00	0.04	0.00	0.04	0.00	0.03	0.00	0.03	0.00	0.04	0.00	0.05	0.00	0.05	0.00
Vanillic acid	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.00	0.01	0.00	0.01	0.00
2,6-Dihydroxy benzoic acid	0.11^{bc}	0.01	0.12^{c}	0.02	0.04^{a}	0.00	0.19^{d}	0.01	0.18^{d}	0.01	0.22^{d}	0.02	0.06^{ab}	0.00	0.07^{ab}	0.00	0.08^{abc}	0.01	0.13^{c}	0.00	0.12^{c}	0.01	0.13^{c}	0.01
Methyl gallate	0.04^{bc}	0.00	0.04^{ab}	0.00	0.10^{d}	0.01	0.05^{c}	0.00	0.05^{c}	0.01	0.04^{abc}	0.01	0.01^{a}	0.00	0.01^{a}	0.00	0.01^{a}	0.00	0.02^{ab}	0.00	0.02^{ab}	0.00	0.02^{ab}	0.00
Catechol	0.64^{c}	0.08	0.72^{cde}	0.05	0.41^{ab}	0.06	0.61^{bc}	0.11	0.65^{cd}	0.10	0.90^{e}	0.10	0.22^{a}	0.03	0.17^{a}	0.05	0.18^{a}	0.04	0.27^{a}	0.04	0.22^{a}	0.06	0.25^{a}	0.05
TOTAL BENZOIC ACID & DERIVATIVES	1.26^{cd}	0.08	1.29^{cd}	0.06	0.87^{ab}	0.06	1.32^{d}	0.12	1.45^{de}	0.11	1.72^{e}	0.14	0.61^{a}	0.04	0.59^{a}	0.06	0.66^{a}	0.04	0.92^{ab}	0.03	0.77^{ab}	0.07	0.99^{bc}	0.06
Agrimoniin	241.72^{e}	3.15	205.82^{d}	5.24	209.80^{de}	9.07	134.39^{bc}	4.12	195.42^{d}	11.63	197.12^{d}	4.60	146.67^{c}	4.60	147.43^{c}	3.54	102.82^{ab}	2.01	103.56^{ab}	7.13	98.60^{ab}	5.17	83.06^{a}	3.63
Casuarictin	880.67^{d}	19.52	675.62^{bc}	30.90	748.90^{cd}	24.60	246.98^{a}	18.48	529.10^{b}	59.91	875.99^{d}	75.10	513.67^{b}	22.17	612.58^{bc}	24.08	213.86^{a}	22.39	152.64^{a}	18.04	147.16^{a}	7.67	125.93^{a}	5.61
Ellagic acid	55.63	1.14	60.34	3.01	62.35	1.35	71.06	3.19	77.61	2.71	67.82	2.61	49.47	1.58	60.31	1.09	55.32	1.09	73.05	1.79	57.24	2.52	59.19	2.07
TOTAL ELLAGITANNINS	1178.01^{e}	19.92	941.77^{cd}	38.09	1021.05^{cde}	27.29	452.43^{a}	24.02	802.13^{bc}	72.86	1140.93^{de}	77.94	709.80^{b}	25.80	820.32^{c}	26.45	372.00^{a}	24.63	329.25^{a}	26.64	303.01^{a}	12.71	268.18^{a}	9.48
TOTAL POLYPHENOLS	2884.35	161.79	2924.90	169.12	2979.66	177.76	2687.86	159.41	3780.54	232.12	3732.15	205.72	2567.17	115.26	2886.60	130.98	2697.48	138.97	2883.85	152.85	2753.08	145.17	2519.93	132.68

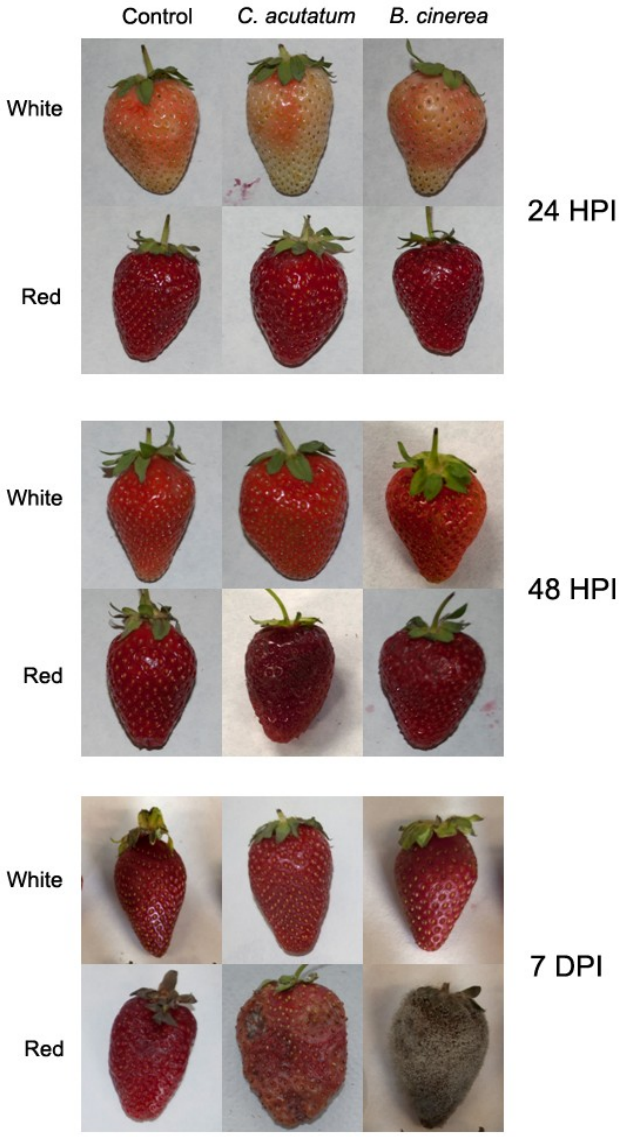
**High molecular weight proanthocyanidins*

Supplemental Table 3. Statistical variation of polyphenols as influenced by pathogen (P), ripening stage of strawberry fruits (R), and post-inoculation time (T) analyzed with factorial ANOVA. Main effects (P, R, T) and interaction (PxR, PxT, RxT, PxRxT) between factor/s displays statistically significant differences at *p<0.05 and at **p<0.01 value. NS indicates non significant variations. with factorial ANOVA. Main effects (P, R, T) and interaction (PxR, PxT, RxT, PxRxT) between factor/s show statistically significant differences at *p<0.05 and at **p<0.01 value.

