Final Report

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Student Project No. SF TF 170

Title: Identifying novel powdery mildew susceptibility and resistance genes in strawberry

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CONTENTS (right-click and select ‘update field’ and ‘update entire table’ to update table of contents)

1. Industry Summary

Strawberry powdery mildew was first identified in the UK by Berkeley in 1854, and since then, the prevalence of the pathogen has expanded and the fungus has become a global economic problem for strawberry growers. Strawberry powdery mildew is caused by the fungus *Podosphaera aphanis*. The symptoms of the disease can be a powdery white mycelium growth, which can cover the leaf and fruit, as well as upward curling of the leaf edges and discoloration. Infection of the powdery mildew can affect strawberry development, leading to severe yield loss due to unmarketable fruit. In the UK untreated disease outbreaks can result in severe yield losses. In 2022, a 20% yield loss is estimated to be around £76 million.

The powdery mildew spores overwinter in old, dead leaves, which are a source of inoculum for the spring. The spores are dispersed by the wind infecting neighboring strawberries. Commercial control of the disease includes the use of fungicidal sprays; however, continued usage has led to evolution of resistance to these chemicals. This is why an alternative solution to the problem is required. Focusing on resistant varieties offers a favorable solution to reduce the impact of powdery mildew and the need for fungicide application.

2. Introduction

Strawberries are the major soft fruit produced in the UK, favored for its sweet fragrant taste and contains compounds that have potential health benefits [1]. The introduction of tunnels has benefited strawberry crop production through protection from extreme weather conditions, extending seasonal production and improving yield. However in a warmer, more humid environment provided by the polytunnel, diseases such as powdery mildew can be more prevalent [2,3].

Podosphaera aphanis is a global pathogen, causing disease on strawberry. The symptoms of the disease include upward curling of the leaf edges and powdery white mycelium growth which can cover the leaf, flowers and fruit. The mycelium is comprised of branching hyphae and a conidial chain termed conidium. The conidia are dispersed locally, by the wind, and germinate upon contact with a neighbouring host plant. The dense layer of mycelium growth can lead to a decrease of photosynthesis leading to a reduction in crop yield. Infection of

the fungus can impact on strawberry development causing misshapen and damaged fruit, leading to severe yield loss due to unmarketable fruit [2,4].

Powdery mildew is maintained by the application of rigorous crop management practices and fungicide sprays. The fungicide sprays are applied at regular intervals, example fungicides applied include Myclobutanil, Penconazole and Bupirimate [5,6]. However, the frequency of fungicide application can be restricted by regulations, as some products such as Penconazole or Proquinazid can only be applied a few times a year. The restriction on applications reduces the possibility of insensitivity and fungicide resistance [2]. For instance, sterol demethylation inhibitors (DMIs) fungicides like Penconazole and Myclobutanil are highly effective at controlling the disease and are commonly used in commercial fields. However, over time the powdery mildew fungus has developed resistance to these fungicides, current evidence suggests one mode of resistance that has occurred in the fungi, is caused by mutations in the *CYP51* gene [7]. Currently there is a limited number of commercially available powdery mildew resistant strawberry cultivars [8]. The standard breeding practice to improve disease resistance is to use a slow and extensive disease screening assay [9]. By identifying genetic components controlling disease resistance we can use advanced genetic techniques to identify resistant individuals and upgrade the current system to enable genetic-informed breeding.

The fuller scientific record is in the PhD thesis.

3. Materials and methods

3.1. Genome wide association study (GWAS)

3.1.1. Field experiment

In year 1, five replicates of 331 different cultivars were collected from stock plants kept under polytunnel conditions (located at NIAB EMR, Kent). Propagation of experimental plants was achieved through the generation of misted tips. Individual runners were trimmed down to one leaf, pinned in module trays (Plantpak Jumbo Tray 54 Cell) containing 50 grams of standard compost and grown in the glasshouse for 6 weeks with misting. After two weeks the misting was reduced, and Universal Green nutrient feed was introduced. The plants were then transported to the field and planted in a randomised block design.

Preliminary phenotypic data was obtained in October year 1. Data on leaves was collected in June, July, August and September in year 2 and 3. Year 3 incorporated mildew fruit symptoms and flower number assessments. Phenotypic leaf data for mildew was obtained through disease symptom scoring on a five point scale (Table 1) [10].

