University of Reading

Identifying Novel Powdery Mildew Susceptibility and Resistance Genes in *Fragaria × ananassa*

Thesis submitted for the degree of Doctor of Philosophy

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Declaration

Declaration: I confirm this is all my own work and the use of all materials from other sources has been properly and fully acknowledged

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Abstract

The biotrophic fungal pathogen *Podosphaera aphanis* causes powdery mildew (PM) disease on strawberry. Strawberry PM is a global problem, infecting all above ground plant organs. The infection of PM leads to lower yields and unmarketable fruit and thus pathogen infections result in high economic losses. Infection of the foliage can lead to a reduction in photosynthesis, leading to decreased CO_2 assimilation and ultimately a decrease in yield. When the PM fungus infects strawberry reproductive tissue, it can cause misshapen or stunted fruit. Control of PM disease is predominantly achieved by the application of fungicides; however, overreliance on chemical application has led to the evolution of fungicide resistant strains. Generating disease resistant cultivars offers a highly favourable solution to reduce the impact of PM on strawberries. Thus far, there are no public, validated, large effect genetic markers for use in improving PM disease resistance in strawberry. This project focused on characterising the genetic components underlying tissue specific resistance to PM disease, whilst ultimately identifying putative resistance genes. To achieve this goal, analysis was conducted across different strawberry genotypes through a genome-wide association study (GWAS) using phenotyping and genotyping data Multiple genetic loci associated with PM resistance in foliage were identified using a GWAS, with several loci displaying a high effect size of over 50%. Moreover, six stable Quantitative Trait Nucleotides were identified across both years of assessment. In a separate experiment RNA sequencing was used to identify differentially expressed genes in the presence of PM across three resistance types: tissue, cultivar and ontogenic resistance. The RNA sequencing analysis revealed a diverse immunogenetic resistance response associated with each different resistance type. Across these resistance types, one gene (*CAF1-11*) was detected throughout, offering a promising candidate for PM resistance. Another highly important trait that can lead to increased strawberry production is fruit number, a component of yield. A GWAS was also conducted to identify genetic loci that may be associated with fruit number. The analysis identified five genes that may be involved in controlling fruit number. Finally, a genomic prediction approach was used to determine the predictive accuracy associated with improving both strawberry PM resistance and fruit number. This study has shown there is a large potential for using genomic selection to increase cultivar foliar PM resistance; however, fruit PM resistance or fruit number were not predicted to lead to an improvement in phenotype using a genomic prediction approach**.** Overall, this study has identified multiple novel genes potentially associated with PM resistance and fruit number. As such this work provides a greater understanding of the complex mechanisms associated with these traits whilst offering a steppingstone towards the development of genetic molecular markers for use in breeding elite strawberry lines.

Contents

List of Figures

Introduction

Chapter 2

Chapter 3

List of tables

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Abbreviations

ABA – Abscisic Acid

AFLP – Amplified Fragment Length Polymorphism

ATP – Adenosine Triphosphate

AUDPC – Area Under the Disease Progression Curve

BLUE – Best Linear Unbiased Estimates (BLUE)