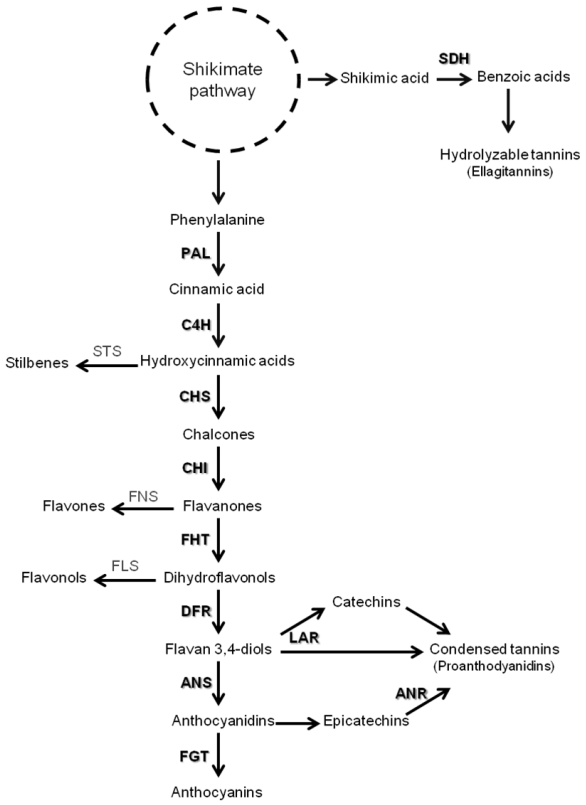
Compound	P	R	T	PxR	PxT	RxT	PxRxT
p-Coumaric acid	*	**	**	NS	NS	*	NS
Ferulic acid	NS	**	*	**	NS	*	NS
Chlorogenic acid	NS	**	NS	**	NS	NS	NS
TOTAL PHENYLPROPANOIDS	**NS**	****	*****	****	**NS**	*****	**NS**
trans-Piceide	NS	**	**	NS	NS	NS	NS
cis-Piceide	NS	**	NS	NS	NS	NS	NS
TOTAL STILBENES	**NS**	**NS**	****	**NS**	*****	**NS**	**NS**
Phloretin	NS	NS	NS	NS	NS	NS	NS
Phlorizin	*	**	*	NS	**	**	NS
Trilobatin	*	**	**	NS	NS	**	NS
TOTAL DIHYDROCHALCONES	*****	****	****	**NS**	****	****	**NS**
Luteolin	NS	**	NS	NS	NS	NS	NS
Luteolin-7-O-Glc	NS	NS	NS	**	NS	NS	NS
TOTAL FLAVONES	**NS**	****	**NS**	**NS**	**NS**	**NS**	**NS**
Naringenin	NS	**	*	**	NS	**	NS
TOTAL FLAVONONE	**NS**	****	*****	****	**NS**	****	**NS**
Kaempferol	*	**	**	**	**	**	NS
Quercetin	*	**	**	NS	NS	NS	NS
Taxifolin	**	**	NS	*	**	**	NS
Kaempferol-3-glucoside	NS	**	NS	NS	NS	NS	NS
Quercetin-3-glucoside	NS	*	NS	NS	NS	NS	NS
Isorhamnetin-3-glucoside	NS	**	NS	NS	NS	NS	NS
Quercetin-3-acetylglucoside	NS	**	NS	NS	NS	NS	NS
Kaempferol-3-rutinoside	NS	**	**	NS	**	NS	*
Quercetin-3-glucosilarabinoside	NS	*	NS	NS	NS	NS	NS
Rutin	NS	**	NS	NS	NS	NS	NS
Isorhamnetin-3-rutinoside	NS	**	NS	NS	**	NS	*
Quercetin-3,4-diglucoside	NS	NS	NS	NS	NS	NS	NS
Dihydrokaempferol	NS	**	**	NS	NS	NS	NS
Quercetin-3-glucuronide	NS	**	**	NS	NS	NS	NS
Kaempferol-3-glucuronide	NS	NS	NS	NS	NS	NS	NS
TOTAL FLAVONOLS	**NS**	****	****	**NS**	**NS**	**NS**	**NS**
Catechin	NS	**	*	NS	*	NS	*
Procyanidin B1	NS	**	NS	NS	*	NS	**
Procyanidin B3	NS	**	NS	NS	*	NS	**
TOTAL FLAVAN-3-OLS	**NS**	****	**NS**	**NS**	*****	**NS**	****
TOTAL PROANTHOCYANIDINS	****	****	****	*****	*****	**NS**	****

Pelargonidin-3-glucoside	NS	**	**	NS	NS	*	NS
Cyanidin-3-galactoside	NS	**	**	NS	NS	**	NS
Peonidin-3-galactoside	NS	NS	NS	NS	NS	NS	NS
Cyanadin-3-rutinoside	NS	NS	NS	NS	NS	NS	NS
Pelargonidin-3-rutinoside	NS	NS	NS	NS	NS	NS	NS
Peonidin-3-rutinoside	NS	NS	NS	NS	NS	NS	NS
TOTAL ANTHOCYANINS	**NS**	****	****	**NS**	**NS**	*****	**NS**
Anthranilic acid	NS	NS	NS	NS	NS	NS	
p-Hydroxybenzoic acid	*	**	**	**	*	NS	**
Cinnamic acid	NS	NS	NS	NS	NS	*	NS
Vanillin	NS	**	**	NS	NS	**	NS
Vanillic acid	NS	**	**	NS	NS	**	NS
2,6-Dihydroxy benzoic acid	*	**	NS	*	**	*	**
Methyl gallate	**	**	NS	**	**	*	**
Catechol	NS	**	*	NS	*	NS	*
TOTAL BENZOIC ACID & DERIVATIVES	**NS**	****	****	**NS**	*****	**NS**	*****
Agrimoniin	NS	**	**	*	**	NS	*
Casuarictin	NS	**	**	**	**	NS	**
Ellagic acid	NS	**	**	NS	*	NS	NS
TOTAL ELLAGITANNINS	**NS**	****	****	****	****	**NS**	****

Supplemental Figure 1. White and red fruits of Alba strawberry inoculated with *C. acutatum*, *or B. cinerea*. Pictures were taken at 24, 48 HPI and at 7 DPI.



Supplemental Figure 2. The shikimate pathway leading to the synthesis of hydrolysable tannins and to the phenylpropanoid and flavonoid pathways.



Chapter 4

Agrobacterium-mediated transformation of *FaMBL1* in strawberry: *in vitro* propagation of cisgenic lines for plant defense study

***Agrobacterium*-mediated transformation of *FaMBL1* in strawberry: *in vitro* propagation of cisgenic lines for plant defense study**

ABSTRACT

FaMBL1 is a gene encoding mannose-binding lectin (MBL) protein and was found to be up-regulated in white fruits of strawberry (*Fragaria* x *ananassa*) during early infection of *Colletotrichum acutatum*. *FaMBL1* along with other *MBLs* have been recognized to have a role in plant defense either through signal transduction or direct anti-fungal activity. In order to have a full concept on the mechanism and mode of action of *FaMBL1* in strawberry defense, the development of a model plant through a stable transformation was carried-out. In this study, the over-expression and silencing of *FaMBL1* through an *Agrobacterium*-mediated transformation of the strawberry cv. Calypso is presented, together with the initial report on the regeneration and validation of transformed lines.

KEYWORDS: mannose-binding lectin, stable transformation, over-expression, silencing, micropropagation

4. 1. INTRODUCTION

Fruit ripening is characterized as the changes in color, texture, aroma, and nutritional quality of mature seed-bearing plant organs, which is brought by the dynamic coordination of developmental and biochemical pathways in the organ[1]. In most fleshy fruits such as strawberry, ripening is defined with development of red color, modification of cell turgor and cell wall structure, and the accumulation of sugars, acids and volatiles[2]. The overall fruit eating quality of strawberry generally improves with ripening; however, these changes also influence the resistance or susceptibility of strawberry to diseases.

The different ripening stages of strawberry fruits respond differently to pathogen attack. Berries in the unripe stages (green and white) usually demonstrate disease resistance as compared to fruits in the ripe stage (red) as reported in several studies[3-6].