1.	No visual symptoms
2.	Slight leaf curling, no visual mycelia
3.	Leaf curling and mottling
4.	Severe leaf curling, discoloration and visible damage to the leaf surface
5.	Severe necrosis and partial leaf death

Table 1. Strawberry powdery mildew disease symptoms five point scale

Genotypic data has been generated via DNA extractions using Qiagen DNAeasy plant mini extraction kit (Qiagen Ltd., Manchester, UK) and sequencing with Affymetrix Istraw90 Axiom® array (90k) containing approximately 90,000 potential genetic markers [11]. This data was used to conduct a genome wide association analysis in order to identify genetic alleles associated with powdery mildew resistance. The disease phenotype was calculated for each individual using the Area Under the Disease Progression Curve (AUDPC) [12] and auto spatial correlation was assessed using the R package 'SpATS' [13]. Best Linear Unbiased Predication (BLUP) allowed the prediction of genotypic scores for Genome wide association study (GWAS) analysis [13].

Full GWAS methods can be obtained from PhD thesis.

3.2. Gene deletion and silencing

3.2.1. Development of vectors

CRISPR/Cas9

The deletion of candidate genes involved with powdery mildew infection was conducted using the CRISPR/Cas 9 system. CRISPR technology requires the design of the specific sgRNA primers that meet precise parameters in order to ensure accuracy during gene editing. This was achieved by using bioinformatic tools to identify potential secondary problems such as possibly off target modification sites in the genome [14–16]. The initial

step of sgRNA primer design was achieved using the software Geneious [15,17–19]. The algorithms used by Geneious 10 software were used to design potential sgRNA primers, using the reference genome 'Camarosa' obtained and MLO gene sequences from GDR [20,21]. Parameters such as on-target sequence scores were generated based on Doench *et al* algorithms and off-target scores based on Cho *et al* algorithms [22,23]. As well as additional online bioinformatic tools for more accurate design (Table 2).

Web tool name	Website
Crisprater	https://crispr.cos.uni-heidelberg.de/ -
Micorhomology	http://www.rgenome.net/mich-calculator/
Out of frame score	http://www.rgenome.net/mich-calculator/
Secondary structure	http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html
Secondary structure	https://en.vectorbuilder.com/tool/dna-secondary-structure.html
Cripsrdirect	https://crispr.dbcls.jp/
GDR	https://www.rosaceae.org/search/genes

Table 2. Online Bioinformatic tools

RNA interference

For Silencing of the genes RNA interference was employed. Polymerase chain reaction (PCR) was used to generate product matching a 400bp section of the MLO target genes. HIGS primers and 'Hapil' DNA were used as specified in the protocol in table 2 using Q5® High-Fidelity DNA Polymerase. The pENTR™/D-TOPO™ Cloning Kit was used for the cloning reaction performed as detailed in table 3. The solution was then mixed gently and incubated for 5 min at room temp. The TOPO cloning reaction was placed on ice ready for one shot competent *E. coli*. 2 µl of TOPO cloning reaction was added into a vial of competent *E. coli* and mixed gently. The solution was incubated on ice for 5 min, then heat-shocked for 30 sec at 42 °C without shaking. 250 µl S.O.C Medium was added and put on the shaker at 37 °C, 200 rpm for 1 hour. The transformed *E. coli* was then loaded onto LB medium plates containing 50 µg/ml Kanamycin and incubated overnight at 37 °c. The next day 5 colonies were picked to confirm transformants. Successful colonies were stored in 50% glycerol and kept at -80 °C.

3.2.2. In vitro micropropagation

Fragaria x ananassa cultivars ‘Hapil’, ‘Calypso’ and the *Fragaria vesca* cultivar ‘Hawaii 4’ were chosen for transformation. ‘Calypso’ and ‘Hawaii 4’ was selected for its high transformation rate [18] and ‘Hapil’ due to its susceptibility to mildew [24]. The strawberry plants used for transformations were sub-cultured and maintained on a monthly basis to generate the required numbers of plants for transformation.

3.2.3. Strawberry transformations

Selected colonies of agrobacterium containing the construct of interest were grown overnight in low salt LB media with antibiotics. In sterile conditions young leaves were collected and submerged in inoculum for 15 mins. Leaves were removed scored and left to dry on blotting paper, then placed on shoot regeneration media (SRM – MS medium supplemented with α-naphthaleneacetic acid (NAA) and thidiazuron (TDZ). The pH was adjusted to 5.8, Agargel was added before autoclaving, then supplemented with filter sterilised glucose) plates and placed in darkness for 4 days. Leaves were then washed and placed on fresh SRM media with antibiotics and left to produce callus. Plants were moved onto fresh media every three weeks.

3.3. RNA Sequencing

Different tissue samples were collected from the polytunnel with and without natural powdery mildew infection. Samples were flash frozen in liquid nitrogen and stored at -80 ^{o}c. RNA was extracted using the Qiagen RNA extraction Kit. Quality and concentration was confirmed on a nanodrop and the Agilent TapeStation system, samples were then sent for sequencing at Novogene. Analysis was performed using the online platform iDEP [25].

Full RNA sequencing methods can be obtained from PhD thesis.