BED – Browser Extensible Data

CAF1 – CCR4 associated factor

CCR4 – Carbon Catabolite Repressor Protein 4

CC – Coiled Coil

CRISPR – Clustered Regularly Interspaced Short Palindromic repeat

DEG – Differentially Expressed Genes

DMIs - Sterol Demethylation Inhibitors

DNA – Deoxyribonucleic Acid

ER – Endoplasmic reticulum

ERF – Ethylene Response Factors

ET – Ethylene

ETI – Effector Triggered Immunity

GDR - Genome Database for Rosacea

GO – Gene Ontology

GS – Genomic Selection

GTP – Guanosine 5 triphosphate

GWAS – Genome Wide Association Study

H^2 – Broad Sense Heritability

HIGS - Host Induced Gene Silencing

HR – Hypersensitive Response

JA – Jasmonic Acid

LD – Linkage Disequilibrium

LRR – Leucine Rich Repeat

QTN – Quantitative Trait Nucleotide

QLT - Quantitative Trait Loci

MAS – Marker Assisted Breeding

MLO - Mildew Loci 0

NBS – Nuclear Binding Site

NHR – Non-Host Resistance

PAMPs - Pathogen-Associated Molecular Patterns

PRGdb – Pathogen Receptor Gene Data Base

PFRU – Perpetual Flowering and Runner

PM – Powdery mildew

PRR – Pattern Recognition Receptors

PS – Phenotype Selection

PTI – PAMP Triggered Immunity

R gene – Resistance gene

RAPD – Random Amplified Polymorphic DNA

RFLP – Restriction Fragment Length Polymorphism

RLK – Receptor Like Kinase

RLP – Receptor Like Protein

RNA – Ribonucleic Acid

SA – Salicylic Acid

S gene – Susceptibility Gene

sgRNA – Single Guided RNA

SpATS – Spatial Analysis of Field Trials with Splines

SNP – Single Nucleotide Polymorphism

SSR – Simple Sequence Repeats

TM – Transmembrane

Chapter 1. Introduction

1.1 *Fragaria spp.*

The Rosaceae family encompasses more than 2500 species, comprising of nuts, ornamentals, pome fruits and berries. The dicotyledonous, non-deciduous strawberry, genus *Fragaria* (Latin 'fragrans' for sweet-scented) is composed of 22 wild species ranging from diploid to decaploid [1], [2]. The diploid genome (2n=2x14) consists of seven pairs of chromosomes and has a genome size of 249 Mega bases (Mbp) [3]. In 1766, the French botanist Duchene was the first to characterise *Fragaria x ananassa*, which resulted from a hybridisation event between two octoploid species [118]. The commercially cultivated octoploid *Fragaria* × *ananassa* contains eight sets of chromosomes (56 in total) with a genome size of 813.4 Mbp. Investigation into the origin of *Fragaria* × *ananassa* led to the determination of the diploid progenitor sub genomes to be *F. vesca*, *F. iinumae, F. viridis* and *F. nipponica*. [1], [2], [4], [5]. Out of the four sub-genomes contained in the octoploid, the *F. vesca* subsp. *bracteata* is considered the most recent addition and represents the most dominant genome present in the octoploid [2], [6]

1.1.1 Commercial strawberry

Strawberry is a globally important crop, favored for the sweet fragrant taste and potential health benefits, due to high vitamin C content and antioxidant acting phenolic compounds [7], [8], [9]. In 2022, the commercial strawberry industry in the UK, was valued at £377 million with strawberry being the highest grossing soft fruit [10]. Breeding efforts have led to the development of more robust commercial cultivars. Many strawberries varieties now possess more desirable agronomic traits which has enabled such flavour or stress endurance [6]. The structure of the strawberry plant comprises of trifoliate leaves, crown, root system and stolons that produce daughter plants (Figure 1). There are two types of commercial strawberries: the June bearer (short day flowering plant) and the everbearer (long day/day neutral flowering plant). The growth of strawberries is contingent on several factors such as temperature, light intensity and daylight [11]. This is important for flower initiation, which is controlled by a complex system induced by temperature and light, referred to as photoperiodism. In photoperiod-sensitive June bearers, the flower buds are induced when exposed to less than 12 hours (short day) of light or low temperatures below 15 ^{o}C. The June bearer induction phase required for flower initiation can be around one to two weeks, though commercial greenhouses usually apply light conditions for three to four weeks. The June bearers are known to experience only one cropping in a season [12]. In contrast, perpetual flowering everbearers produce flowers from spring and late into summer, which is a highly desirable trait

for a crop. The continuous fruit production in everbearers is due to photoperiod insensitivity and is considered to be attributed to a single dominant gene in *F. vesca* [13]. However the concept of a single continuous flowering gene regulating flowering time in *F.* × *ananassa* has been disputed, with evidence suggesting that it is actually a polygenic trait in octoploids [14], [15].