A transcriptomic analysis revealed the molecular profile of unripe and ripe strawberry fruits upon infection of *C. acutatum*. A mannose-binding lectin (*MBL*) gene, *FaMBL1,* was identified as the most up-regulated after 24 h of *C. acutatum* infection in white strawberry fruits where the pathogen is at a quiescent state[3,7] The expression of *FaMBL1* in white and red strawberry fruits was further investigated and it was found that the lectin gene was expressed exclusively in white fruits at 24 h post infection with *C. acutatum*[7]. The involvement of *MBL*s in plant defense against different pathogens has been demonstrated in other plant species such as *Capsicum annuum*, *Capsicum frutescens*, *Dioclea guianensis* and several orchid species, either through signal transduction or direct antifungal activity[8-12]. While several studies have already reported on the role of *MBL*s in plant defense, the mode of action and mechanism of the lectin group is not yet fully understood, as in the case of strawberry. To date, the most recent report on the possible involvement of *FaMBL1* on the resistance of white strawberry fruits to anthracnose was demonstrated through *Agrobacterium*-mediated transient gene

expression[7]. White and red fruits of strawberry were transformed through silencing and over-expression of *FaMBL1,* respectively and it was found that *FaMBL1*-silenced white fruits exhibited an increase in susceptibility to *C. acutatum*, while over-expression of *FaMBL1* in red fruits showed a lower susceptibility. Although the transient transformation of the strawberry fruits provided evidence on the involvement of *FaMBL1* in the resistance of white fruits against *C. acutatum*, a full understanding of the systemic response of strawberry plants with silenced and overexpressed *FaMBL1* is desired. This can be achieved by developing a stable transformation of *FaMBL1* in strawberry. In fact, in transient transformation, the foreign gene is expressed only for a finite period of time since they are not integrated into the genome. This limits the possibility of studying the mechanism and mode of action of *FaMBL1* on strawberry defense against *C. acutatum*. Moreover, the transfection of *A. tumefaciens* during transient transformation could interfere with the general physiology of the plant. To address these gaps, the development of a model plant through stable transformation was performed on the strawberry cultivar Calypso. Following the preparation of plasmids and competent *Agrobacterium* cells for the silencing and over-expression of the mannose-binding lectin gene, the regeneration of cisgenic lines is necessary to obtain stably transformed plants. In this study, the initial results of *in vitro* culture of strawberry and infection of *A. tumefaciens* along with the methods employed to validate transformation is presented.

4. 2. MATERIALS AND METHODS

The compositions of the media with their corresponding supplements used in this study were based from the publication "*Fragaria* x *ananassa* transformation" by Cappelletti et

al.[13]. All compounds and reagents in the study are purchased from Duchefa Biochemie, unless indicated otherwise. Details on the protocols and preparation of the media, antibiotics and other reagents used in the study are annexed as supporting information. All glasswares and laboratory equipment were sterilized for 20 min at 1 bar prior to use. As the experiment requires aseptic conditions, the procedures were performed under a laminar flow hood.

4.2.1. Plant materials

Micropropagated plants of the strawberry cultivar Calypso were kindly provided by the group of Dr. Bruno Mezzetti from the University of Ancona, Italy. These were multiplied in a basal media of MS salts supplemented with 30g/L sucrose and 7.5g/L plant agar (Supplemental Table 1). These *in-vitro* cultured strawberries were grown as a source of explants for the transformation and were sub-cultured regularly every four weeks. Newly expanded leaves coming from four-week old *in-vitro* grown Calypso were incised and lightly wounded by doing transverse cuts on the leaf surface (Figures 1a and 1b). The leaves were placed in sterile water until the commencement of the transformation experiment (Figure 1c). Around 100 leaves for each construct were used for the infection with *A. tumefaciens*.

4.2.2 Preparation of *Agrobacterium* strains and infection solution

The vectors pK7WG2 and pk7GWIWG2 (II) were used for the over-expression and silencing of *FaMBL1*, respectively, with kanamycin as the plant selectable marker. The procedures in plasmid construction are described in Guidarelli et al., 2014. Resulting plasmids were labeled as 35s:*FaMBL1* for over-expression and pK7:*FaMBL1* for

silencing and were introduced into *A. tumefaciens* strain EHA105 using the freeze-thaw shock method[14] (Supplemental Information 1).

A small colony of the *A. tumefaciens* strain containing 35s:*FaMBL1* and pk7:*FaMBL1* were grown in 10 mL LB liquid medium with 40µl rifampicin (25mg/ml) and 15µL spectinomycin (50mg/ml) (Supplemental Table 2) in a 50 mL flask. The cultures were covered with a cotton plug and sealed with parafilm and incubated overnight at 28°C on a shaker at 100-150 rpm. Once the culture reached the optical density (OD) of 0.6 at 600nm, the cells were pelleted at 2,500 g for 15 min and re-suspended in 40 mL leaf infection solution (Supplemental Table 1). Acetosyringone and proline were further added to the leaf infection solution and were mixed vigorously on a shaker (100-150 rpm) at 28°C on for 5 h to activate *Agrobacterium* cell division and virulence.

4.2.3. Infection and co-culturing of leaf explants

The incised leaves were retrieved from the sterile water and transferred to a falcon tube with 30 mL leaf infection solution (Figure 2a). The tubes were left for 15 minutes with shaking at 100-150 rpm (Figure 2b). For each construct, 100 leaf explants were used. Following the infection, the leaves were blotted onto sterile filter papers, and transferred in Petri dishes with *Agrobacterium*-plant co-culturing medium (Supplemental Table 1), with the abaxial side of the leaf touching the medium (Figures 3a-3c). A 500 µL liquid solution comprising the same components of the solid medium was sprayed onto the leaflets in each plate (Figure 3d). Ten Petri dishes of the were prepared for each construct and were incubated for 48 h at 25°C in dark conditions. After incubation, the infection was suspended by transferring the leaves in a falcon tube containing 50 mL of washing solution (Supplemental Table 1) and was left for five hours at 25°C with shaking at 100-150 rpm. Leaves were again blotted on a sterile filter paper after washing.

4.2.4. Leaf tissue regeneration and selection

The leaves infected with *A. tumefaciens* were transferred on a selection medium supplemented with plant hormones, the antibiotic kanamycin (50mg/L) as the selective agent, and cefotaxime (200mg/L) as a decontaminant (Supplemental Table 1). The abaxial side of the leaf in was placed in contact with the medium. The leaf tissues were sub-cultured every two weeks on freshly prepared selection media until differentiation. The explants were initially incubated for two weeks at continuous dark, then at a photoperiod of 16 h light and 8 h dark at 24°C. Upon emergence of green and elongated shoots, the regenerated tissue were excised from the callus and transferred in test tube containing shoot regeneration medium (Supplemental Tables 1&2). Isolated shoots were kept in growth chambers and sub-cultured every two weeks in freshly prepared shoot regeneration medium until the shoots proliferated.

4.2.5. PCR analysis for confirmation of transformation

PCR analysis was performed to test if the regenerated plant lines are genetically transformed. Two PCR protocols were used for the purpose of result validation. The direct protocol of the Phire Plant Direct PCR kit (Thermo Scientific, Massachusets, USA) was first tested following the procedures provided by the manufacturer (Supplemental Information 2). The regenerated plants were also tested with a protocol on PCR amplification of DNA from plants[15] (Supplemental Information 3).

To verify plant transformation, the plasmid region between the promoter P35S and the ATTR2 cassette in which *FaMBL1* was cloned, was amplified using P35S2 and ATTB2 primers (Figure 4). An amplicon of 1,481 bp and 452 bp, was expected for the over-expression and silencing, respectively. Additional analysis was performed with the use of

specific primers for control (Supplemental Table 3). PCR products were visualized on a 1% agarose gel, containing gel red (5 µl/50 ml 1xTAE) and visualized under UV light.

4.3. RESULTS AND DISCUSSION

The importance of genetic analysis in understanding the role of genes in biological process is gaining momentum, especially with the advent of new sequencing technologies where a specific gene with a known sequence is modified and the phenotype is analyzed to determine corresponding gene function. This approach, known as reverse genetics, is accomplished though stable transformation or transient expression of a target gene[16]. In the present study, a stable transformation through leaf regeneration was carried out to develop model plants with over-expressed and silenced *FaMBL1,* a gene which is found to be correlated with the resistance of white strawberry fruits against *C. acutatum*[3]. *Agrobacterium tumefaciens*-mediated transformation was employed as it was reported to be the most effective and widely used method for strawberry gene engineering[17]. Meanwhile, plant regeneration through i*n vitro* technique are important tools for modern plant improvement programs to introduce new traits into selected plants in a minimum time[18]. In order to successfully develop transformed lines, an efficient regeneration and transformation system that provides the protocol for the selection and recovery of genetically modified plants is essential[17]. The importance of maintaining aseptic conditions in transformation is also essential to achieve successful regeneration[19].