4. Results

4.1. Genome wide association study (GWAS)

The phenotype scores were obtained on a monthly basis during the summer period from July to September in the years 2021 and 2022. The frequency plot represents the phenotyping sampling in 2021 (Figure 4.1.1). The scores in July show low levels of powdery mildew symptoms compared to the phenotyping scores in September which show over 50 % of the cultivars presented strong powdery mildew disease symptoms scores of over 3 (Figure 4.1.1). The levels of resistance for cultivars were determined through collating disease data from replicated plants across the plot. The boxplot shows a selection of the

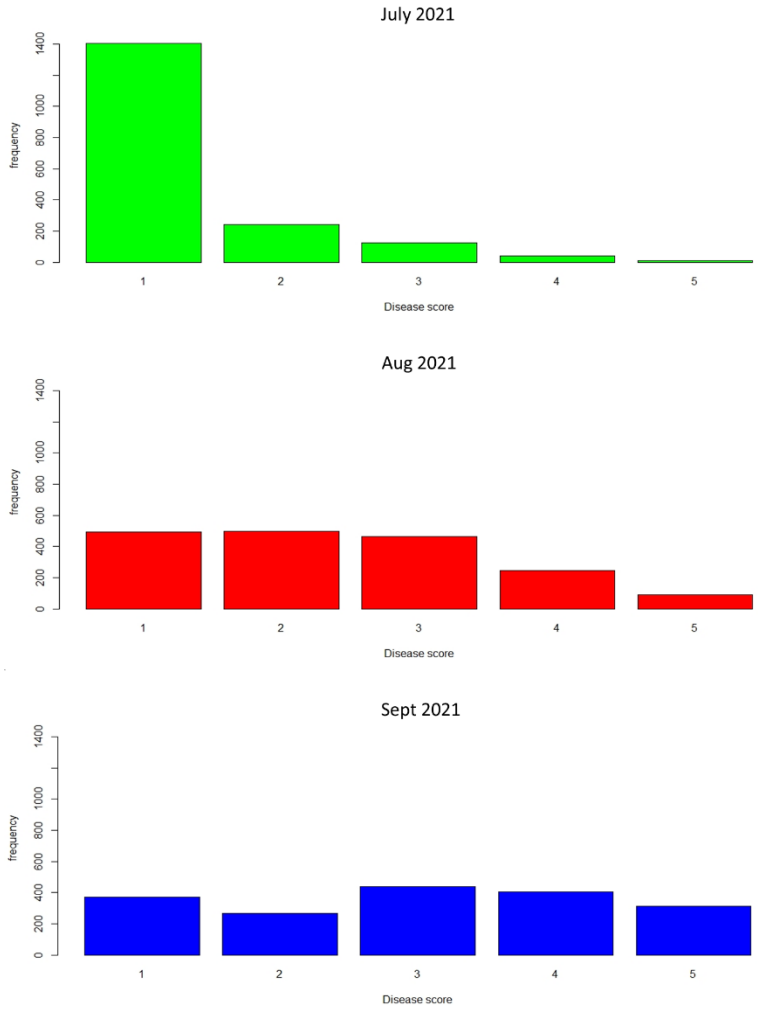

Figure 4.1.1. Phenotype data of strawberry powdery mildew from 2021

level of susceptibility and resistances of cultivars used in the GWAS, with ‘San Andreas’ exhibiting high resistance and ‘Korona’ high susceptibility to infection (Figure 4.1.2). The disease profile was observed for leaves in 2021 and 2022, with Broad sense heritability

observed to be 0.83 and 0.87 respectively suggesting a strong genetic component controlling powdery mildew disease severity.

Multiple qualitative trait nucleotides (QTN) were identified for both years, with six stable QTN identified for both consecutive years of assessment. Stable QTN were identified on chromosomes 1B, 3D, 4A, 5A, 6A and 7D. The most significant QTN was located on 7D, shown to have an allele associated with a Receptor like Kinase and Nuclear binding domain. The result of this allele produced a significant effect size of 61 % (Figure 4.1.3).