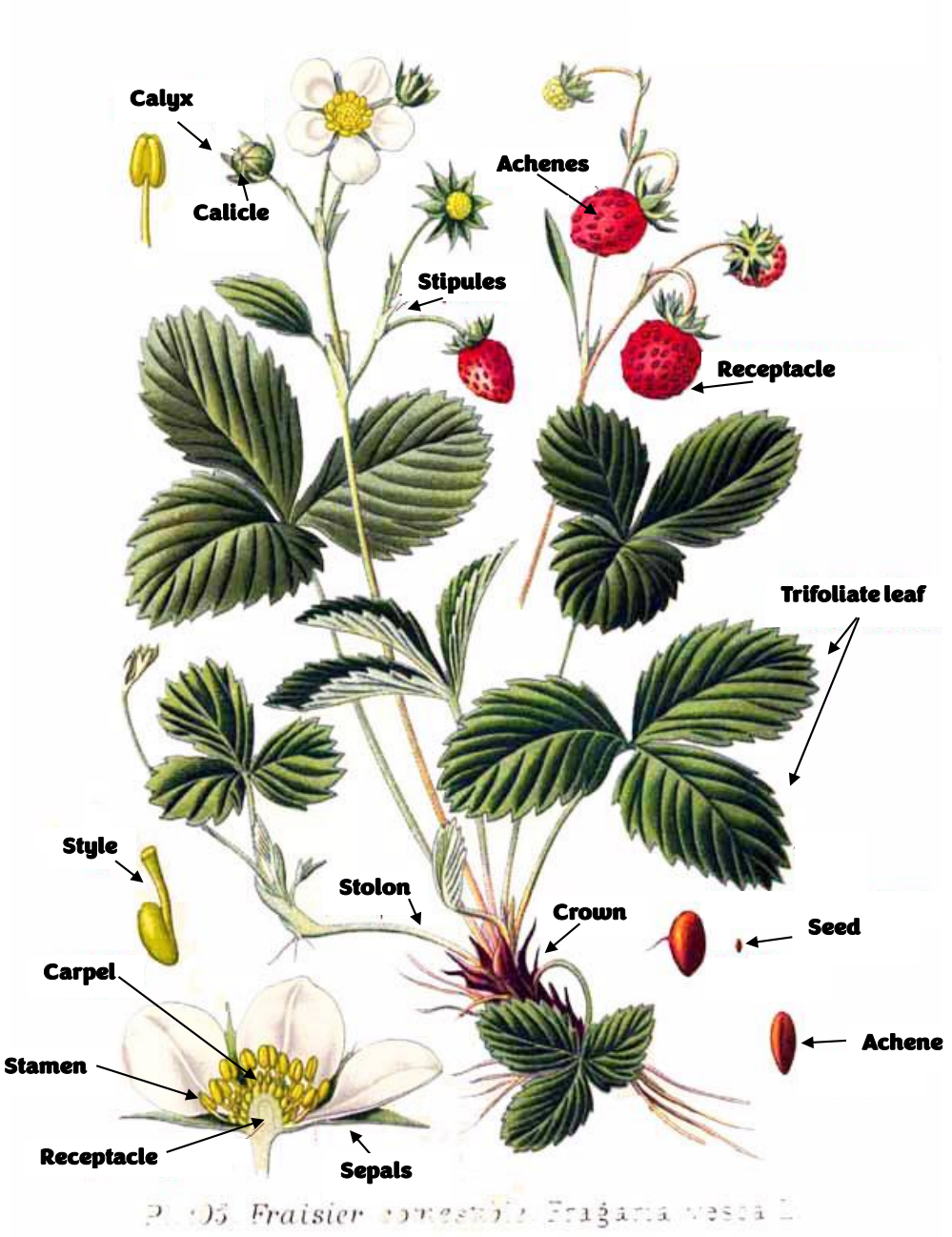

Figure 1. *Fragaria vesca* illustration – A. Masclef (1891) Atlas des Plantes de France

Although June bearers and long day everbearers have different flowering habits, they both require autumnal conditions for floral primordia initiation. In contrast, floral initiation in day neutral cultivars is controlled exclusively by temperature, with flowering being inhibited at low temperatures [14]. The June bearers and everbearers also have different vegetative and growth development. The everbearers are predominantly smaller with more crowns and produce less runners compared with June bearers [16].

The strawberry plant propagates either sexually by producing seeds or through a vegetative state, producing daughter plants via the stolons (runners) [14]. The blossom of the strawberry can have between 20 – 400 pistils that develop into achenes (the true fruit) that contain one seed. The achenes are attached to the surface of the receptacle, the fleshy tissue [17]. Fruit size corresponds to flower size, with larger flowers producing larger fruit as seen in *F. chiloensis* [14]. After approximately 25-30 days after pollination the fruit ripens under temperatures of 18-25 ^{o}C [18]. Water and nutrients also play a larger role in flowering and fruit development; for example, increased nutrients can improve fruit yield. However, high levels of nitrogen can inhibit flower production, indicating that a fine balance of nutrients is required for increased yield to flower production ratio [14].