4.3.1. Strawberry *in vitro* culture of plant materials

Plant regeneration is said to be a crucial aspect of biotechnology-related tissue culture as it facilitates the production of genetically engineered plants[19]. There are three steps of

adventitious tissue regeneration from strawberry explants: i) formation of viable adventitious bud on explants, ii) elongation of buds into shoots, and iii) rooting of the shoots to form whole plants. Factors such as genotype, culture medium, physical conditions determine the success of adventitious shoot regeneration. In our study, the strawberry cultivar Calypso was used in the experiment due to reports of successful genetic transformation with the cultivar in the past[20,21]. The leaves of Calypso were used as explants in adventitious tissue regeneration as it gives 40-60% more regeneration level compared to other organs such as petioles, stipules and roots[22].

4.3.2. *Agrobacterium* strains and leaf infection solution

The infection of the leaf explants with *Agrobacterium* is a major determinant of the success of plant transformation. Fresh cultures of *A. tumefaciens* (not older than 72 h) harboring the plasmid must be used in the preparation of the inoculation suspension to ensure viability of the strain. Prior to the preparation of the *Agrobacterium* suspension, colonies of the strains were already re-cultured two days ahead of the scheduled transformation.

To induce the virulence of *A. tumefaciens,* the importance of adding acetosyringone[23] and the proline to the leaf infection solution should also be noted. The effects of the plant signal molecule acetosyringone and the osmoprotectant proline have been reported to increase the transformation efficiency of *A. tumefaciens*[24].

4.3.3. Explant differentiation and shoot regeneration

Agrobacterium infected leaves were transferred in a selection medium supplemented with cytokinin-like hormone (thidiazuron) and auxin (2,4-D) to facilitate cell division and differentiation. Callus formation from the explants was observed after the first sub-

culturing. On the third sub-culturing, small, green protrusions were observed from the mass of the yellowish callus, which were identified as the regenerated shoots. The regenerated shoots, which are usually of 2-4 cm length, were detached from the callus and transferred to test tubes containing shoot proliferation medium. One important factor in successful transformation is the selection and recovery of cells following organogenesis[19], hence it is crucial to select only differentiated shoots for subsequent culturing in shoot proliferation medium.

The adventitious shoots were sub-cultured every 2 weeks in a new shoot proliferation medium until they were transformed. For the cultivar Calypso, previous studies indicated that successful regenerated lines could be visually judged when plantlets are showing more homogenous green color and could be achieved after 2-3 sub-cultures[13]. As observed in our study, newly proliferated leaves from the first sub-culture were initially translucent (Figure 5a), and eventually became darker and more opaque after sub-culturing the plants for the fourth time (Figure 5b).

4.3.4. Kanamycin as selection agent

The plasmids used in this study possess the *R-KAN* gene, which confers resistance to the antibiotic kanamycin. The introduction of kanamycin in the selection and shoot proliferation media facilitates the selection of cisgenic cells by inhibiting the growth of those that are not resistant to the antibiotic and allowing only the growth of transformed cells.

Kanamycin selection of cisgenic cells is either performed iteratively or non-iteratively. Iterative selection is characterized by a progressive increasing of kanamycin concentration, while non-iterative selection was represented by a constant kanamycin concentration of according to a preliminary toxicity threshold test. In our study, a non-

iterative selection was performed using kanamycin concentration at 50mg/L in both selection and shoot proliferation medium. This choice was based on previous *Agrobacterium* transformation in strawberry using kanamycin as the selection agent[13].

4.3.5. PCR analysis of cisgenic lines

Emergence of green, opaque leaves from the regenerated plants was considered as a sign of a probable successful transformation[13]. In order to validate the transformation, PCR test was performed on lines that were visually identified as transformed.

A total of eight regenerated lines were tested with PCR analysis and gel electrophoresis. Of the eight, two lines were over-expressing *FaMBL1* and six were silencing it. Earlier during the sub-culturing of adventitious shoots, problems with contamination of the cultures especially, with *Penicillium* spp*.,* were encountered. A substantial number of possible cisgenic lines were lost due to the contamination without being genetically analyzed.

The PCR analysis conducted for the verification of transformed lines were tested with two protocols. Initially, the Phire Plant Direct PCR kit (Thermo Scientific) was used with the target primers (P35S2 and ATTB2) (Figure 4). Although the leaf samples collected were opaque and dark green in color, a negative result was obtained from the lines tested. With the same kit, another PCR analysis was performed on the same samples using the overLEC and RKan (Figure 4) as primers for control. The overLEC are specific primers used to amplify the intrinsic lectin in the strawberry plant and was used as a control for the protocol followed. On the other hand, RKan was used to amplify the kanamycin resistance gene that was localized together with *FaMBL1* during the transformation, thus a positive PCR result will verify that lines are transformed. The PCR of samples with specific primers for strawberry lectin (overLEC) resulted in positive amplifications

(Figure 5a), demonstrating that the PCR protocol followed is working. However, no PCR product was visualized after amplifying the kanamycin resistance gene with the RKan primers, indicating that the regenerated lines are not transformed. To validate these findings, another PCR protocol was used which involves direct DNA extraction[15]. Similar with the Phire Plant Direct PCR kit, a positive result was observed when the samples were analyzed with the specific overLEC primers (Figure 6b). In the same way, the analysis with the target primers and RKan primers did not give a positive indication that the plants tested are indeed transformed.

4.4. SUMMARY AND RECOMMENDATIONS

The transformation of strawberry plants was carried out to produce cisgenic strawberry lines with silenced and over-expressed *FaMBL1,* which will allow the study of the mechanism of the gene in strawberry, specifically during infection of *C. acutatum.* The *FaMBL1* was demonstrated to be involved in the resistance of white strawberry fruits to fungal infection.

The experiments performed were focused on the regeneration of transformed Calypso strawberry following three major procedures: 1) proliferation of plant source material; 2) infection of leaf explants with *A. tumefaciens,* and; 3) PCR analysis for the genetic validation of transformed plants. A total of eight cisgenic lines were regenerated *in vitro* (two over-expressed and six silenced *FaMBL1*).

Despite the development of opaque and dark green leaves, the PCR analysis of the lines produced from the silencing and over-expression of *FaMBL1* gave negative results. The samples collected for the extraction of the genomic DNA could affect the PCR result due to the possibility that only a small portion of the plant has been transformed at the time

of analysis. Another probable reason could be the low quantity of transformed cells and is not sufficient for PCR detection.

In order to overcome the problem on the validation of transformed line, the use of fluorescent marker such as the Green Fluorescent Protein (GFP) will be integrated in future transformations. The GFP will allow the verification of transformed plants through fluorescent stereomicroscope allowing a first visible selection of transformants.

Aside from Calypso which is an everbearing strawberry cultivar, performing the transformation on short-day cultivar such as Sveva is also considered for a differential comparison on *FaMBL1* mode of action against *C. acutatum.*

Finally, kanamycin sensitivity screening for the next transformations is also recommended in order to determine the optimum kanamycin concentration for our experiment. The iterative selection is considered as this allows rapid growth of callus and early shoot initiation, while the eventual higher concentrations eliminate non-cisgenic shoots from surviving[25].