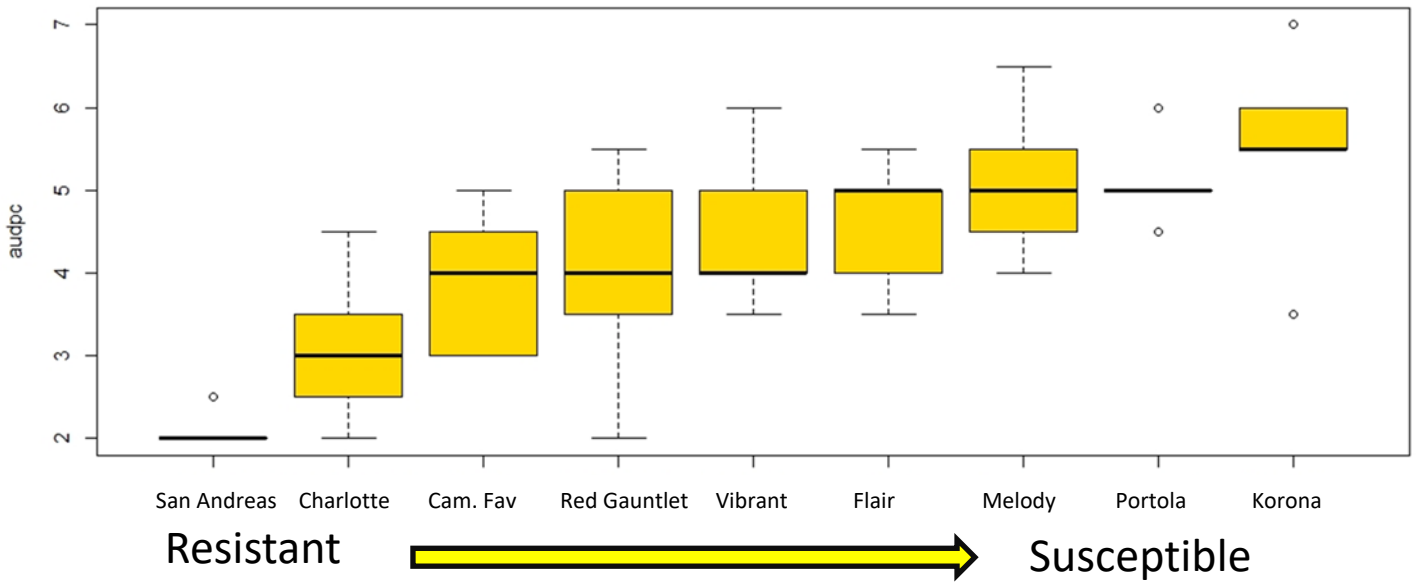

Figure 4.1.2. Boxplot highlighting different resistance levels of selected strawberry cultivars. Arrow represents resistance to susceptible levels.