The commercial industry has driven the improvement of cultivars to enhance fruit quality and yield. Many studies have investigated attributes such as taste, ripening, yield and nutritional content [19], [20], [21]. Another important attribute is abiotic and biotic stress that can be a limiting factor for fruit production. Studies comparing traits of diploid variation found that *F. iinumae, F. vesca* and *F. nipponica* all exhibit traits of cold tolerance. Moreover, wild *F. vesca* demonstrates superior heat and drought tolerance, along with disease resistance to pathogens such as powdery mildew [18]. Desired traits once identified in wild populations can then be introduced into breeding lines. Understanding the genetic components underlying traits of interest can allow the development of genetic markers to develop more robust commercial cultivars. While some traits are controlled by a single genetic factor (monogenic), others are influenced by multiple genetic factors (polygenic) and require intensive investigations to identify the genetic controls responsible for the desired traits, such as disease resistance [22]. Additionally, while monogenic resistance has been introduced into crops, reports of breakdown in major resistance have occurred over time. This is attributed to a single genetic factor exerting high selection pressure on the pathogen [23], [24], [25].

1.2 Plant immunity

Plants have evolved three main types of defence against pathogens and pests. The first is passive resistance enabled by physiological elements such as wax cuticles or cell walls, repelling initial infection. The plant's second defence mechanism is non-host resistance (NHR) protecting against specific pathogens at the membrane [26]. This type of defence uses transmembrane pattern recognition receptors (PRRs) that are triggered by pathogen-associated molecular patterns (PAMPs). The immune response is amplified by an increase in stress-related hormones leading to reinforcement in transcriptome reprogramming and a reduction in growth and photosynthesis [27]. Early cell response to infection involves an influx of calcium (Ca^{2+}), MAPK cascade activation and the production of reactive oxygen species. As well as negative regulation of phosphatase and ubiquitination-mediated proteins act as a control to reduce an overexpressed immunity response [28]. The third type of defence is effector-triggered immunity (ETI). This ETI response mounts a specific resistant gene (R gene) mediated resistance activated by pathogen effectors [29], [30].

1.2.1 Resistance Genes (R-Genes)

R genes play an important part in the ETI response. They get activated in response to signals produced by pathogens effectors and lead to modifications instigated in the cell [31]. The R genes can be effective through different mechanisms. One method is through interacting directly with effectors by incapacitating them. Another operates when pathogen effectors modify protected proteins in the plant, prompting downstream signals that in turn, initiate an R gene response [32].

Resistance genes (R-genes) all comprise of a leucine rich-repeat (LRR) domain in the C terminal, yet differ in their N terminal domains, which are specialized for specific disease resistance (Figure 2). The nuclear binding site (NBS) and LRR proteins play important roles in detecting effectors and initiating resistance to pathogen infection. The N-terminal NBS domain has a highly conserved region that hydrolyses ATP and GTP, whereas the C-terminal LRR domain is involved with protein to protein interactions [32], [33]. Avirulence (Avr) proteins secreted by the pathogen can inadvertently induce a mild to severe immune response in the cell, which can be detected by the LRR domain in the host plant R genes [34], [35].