4.5. REFERENCES

1. Barry, C. S.; Giovannoni, J. J., Ethylene and Fruit Ripening. *Journal of Plant Growth Regulation* **2007,** *26* (2), 143-159.
2. Giovannoni, J. J., Genetic regulation of fruit development and ripening. *The plant cell* **2004,** *16* (suppl 1), S170-S180.
3. Guidarelli, M.; Carbone, F.; Mourgues, F.; Perrotta, G.; Rosati, C.; Bertolini, P.; Baraldi, E., Colletotrichum acutatum interactions with unripe and ripe strawberry fruits and differential responses at histological and transcriptional levels. *Plant Pathology* **2011,** *60* (4), 685-697.
4. Li, Q.; Ji, K.; Sun, Y.; Luo, H.; Wang, H.; Leng, P., The role of FaBG3 in fruit ripening and B. cinerea fungal infection of strawberry. *The Plant Journal* **2013,** *76* (1), 24-35.
5. Neri, F.; Cappellin, L.; Spadoni, A.; Cameldi, I.; Algarra Alarcon, A.; Aprea, E.; Romano, A.; Gasperi, F.; Biasioli, F., Role of strawberry volatile organic compounds in the development of Botrytis cinerea infection. *Plant Pathology* **2015,** *64* (3), 709-717.
6. Weber, N.; Veberic, R.; Mikulic-Petkovsek, M.; Stampar, F.; Koron, D.; Munda, A.; Jakopic, J., Metabolite accumulation in strawberry (Fragaria × ananassa Duch.) fruits and runners in response to Colletotrichum nymphaeae infection. *Physiological and Molecular Plant Pathology* **2015,** *92*, 119-129.
7. Guidarelli, M.; Zoli, L.; Orlandini, A.; Bertolini, P.; Baraldi, E., The mannose-binding lectin gene FaMBL1 is involved in the resistance of unripe strawberry fruits to Colletotrichum acutatum. *Molecular plant pathology* **2014,** *15* (8), 832-840.
8. Hwang, I. S.; Hwang, B. K., The pepper mannose-binding lectin gene CaMBL1 is required to regulate cell death and defense responses to microbial pathogens. *Plant physiology* **2011,** *155* (1), 447-463.
9. Ngai, P. H. K.; Ng, T. B., A lectin with antifungal and mitogenic activities from red cluster pepper (Capsicum frutescens) seeds. *Applied microbiology and biotechnology* **2007,** *74* (2), 366-371.
10. Araújo-Filho, J. H.; Vasconcelos, I. M.; Martins-Miranda, A. S.; Gondim, D. M. F.; Oliveira, J. T. A., A ConA-like lectin from Dioclea guianensis Benth. has antifungal activity against Colletotrichum gloeosporioides, unlike its homologues, ConM and ConA. *Journal of agricultural and food chemistry* **2010,** *58* (7), 4090-4096.
11. Wang, X.; Bauw, G.; Van Damme, E. J. M.; Peumans, W. J.; Chen, Z. L.; Van Montagu, M.; Angenon, G.; Dillen, W., Gastrodianin-like mannose-binding proteins: a novel class of plant proteins with antifungal properties. *The Plant Journal* **2001,** *25* (6), 651-661.
12. Sudmoon, R.; Sattayasai, N.; Bunyatratchata, W.; Chaveerach, A.; Nuchadomrong, S., Thermostable mannose-binding lectin from Dendrobium findleyanum with activities dependent on sulfhydryl content. *Acta biochimica et biophysica Sinica* **2008,** *40* (9), 811-818.
13. Cappelletti, R.; Sabbadini, S.; Mezzetti, B., Strawberry (Fragaria × ananassa). In *Agrobacterium Protocols*, Wang, K., Ed. Springer New York: 2015; Vol. 1224, pp 217-227.
14. Holsters, M.; Waele, D.; Depicker, A.; Messens, E.; Montagu, M.; Schell, J., Transfection and transformation of Agrobacterium tumefaciens. *Molecular and General Genetics MGG 163* (2), 181-187.
15. Bellstedt, D. U.; Pirie, M. D.; Visser, J. C.; de Villiers, M. J.; Gehrke, B., A rapid and inexpensive method for the direct PCR amplification of DNA from plants. *American Journal of Botany* **2010,** *97* (7), e65-e68.
16. Guidarelli, M.; Baraldi, E., Transient transformation meets gene function discovery: the strawberry fruit case. *Name: Frontiers in Plant Science* **2015,** *6*, 444.
17. Qin, Y.; Teixeira da Silva, J. A.; Zhang, L.; Zhang, S., Transgenic strawberry: State of the art for improved traits. *Biotechnology Advances* **2008,** *26* (3), 219-232.
18. Taji, A.; Kumar, P. P.; Lakshmanan, P.; Taji, A.; Lakshmanan, P., *In vitro plant breeding*. Food Products Press: 2002.
19. Debnath, S. C.; Teixeira da Silva, J. A., Strawberry culture in vitro: applications in genetic transformation and biotechnology. *Fruit, Vegetable and Cereal Science and Biotechnology* **2007,** *1* (1), 1-12.

20. Folta, K. M.; Dhingra, A., Invited review: Transformation of strawberry: The basis for translational genomics in Rosaceae. *In Vitro Cellular & Developmental Biology-Plant* **2006,** *42* (6), 482-490.
21. Lunkenbein, S.; Coiner, H.; de Vos, C. H. R.; Schaart, J. G.; Boone, M. J.; Krens, F. A.; Schwab, W.; Salentijn, E. M. J., Molecular characterization of a stable antisense chalcone synthase phenotype in strawberry (Fragaria× ananassa). *Journal of Agricultural and Food Chemistry* **2006,** *54* (6), 2145-2153.
22. Passey, A.; Barrett, K.; James, D., Adventitious shoot regeneration from seven commercial strawberry cultivars (Fragaria× ananassa Duch.) using a range of explant types. *Plant cell reports* **2003,** *21* (5), 397-401.
23. Gelvin, S. B., Agrobacterium-mediated plant transformation: the biology behind the "gene-jockeying" tool. *Microbiology and molecular biology reviews* **2003,** *67* (1), 16-37.
24. James, D. J.; Uratsu, S.; Cheng, J.; Negri, P.; Viss, P.; Dandekar, A. M., Acetosyringone and osmoprotectants like betaine or proline synergistically enhance Agrobacterium-mediated transformation of apple. *Plant cell reports* **1993,** *12* (10), 559-563.
25. Mathews, H.; Dewey, V.; Wagoner, W.; Bestwick, R. K., Molecular and cellular evidence of chimaeric tissues in primary transgenics and elimination of chimaerism through improved selection protocols. *Transgenic Research* **1998,** *7* (2), 123-129.

4.6. FIGURES

Figure 1. Preparation of leaf explants: A) Incision of leaves from in-vitro cultured 'Calypso' strawberry; B) Wounding of leaves with a scalpel (inset: leaflets showing transverse cuts on surface; C) Soaking of leaflets in sterile water.

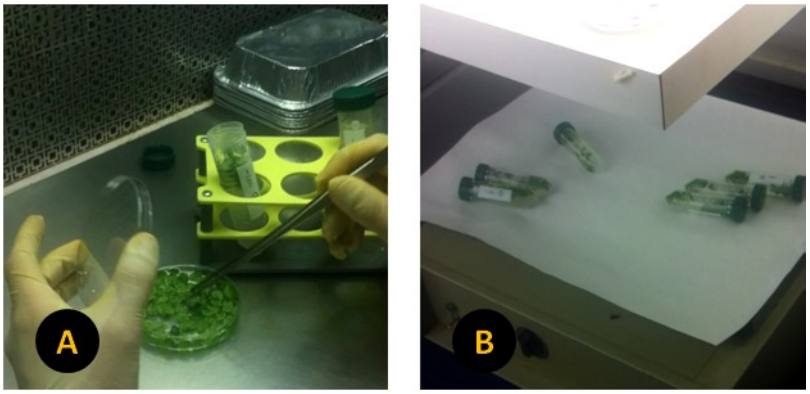


Figure 2. Infection of leaflets with *A. tumifaciens:* **A)** Transferring of strawberry into a falcon tube containing leaf infection solution; **B)** On-going infection, facilitated by shaking for 15 min at 100-150 rpm.