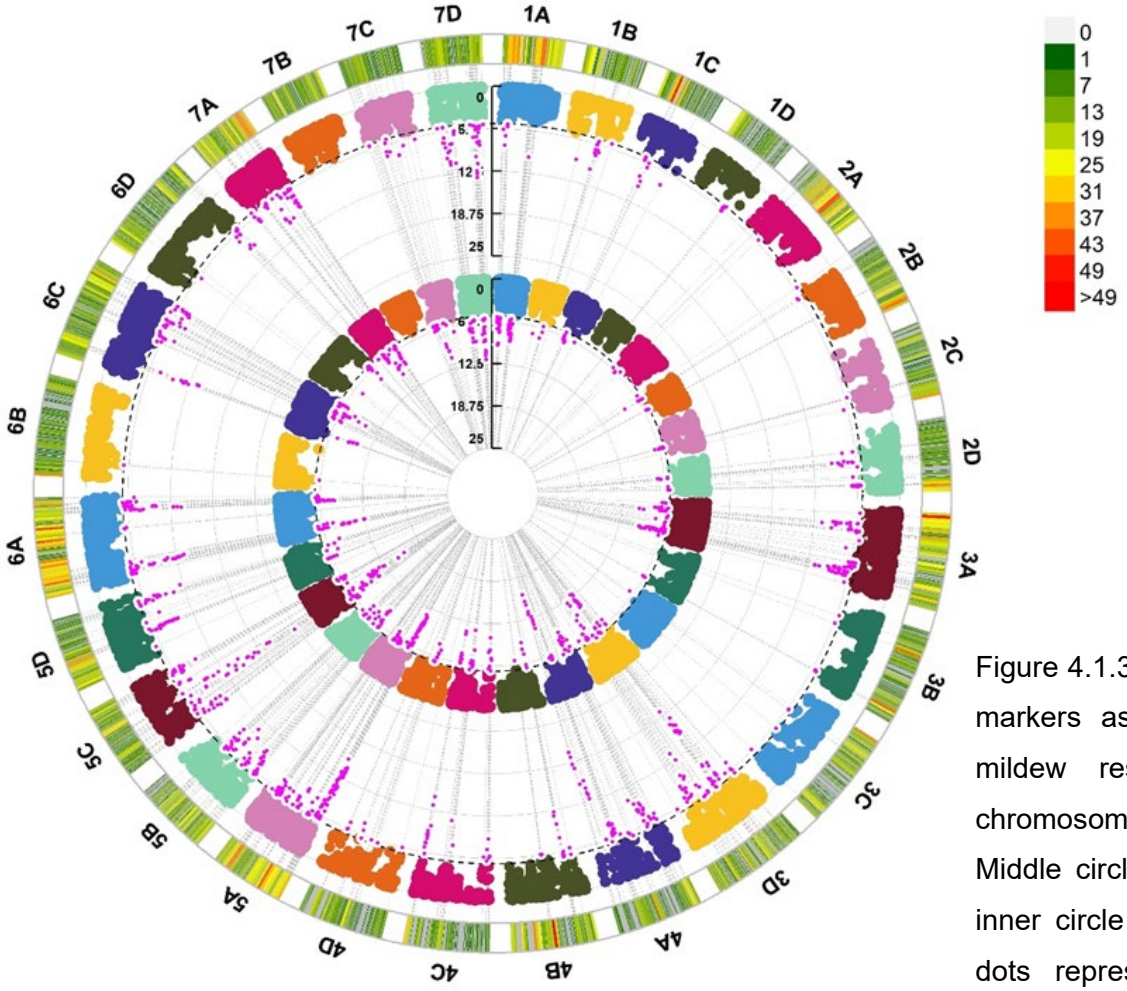

Figure 4.1.3. Manhatton plot of SNP markers associated with Powdery mildew resistance. Outer circle chromosome SNPs are located. Middle circle SNPs from 2021 and inner circle SNPs from 2022. Pink dots represent SNPs above the significant threshold.

4.2. Gene deletion and silencing

In order to assess the MLO genes for gene editing, tblastx was used to perform a BLAST search against the complete octoploid genome sequence of *Fragaria x ananassa* cv 'Camarosa' from GDR [21]. The results demonstrated a 95% similarity to the reference genome, establishing a high conservation of these genes in the Fragaria genome. Additionally, a BLAST search was performed using the Genome Database for Rosacea (GDR) for wild diploid *Fragaria vesca, F. nubicola, F. nipponica and F. innumae.* Results showed 95% conservation with all *Fragaria* diploid sub species [21,26]. Overall, the conservation in these two genes selected were determined to be good candidates for gene editing.

CRISPR/CAS 9 cloning

MLO candidate 1 vesca						
Name	**GC %**	**G or A**	**Microhomology Score**	**Out-of-frame Score**	**Efficiency score**	**Secondary structure**
CRISPR guide 91	43.5	Y	667.1	77.784	0.63 MEDIUM	G:-0.50
CRISPR guide 25	39.1	Y	591.5	65.477	0.76 HIGH	G:-0.50
CRISPR guide 5	39.1	Y	1018.4	66.04477612	0.75 HIGH	G:0.00
MLO candidate 1 Camarosa						
Name						
CRISPR guide 48	43.5	Y	667.1	77.784	0.63 MEDIUM	G:-0.50
CRISPR guide 42	43.5	Y	591.5	65.477	0.76 HIGH	G:-0.50
CRISPR guide 36	39.1	Y	1018.4	66.04477612	0.75 HIGH	G:0.00
MLO candidate 2 vesca						
Name						
CRISPR guide 12	43.5	Y	340.4	58.401	0.65 MEDIUM	G:-1.10
CRISPR guide 63	34.8					
CRISPR guide 4	39.1	Y	1233.2	77.1326	0.61 MEDIUM	G:0.00
MLO candidate 2 Camarosa						
Name						
CRISPR guide 47	43.5	Y	340.4	58.401	0.65 MEDIUM	G:-1.10
CRISPR guide 93	34.8	Y	837.8	82.310814	0.61 MEDIUM	G:-0.50
CRISPR guide 50	39.1	Y	1233.2	77.1326	0.61 MEDIUM	G:0.00

Table 4.2.1. Parameters for selecting sgRNA primers to use for CRISPR Cas9 gene editing

The selected sgRNA were then sent for synthesising by Eurofins. Using the golden gate approach level 1 components (Cas9, antibiotic resistance, sgRNA) were individually incorporated into vector backbone using restriction enzymes. This could then be assembled into a level 2 destination vector, incorporating all components ready for strawberry transformations.

HIGS cloning

The HIGS cloning primers were designed to match approximately 400 bp of the MLO candidate genes, with an additional 4 bp CACC overhang, recommended for the pENTR TOPO cloning kit. Using 'Hapil' DNA a blunt end product was produced via PCR (Figure 4.2.1).