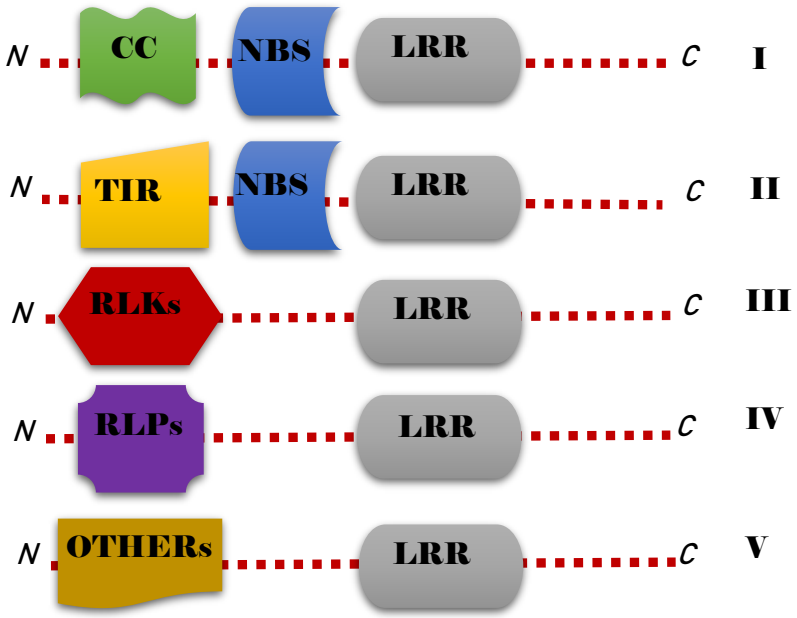

Figure 2. Resistance genes - five categorises of R-genes based on their arrangement of domains found in plants. NBS – Nuclear binding site, TIR – Toll/Interleuking-1 receptor, RLK- Receptor like kinase, RLP – Receptor like protein and CC- Coiled coil and LRR – Leucine rich repeat. Source S. C. Lynn (author)

The NBS-LRR with the combination of variants, are collectively called nucleotide-binding domain and leucine-rich repeat-containing (NLR) genes [36]. NLR genes trigger ETI and are continually expressed at low levels until activated by an immune response [37]. The activated immune response then leads to localized cell death (hypersensitive response) in the plant, which results in pathogen containment and restricts the pathogen's spread [38]. Several NLR's genes respond to a broad spectrum of stimuli whereas others, for example, *Mla6* or *Mla13* are stimulated by specific pathogen effectors [36]. RNA sequence data from Barbey *et al.* (2019) identified that the majority of NLR genes are expressed in the strawberry root and leaves system and suggests that broad-spectrum resistance NLR genes have adapted specificity to one or two tissue types [37].

1.2.2 Susceptibility genes (S-genes)

In contrast to resistant genes, susceptibility genes exacerbate pathogen infection. For example, in the event of a pathogen attack, the actin cytoskeleton plays an important role in resisting penetration of fungal infection by restructuring of the cytoskeleton. However, protein inhibitors instigated by the pathogens can cause actin to depolymerize, reducing the structural integrity of the cytoskeleton and allowing pathogens to penetrate the cell wall [26].

One important S gene is the trans-membrane Mildew resistant Locus 0 (MLO). The MLO genes were first identified in barley in 1942 and found to act as suppressors of the defence

response [39]. Since then, MLO genes have been identified in several other species such as the 15 MLO genes identified in Arabidopsis, 8 in wheat and 17 in grapevine [40]. Recently, Jambagi and Dunwell (2017) identified 12 MLO genes in *Fragaria vesca* and *vesca* accession 'Hawaii 4'. A further study by Tapia *et al.* (2019), identified additional MLO genes in *Fragaria* by finding 20 MLO genes in *F. vesca* and 68 in *Fragaria* × *ananassa* [41].

MLO genes are highly conserved and the presence of the endogenous MLO genes is considered to be a prerequisite for the mildew infection [42]. MLO genes encode membrane-bound proteins with seven transmembrane domains; the N-terminal is extracellular and the C terminal is cytosolic [40]. The majority of *F. vesca* MLO proteins are predicted to be located in the plasma membrane, the exception being *FvMLO7*, which is located in the extracellular matrix. In contrast, in *F.* × *ananassa* out of the 68 MLO proteins, 61 are in the plasma membrane, nine are located in the organelles, four in the chloroplast, two in the endoplasmic reticulum (ER), two in the nucleus and one in the Golgi bodies [41].