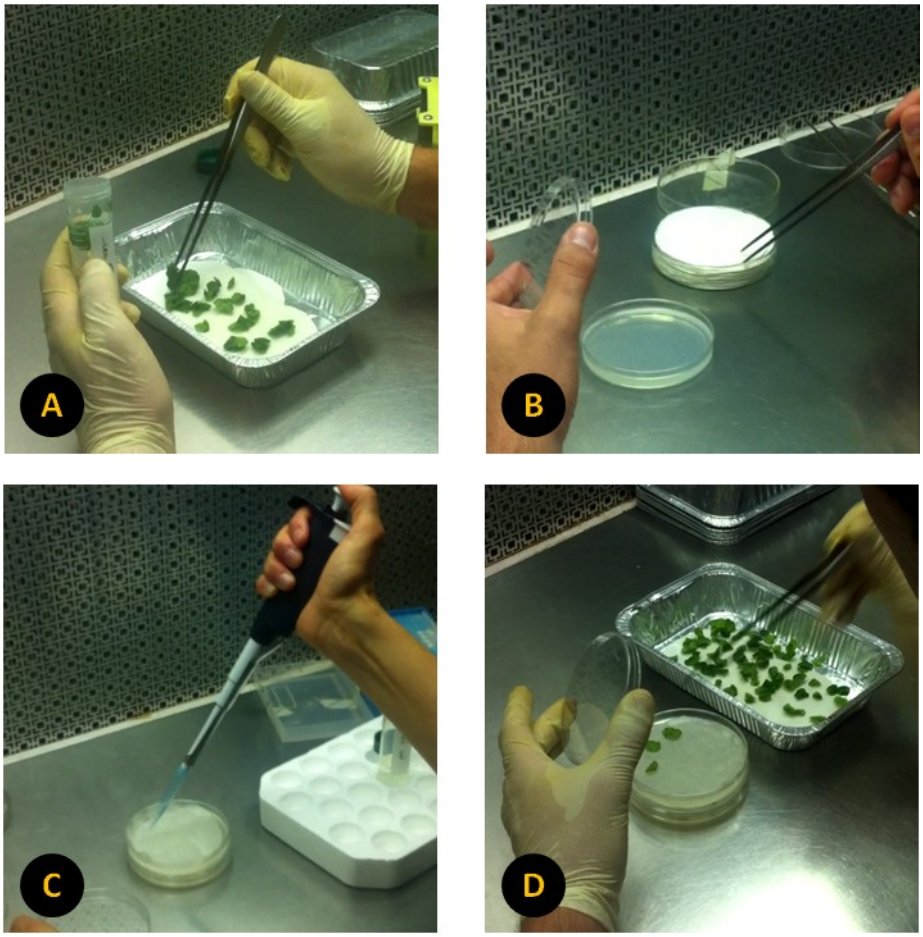


Figure 3. Co-culturing of leaflets: **A)** Laying-out a filter paper over a solid *Agrobacterium*-plant co-culturing medium; **B)** Spraying of *Agrobacterium*-plant co-culturing liquid solution over the filter paper; **C)** Retrieval of leaflets from the leaf infection solution and blotting on sterile paper; **D)** Transferring of leaflets onto the *Agrobacterium*-plant co-culturing medium.

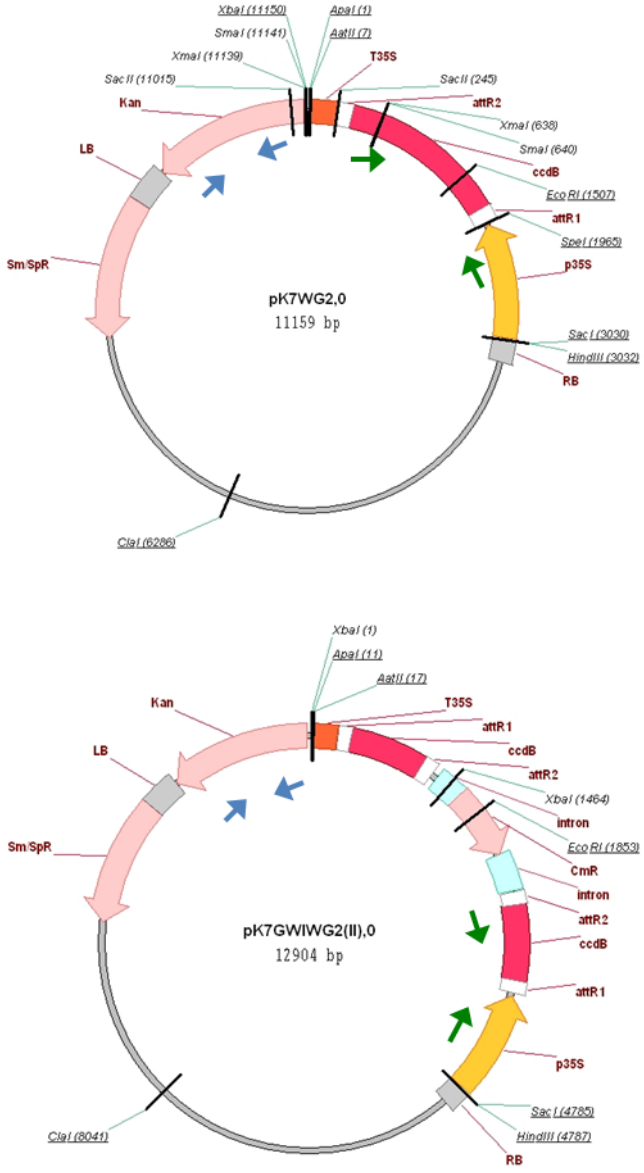


Figure 4. Gateway vectors used for cloning *FaMBL: (top)*—over-expression; *(bottom)*—silencing. Indicated in the maps are the regions in which the primers used the PCR analysis were drawn. The blue arrow indicate the kanamycin resistant region (RKan), while the green arrows indicates plasmid region between the promoter P35S and the ATTR2 cassette in which *FaMBL1* was cloned (P35S2 and ATTB2 primers).

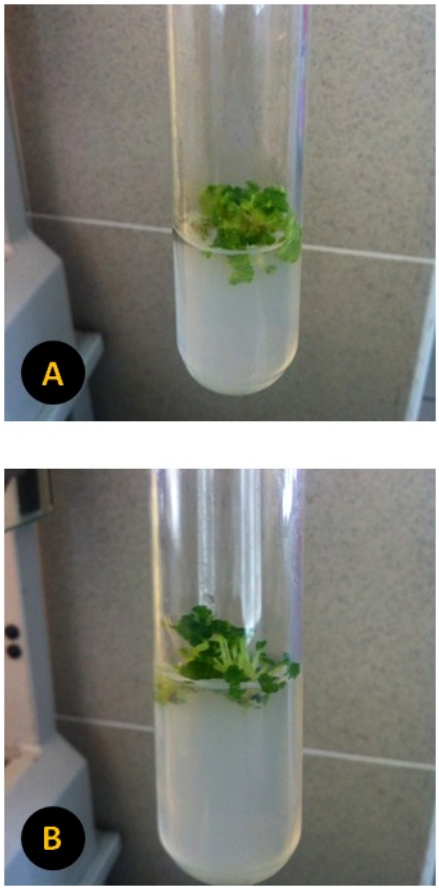


Figure 5. Regenerated plantlets of Calypso with over-expressed *FaMBL1*: **A)** Developing leaflets appear translucent after the initial sub-culturing in shoot regeneration medium; **B)** After the fourth sub-culturing, leaflets become opaque and develop dark green color.