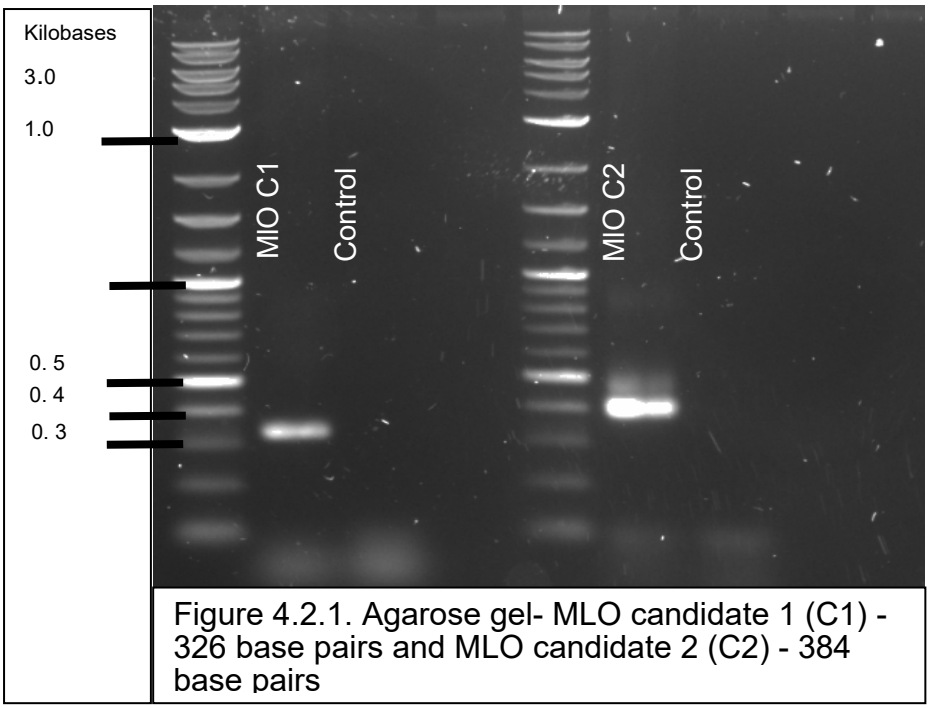

Figure 4.2.1. Agarose gel- MLO candidate 1 (C1) - 326 base pairs and MLO candidate 2 (C2) - 384 base pairs

The PCR product and pENTR/D-Topo vector was transformed into chemically competent *E. coli* cells. They were then plated overnight on LB/Kanamycin plates. Successful colonies were then selected, and the plasmid extracted for PCR confirmation of transformation to ensure the vector containing the product had been successfully transformed. The vectors were transformed into agrobacterium and successful candidates were sent for confirmation to Eurofins for sequencing. Positive cultures were selected and incubated overnight for

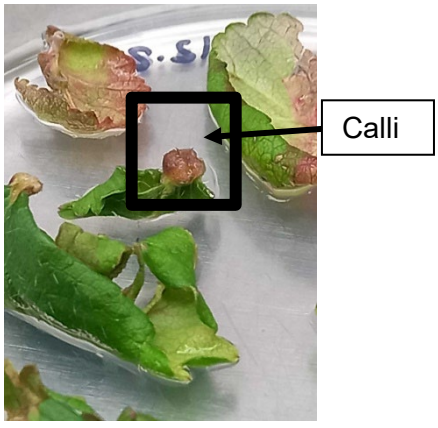

Figure 4.2.2 – Transformed strawberry leaves with calli present.

transformation into young strawberry leaves. The leaves were regularly sub-plated awaiting regeneration of Calli (Figure 4.2.2).