The MLO genes are involved with different tissues responses such as root morphogenesis, for example in Arabidopsis *MLO4* and *MLO11* are involved in regulating thigmotropic root growth and pollen tube reception by the embryo sac [39], [43]. At the PM penetration site, the MLO genes cause a negative regulation of vesicles, this interacts with the *PEN* gene and actin-dependent defence pathways to result in mildew susceptibility [44]. The loss of function of MLO genes can lead to a pathogen's reduced ability to penetrate the epidermal cell wall, due to cell wall remodeling and oxidative cross-linking fortifying the wall at the entry site. [38]. Studies have identified recessive mutants in an MLO gene in barley and Arabidopsis, conferring to the resistance to penetration of mildew due to the inability of the pathogen to enter the cell wall. In addition, similar results have been noted in the tomato recessive allele ol-2, the mutated MLO acts as a non-host resistance (NHR) mechanism to the infection and confers complete mildew resistance [47]. The impact of modification in MLO genes has been seen to be species-dependent, for instance, resistance has been shown to be accomplished by knocking out only one gene in pea and tomato (*SlMlo1*) [40], [47]. However, Pessina *et al.* (2016) state that the knockout of all three MLO genes in grape (*VvMLO6, VvMLO11* and *VvMLO7*) was required to reduce the severity of powdery mildew infection by 77% [48]. This has also been seen in Arabidopsis *AtMLO2, AtMLO6* and *AtMLO12* requiring all three genes to be knocked out for complete resistance to be achieved [40].

The MLO genes are good candidates for gene editing systems like CRISPR-Cas9 for producing broad range resistance to powdery mildew. However, it is prudent to consider that MLO genes may play a role in the regulation of other pathways; therefore, knocking out MLO genes may result in pleiotropic effects such as the inhibition of growth, flowering or fruit

production. For example, barley MLO mutants, early senescence-like chlorosis of leaves has been reported under particular environmental conditions [49], leaf mesophyll cells undergo spontaneous cell death and there is a reduction in grain yield [50]. However, the benefits for potential broad and durable resistance to mildew with MLO genes still makes them a good candidate for gene editing in order to develop powdery mildew resistant strawberry [47], [50]. Also, the potential of knocking out MLO genes in *Fragaria* may, in principle, lead to a better understanding of powdery mildew infection in other Rosaceae tree species [38].

1.3 Strawberry powdery mildew (*Podosphaera aphanis*)

1.3.1 Erysiphaceae

The disease commonly referred to as Powdery Mildew (PM) is caused by a number of heterothallic, obligate, biotrophic fungi in the Erysiphaceae family. As obligate biotrophs, *Erysiphaceae spp.* rely exclusively on their host for survival and thus laboratory cultivation has proven difficult [51]. *Erysiphaceae sp.* reside on plant surfaces, forming a white powdery structure covering exposed tissues such as the leaves, fruit and flowers [52], [53]. The powdery mildew family includes approximately 900 species worldwide, in sixteen genera, effecting and colonizing over 10,000 host plant species, including a variety of crops, all of which are angiosperms [53], [54].

1.3.2 Disease Identification

First identified by Berkeley in 1854, strawberry powdery mildew (PM) *Podosphaera aphanis* (former *Sphaerotheca macularis f. sp. fragariae*) has become a global problem for strawberry crops [55]. It was originally believed that a single type of PM infected all crops, but in1976 it was establish that there was two different causal agents for hop and strawberry infection, leading to the differentiation of PM classes [56]. Symptoms of PM infection include a noticeable white powdery structure of mycelium growth on leaves (particularly the abaxial - underside), flowers and fruit (Figure 3a). The mycelium is composed of branching hyphae and a chain of conidia (Figure 3b). The conidia are dispersed locally by the wind and germinate upon a neighbouring host plant [57]. Spore dispersal in the field can reach between 1.2 to 1.5 meters; notably, a reduction in dispersal distance is found in polytunnels, due to reduced wind spread [58]. The dense layer of mycelium growth can lead to a reduction of photosynthesis and transpiration leading to tissue damage [59], [60], [61]. However, free water, for example rain, can limit dispersal by reducing the number of conidia in the air, washing them off the leaves or by tissue surface moisture that can result in reduced germination and colony growth [52], [62], [63], [64].