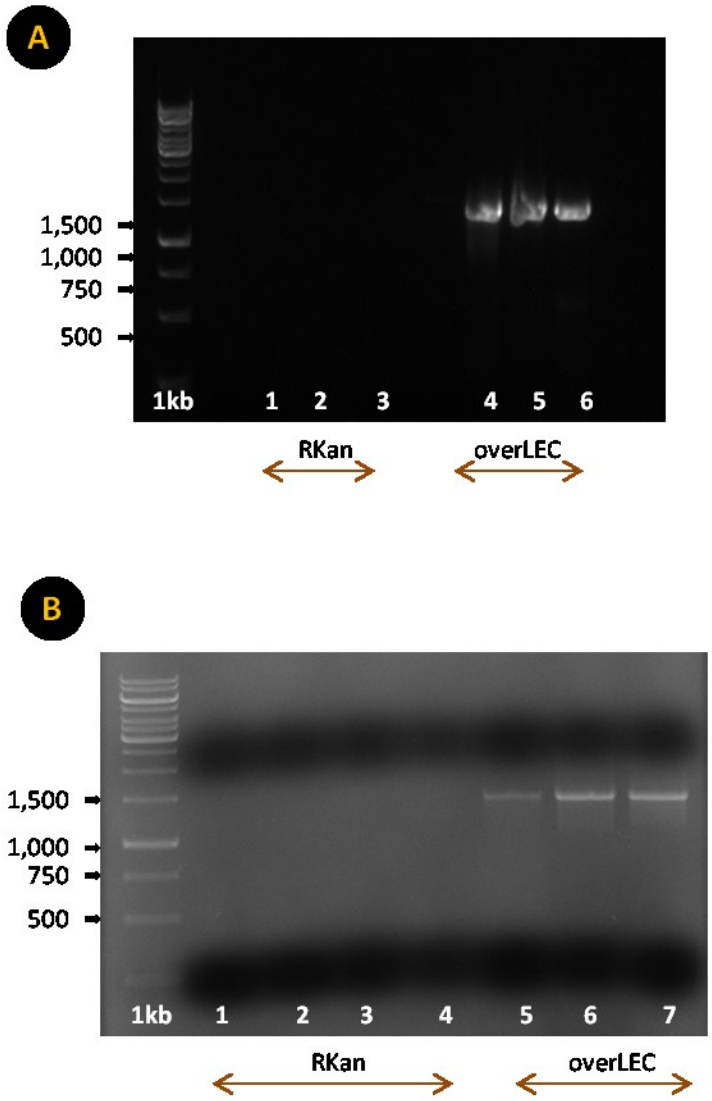


Figure 6. Electrophoresis of genomic DNA extracted from probable cisgenic lines with Rkan and OverLec primer using two protocols: **A)** Phire Plant Direct PCR kit; **B)** Direct PCR amplification of DNA from plants by Bellstedt et al., 2010.

4.7. SUPPORTING INFORMATION

Supplemental Information 1. Freeze and thaw shock transformation for *Agrobacterium* strains (Holsters et al., 1978).

DAY 1

1. Pick a single colony and inoculate 3 mL of LB + 12 µL Rifampicin.
2. Grow at 28°C overnight.

DAY 2

3. Inoculate 50 mL of LB in a 250 mL flask with 1 mL (1/50 volume) of the overnight culture.
4. Grow at 28°C until OD_{600} is between 0.5 and 1.0 (around 3 – 4 hours).
5. Chill culture for 5 – 10 minutes in ice.
6. Centrifuge at 3,000 rpm for 5 minutes.
7. Discard supernatant and re-suspend pellet in 1 mL of ice cold 20 mM $CaCl_2$.
8. Dispense 0.1 mL of bacterial suspension into pre-chilled 1.5 mL centrifuge tubes.
9. Add 1 µg of plasmid DNA to one tube and nothing in another (control).
10. Freeze tubes in liquid N_2; thaw tubes for ca. 5 minutes at 37°C.
11. Add 1 mL of LB to each tube and transfer content to 15 mL tube.
12. Incubate for ca. 2 hours at 30°C.
13. Centrifuge the tubes for 5 minutes at ca. 4,000 rpm to pellet cells.
14. Remove supernatant and re-suspend pellet in 100 µL of LB.
15. Plate all.

Supplemental Information 2. Direct PCR protocol from Phire Plant Direct PCR Kit (Thermo Fisher, Scientific).

A. Plant Material

- Take a 0.5 mm diameter sample from the plant leaf and place it directly into the PCR reaction (20–50 µL in volume).
- **Young leaves are recommended for use.**
- Fresh plant material is usually the best choice, even though plant material stored at 4 °C, frozen or on commercially available cards such as Whatman 903 and FTACards can also be used.
- It is recommended to add sample into a liquid, rather than onto the wall of an empty tube. Make sure that you see the sample in the solution.

B. PCR reaction mix

Component	20 µL reaction	Final concentration
H2O	add to 20 µL	
2X Phire Plant PCR Buffer	10 µL	1X
Primer A	2.5 µ L	0.5 µM
Primer B	2.5 µ L	0.5 µM
Phire Hot Start II DNA Polymerase	0.4 µL	
Plant tissue	0.5 mm diameter leaf sample	

C. PCR Cycling condition

Cycle step	Temperature	Time
Initial denaturation	98°C	5 min
Denaturation	98°C	5 s
Annealing		
Primers:		
1. P35S2 & ATTB2	52°C	5 s
2. Lec	62°C	5s
3. RKan	60°C	5s
Extension	72°C	20 s[a]
		30 s[b]
	40 CYCLES	
Final extension	72°C	1 min

[a] *samples with silenced FaMBL1*

[b] *samples with overexpressed FaMBL1*

Supplemental Information 3. Direct PCR protocol by Bellstedt et al., 2010, with modifications.

A. Preparation of grinding and GES buffer

***** (reagents used in this protocol are from Sigma)

Grinding buffer

15 mM Na 2 CO 3
35 mM NaHCO 3
2% (m/v) PVP 40
0.2% (m/v) BSA
0.05% (v/v) Tween 20
1% (m/v) sodium metabisulphite

- To prepare 100 mL of grinding buffer, dissolve all components except Tween 20 in double-distilled sterile water.
- Stir at low speed to avoid excessive foaming.
- Once all components have dissolved, adjust pH to 9.6 using NaOH solution.
- Add Tween 20.
- Autoclave and store at 4 ° C.

GES buffer

0.1 M glycine-NaOH, pH 9.0
50 mM NaCl
1 mM EDTA, pH 8.0
0.5% (v/v) Triton X-100

To prepare GES buffer, make up the following stock solutions:

- 1 M Glycine, pH 9.00 (200 mL).
  - Dissolve 15.014 g glycine in 200 mL double-distilled sterile water.
  - Adjust pH to 9 using NaOH pellets.
  - Autoclave
  - Store at room temperature.
  - Use 10 mL of 1 M glycine stock per 100 mL GES buffer.
- 5 M NaCl (200 mL)
  - Dissolve 58.44 g NaCl in 200 mL double-distilled sterile water with mild heating.
  - Autoclave.
  - Store at room temperature.
  - Use 1 mL of 5 M NaCl stock per 100 mL GES buffer.
- M EDTA, pH 8.00 (100 mL)
  - Dissolve 3.723 g EDTA in 100 mL double-distilled sterile water.
  - Adjust pH to 8 using NaOH pellets.
  - Autoclave.
  - Store at room temperature.
  - Use 1 mL of 0.1 M EDTA stock per 100 mL GES buffer.

To prepare 100 mL of GES buffer, add the volumes of the above-mentioned components and make up the remaining volume with double-distilled sterile water. Autoclave and pipette aliquots of 500 µ L (500 µ L of GES is enough to treat 20 samples). Store aliquots and stock at -20 ° C.