4.3. RNA sequencing

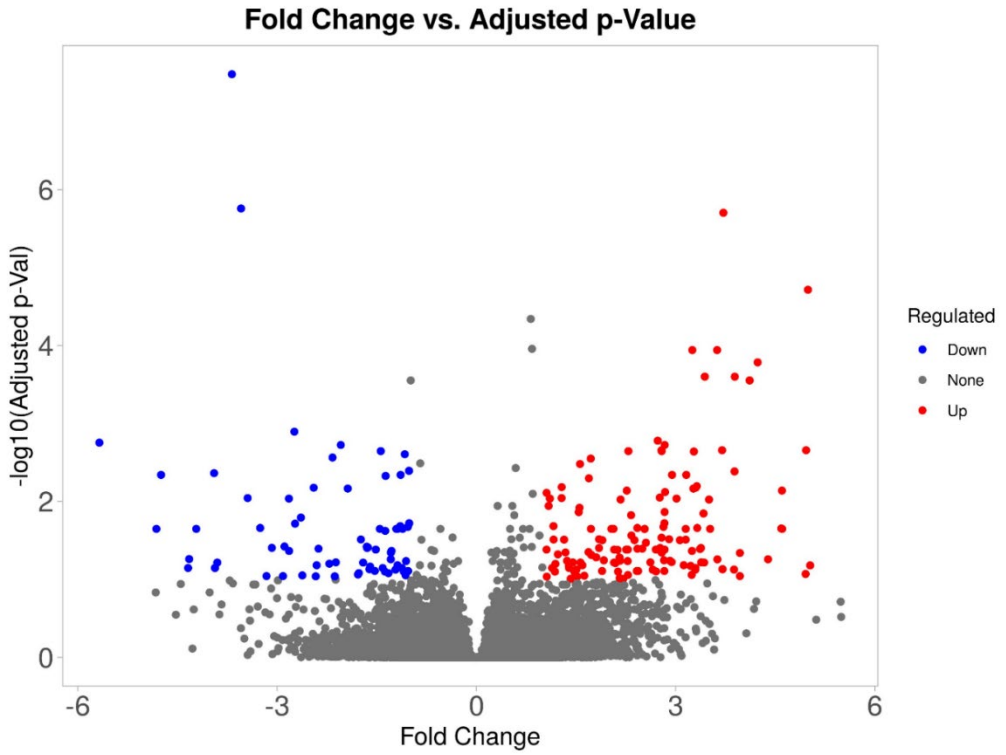

Figure 4.3.1. Volcano plot of differentially expressed genes (DEGs) for all tissue types associated with powdery mildew infection. Scatter plot dots represents each gene, blue dots indicate significantly downregulated genes, red dots significantly upregulated genes and grey dots represent genes with no significant change. The x-axis represents the log2 fold change in expression and the y axis represents the adjusted p -log10 value (padj).

To identify genes in different strawberry tissue that were differentially expressed, samples from infected and non-infected with P. aphanis were collected. Overall, 108087 genes were generated, with 2692 differentially expressed associated with powdery mildew infection on strawberry tissue. The volcano plot represents all the differentially expressed genes (DEGs) of different tissue types analysed (Figure 4.3.1). The blue dots represent the down regulated DEGs involved with powdery mildew infection and the red dots the upregulated. The cluster heat map shows the visual representation of the DEGs for all tissue types from infected and non-infected samples, revealing the changes associated with powdery mildew infection.

Furthermore, the heat map shows the hierarchical clustering in the data matrices (Figure 4.3.2).

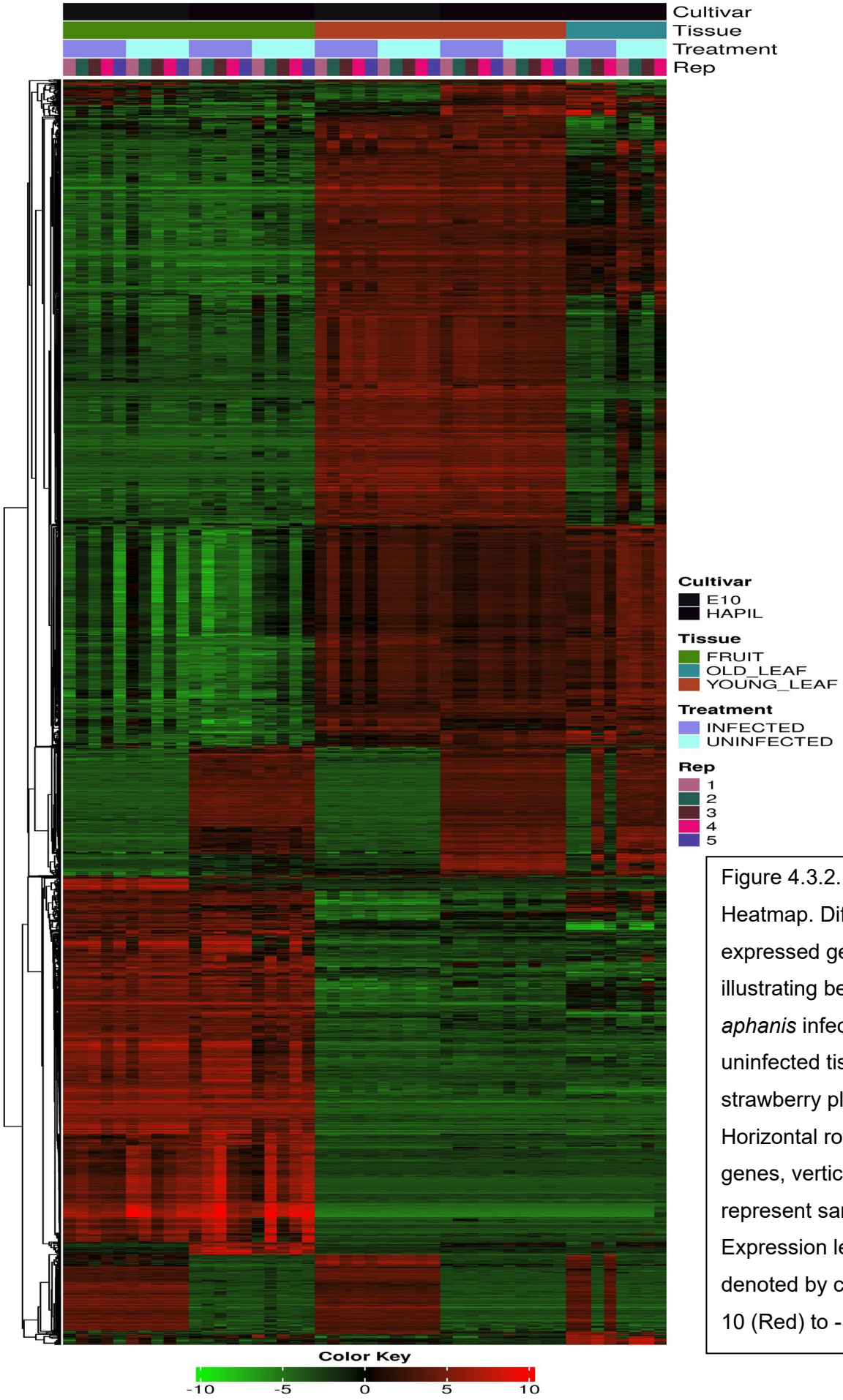

Figure 4.3.2. Cluster Heatmap. Differentially expressed genes (DEGs) illustrating between *P. aphanis* infected and uninfected tissue in strawberry plants. Horizontal rows represent genes, vertical columns represent samples. Expression levels are denoted by colours scale 10 (Red) to - 10 (green).

5. Discussion

5.1. Genome wide association study (GWAS)

In previous studies powdery mildew resistance has been shown to be a polygenic trait [24,27]. In order to provide robust disease resistance markers a GWAS was conducted to assess a large pool of cultivars to assist resistance gene identification. Preliminary results showed presence of powdery mildew symptoms, even at early stages of planting in October 2020. The analysis of the phenotyping results showed a steady progression of powdery mildew infections from July to September, with over 50 % strong powdery mildew presence in September (Figure 4.1.1). The data obtained allowed a good range of coverage across suseptable and resistance levels of cultivars used in the GWAS. For years 2021 and 2022, there was strong broad sense heritability scores calculated for the foliage at 0.83 and 0.87 respectively. The heritability scores suggested a strong genetic link associated with powdery mildew resistance. Over the course of the two years multiple quantative trait loci (QTN) were identified, with six stable QTN identified for both years (Figure 4.1.3). As powdery mildew resistance in strawberry has been determined to be a polygenic trait, identitfying the genes underlining the QTN will be highly beneficial for breeders for use as functional genetic markers. Future work should be performed to validate these genes underlying QTN for breeding applications.

5.2. Gene deletion and silencing

Work produced on MLO genes in apple, barley and other crops has shown that mutant/knock out MLO genes can provide resistance to powdery mildew. In literature research, two candidate genes were identified for gene editing and silencing [20]. The two genes were compared to the octoploid ‘Camarosa’ and diploid vesca sequences obtained from the Genome Database for Rosaceae (GDR) [26,28]. Candidate genes were highly conserved within the Fragaria genus and provided good evidence for candidates for gene editing in commercial (octoploid) strawberry. To provide the most effective method CRISPR/Cas9 was chosen to delete the genes and RNAi was selected to silence the gene function. The design of primers were produced for both CRISPR/Cas 9 and HIGS systems to be run in tandem to validate gene function of the MLO genes. However, the time to construct the CRISPR/Cas 9 sgRNA primers fully, led to the decision to have the main focus on the RNAi method. The RNAi primers were successfully transformed into vectors and then into agrobacterium for strawberry transformations. The vectors successfully produced calli

that was subsequently grown on media for strawberry plant development. However, the calli initially produced did not develop into viable plants to enable validation with pathogenicity tests. Conversely, results found in the other work packages of this project, demonstrated that different MLO genes would be better suited for selection in future functional validation work. In addition, future work should incorporate transient assays as a faster way to identify potential gene function.

5.3. RNA sequencing

RNA sequencing is a powerful tool to investigate differentially expressed genes involved in a desired trait such as fruit development or disease resistance. The information obtained can be used to inform which genes are involved in generating the phenotype for application in breeding more robust crops [29]. This project focused on identifying differences between tissue involved with powdery mildew disease, in view of finding resistance genes. The results provided a more in-depth knowledge of the powdery mildew infections on strawberry and the role in immunity. The results identified several genes of interest, with one found to be involved with resistance in all tissue types.

5.4. Conclusion

The genetic editing of MLO genes is an important avenue to investigate for future breeding. The results from this project have led to the identification of significant MLO genes in the GWAS and RNA sequencing. Manipulating of these MLO genes may provide potential success in resistance to powdery mildew. Furthermore, the GWAS results successfully identify genetic markers that can be implemented into resistance breeding. The RNA sequencing provided a greater mechanistic understanding of powdery mildew infection that has broaden our knowledge of the disease, as well as provided potential gene candidates that may be exploited to achieve resistance in strawberry. Future work should focus on further validation of the genes identified in this project for the advancement of strawberry breeding programmes.

Please refer to PhD thesis for more in depth analysis of the project.

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