B. Plant sample and template preparation

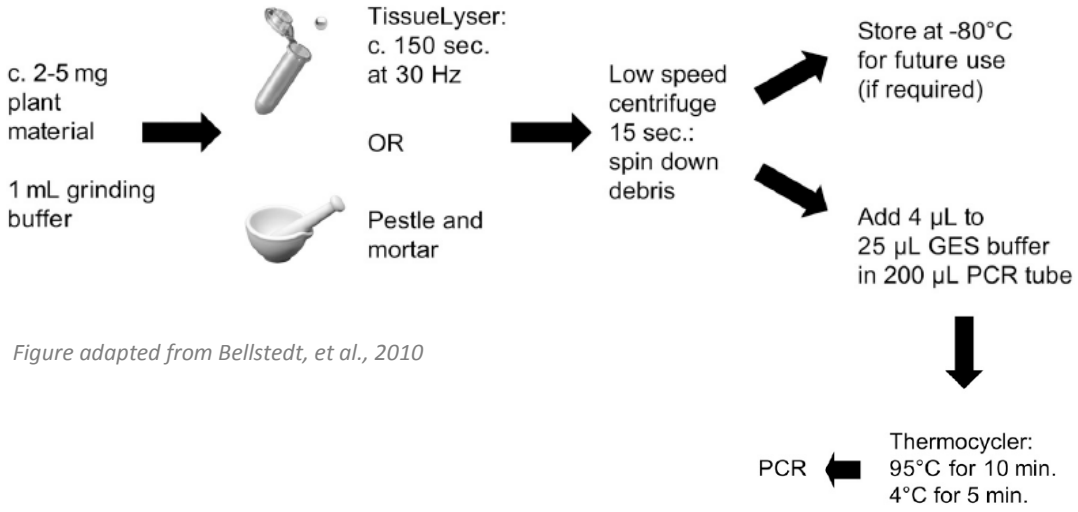


Figure adapted from Bellstedt, et al., 2010

C. PCR reaction mix

Component	50 µL reaction	Final concentration
H2O	add to 50 µL	
$MgCl_2$	5 µL	
Buffer	10 µL	1X
Taq Polymerase	0.25 µL	
dNTPs	4 µL	
Primer A	X µ L	0.5 µM
Primer B	X µ L	0.5 µM
Plant tissue	1 µL	

D. PCR cycling condition

Cycle step	Temperature	Time
Initial denaturation	98°C	5 min
Denaturation	98°C	5 s
Annealing		
Primers:		
1. P35S2 & ATTB2	52°C	5 s
2. Lec	62°C	5s
3. RKan	60°C	5s
Extension	72°C	20 s[a] 30 s[b]
	40 CYCLES	
Final extension	72°C	1 min

[a] *samples with silenced FaMBL1*
[b] *samples with overexpressed FaMBL1*

Supplemental Table 1. List of reagents and procedures in the preparation of media utilized in the study.

Media	Reagents	Remarks
MS basal medium/ MS0	4.4 g/L MS 7.5 g/L plant agar 30 g/L sucrose	-autoclave
LB liquid medium	10 mL of Luria broth	-autoclave
	40 µl rifampicin 15 µl spectinomycin	-add antibiotics only when media bottle is cool to touch
MS20	4.4 g/L MS 20 g/L sucrose	-autoclave
Leaf infection solution	40 mL of MS20 40 µl proline 40 µl acetosyrigone	-proline and acetosyringone to be added after autoclaving MS 20
Agrobacterium-plant co-colturing medium	MS0	-MS0 to be poured on Petri dish
	500 µl leaf infection solution	-to be applied over filter paper
Leaf tissue washing solution	50 mL sterile H2O 250 µl cefotaxime (100 mg/L of 500 mg/L stock)	
Selection medium	7.5 g/L plant agar 30 g/L sucrose 4.4 g/L MS with vitamins 500 µl TDZ (0.5 mg/L of 1 mg/ml stock) 20 µl 2,4D (0.02 mg/L of 1mg/ml stock)	-adjust pH to 5.6-5.7 -autoclave
	1 mL kanamycin (50 mg/L of 50 mg/ml stock) 2 ml cefotaxime (200 mg/L of 100 mg/ml stock)	-perform antibiotics under hood -add antibiotics only when media bottle is cool to touch
Shoot regeneration medium	7.5 g/L plant agar 30 g/L sucrose 4.3 g/L MS 250 µl 6-BAP (0.25 mg/L of 1mg/ml stock)	-autoclave
	1 mL kanamycin (50 mg/L of 50 mg/ml stock) 2 ml cefotaxime (200 mg/L of 100 mg/ml stock)	-perform antibiotics under hood -add antibiotics only when media bottle is cool to touch

Supplemental Table 2. Stock of antibiotics, hormones and other solutions used in the study.

Antibiotic/hormone/solution	Weight/volume	Remarks
THIDIAZURON (TDZ, 1mg/ml)	10 mg TDZ 10 ml sterile H_2O	-store at -20°C
2,4-Dichlorophenoxyacetic acid (2,4-D, 1mg/ml)	50 mg 2,4-D 1 drop ethanol or NaOH, 1N	-arrive at a volume of 50 mL with sterile H_2O -store at 4°C
6-Benzylaminopurine (6-BAP, 1mg/ml)	50 mg 6-BAP 1 small drop KOH, 1M	-arrive at a volume of 50 mL with sterile H_2O -store at -20°C
Kanamycin monosulfate (50mg/ml)	500 mg kanamycin 10 mL sterile H_2O	-aliquot in 1.5 mL tubes - store at -20°C
Cefotaxime (100mg/ml)	1 g cefotaxime 10 mL sterile H_2O	-aliquot in 1.5 mL tubes - store at -20°C
Acetosyringone (20mg/mL)	100 mg acetosyringone 5 ml ethanol	-filter -store at -20°C
Proline (200mg/ml)	1 g proline 5 mL sterile H_2O	-filter -store at -20°C
Spectinomycin (50mg/ml)	500 mg spectinomycin 10 mL sterile H_2O	-aliquot in 1.5 mL tubes - store at -20°C
Rifampicin (25mg/ml)	250 mg rifampicin 10 mL methanol	-aliquot in 1.5 mL tubes - store at -20°C

Supplemental Table 3. Primers used for the PCR analysis.

Gene	Sequence	bp
P35S2 ATTB2	5´-ATTACAATTTACTATTCTAGTCG-3´ 5´-ACCACTTTGTACAAGAAA-3´	1,481 bp for overexpressed 452 bp for silenced
OverLec (Fw) OverLec (Rv)	5´-CACCATGTCTTCTCCTTCATTCACAAT-3´ 5´-TATACTAGTGATTAGGTGCCTTGATGT-3´	1,329 bp
RKan (Fw) RKan (Rv)	5´-ATCTGGTGGAAAATGGCCG-3´ 5´-AGCAATATCACGGGTAGCCA-3´	102 bp

Chapter 5

Conclusion

The study was conducted to investigate on the interaction of unripe and ripe strawberry fruits to *C. acutatum* and *B. cinerea*, at the same time identify the properties that regulate the ontogenic resistance in strawberries.

Three different approaches were studies were performed elucidate the objective:

i). trancriptomic analysis of white and red fruits of woodland strawberry inoculated with *B. cinerea;*

ii). metabolomic analysis of white and red strawberry fruits inoculated with *C. acutatum* and *B. cinerea*; and,

iii). *Agrobacterium*-mediated transformation to silence and over-express *FaMBL1*.

The RNA-Seq analysis provided over 2,000 DEGs in the four conditions studied. The transcriptome profile of white inoculated fruits suggests that the resistance of the unripe stages is due a general up-regulation of defense-related. Meanwhile, in the transcriptome profile of red inoculated fruits, PR-related proteins were found to be regulated, which suggests the activation of the pathogens from quiescence in the ripe fruits. A more comprehensive synthesis of the results is needed in order to exhaust the information obtain from the analysis.

Meanwhile, the metabolomic analysis white and red strawberry fruits revealed that proanthocyanidins, flavan-3-ols and ellagitannins are constitutive of the pre-formed biochemical barriers in unripe fruits. A time specific analysis also revealed the accumulation of condensed and hydrolyzed tannins in whit fruits infected with *C. acutatum* and *B. cinerea* after 48 h of infection.

The attempt to study the mechanism of *FaMBL1* through the regeneration of cisgenic plants is quite a long feat. As the first part of the process, the regeneration of plantets was carried out; however the first lines that were proliferated resulted in a negative

transformation. A new batch of transformation will performed in the future, and this time the kanamycin sensitivity will also be performed.

The results generated in this study, especially from the transciptomic and metabolomic analysis could be used for the development of new cultivars less susceptible to *C. acutatum* and *B. cinerea.*

ANNEX

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