PM leaf symptoms are expressed via upward curling of the leaf edges, with discoloured patches and dark round structures (the cleistothecia) within the mycelium growth (Figure 3C) [52]. The infection and disease is more prominent on the surface of the abaxial (underside) side of the leaf rather than the adaxial (upper side), suggested to be due to the unfolding of early leaves during leaf development and possible exposure to UV radiation as opposed to plant resistance [65]. Flowers infected with *P. aphanis* can cause the fruit to be aborted or produce misshapen and small fruit [48], [52]. *P. aphanis* has a limited host range on strawberry and raspberry; however, the strain isolates are hypothesized to be genetically distinct and only infect their respective hosts [66], [67]. *P. aphanis* can survive the winter period as cleistothecia and mycelium in a dormant state on remaining strawberry leaves [64], [68]. When the warmer weather of spring returns, the mildew re-sporulate initiating new infection amongst the local hosts [68].

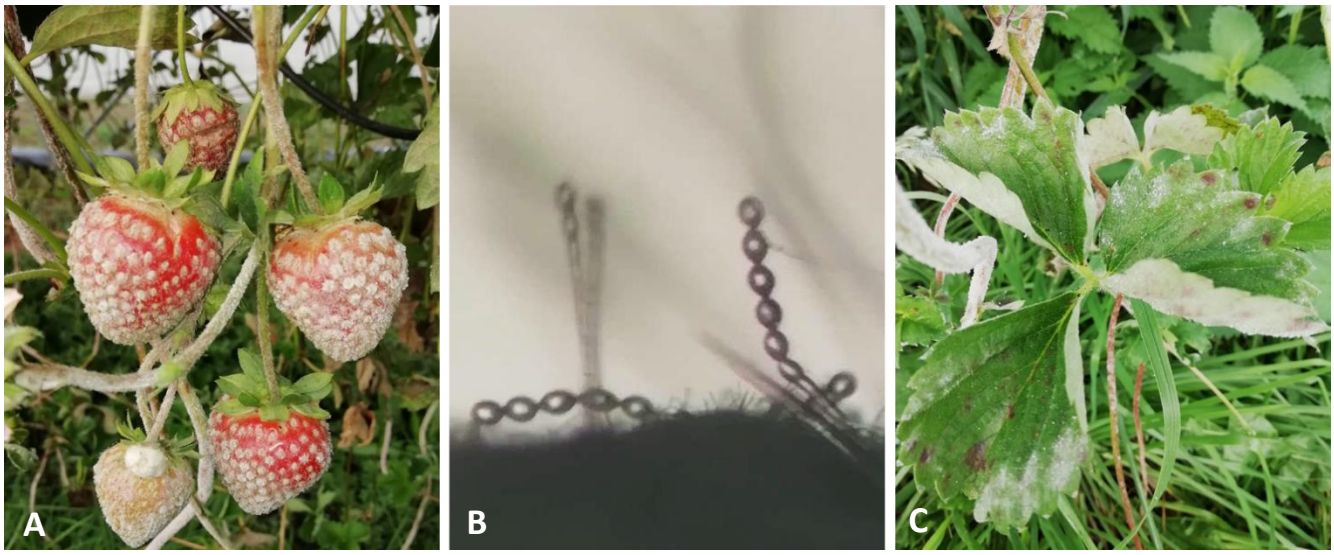

Figure 3 Strawberry powdery mildew infection (*P. aphanis)* A. Mycelium growth on strawberry B. Microscope image of conidia on foliage and C. Mycelium growth and curling on strawberry foliage. Source from S. C. Lynn (author).

1.3.3 Epidemiology

Anamorph (Asexual) cycle

Podosphaera aphanis has both, a teleomorph (sexual) and an anamorph (asexual) phase [57]. In the anamorph phase, mycelium produces long conidium chains of linked conidia. The conidia of *P. aphanis* disperses to neighboring hosts (Figure 4) and can produce many generations over the spring/summer (April to August) growing season. Upon dispersal, the conidia infect new plant tissue, germinate and then produce mycelium [57]. In laboratory conditions approximately 20 conidia have been observed on one conidial chain; the long chain acts to ensure that the conidia are above the trichomes (leaf hairs) to aid successful dispersal. The initiation of germination will only begin once the conidia have detached from the chain and land on a suitable host [51]. The conidia are only viable for a short period of time; however, the duration of cycles on foliage can vary between 4 to 9 days depending on temperature and humidity [59].

Teleomorph (Sexual) cycle

The cleistothecia are the pathogen's resting structures, which can survive in a dormant state in mycelium over winter [68], [69], [70]. The cleistothecia (cleisto = closed – Greek) is referred to as the closed state and chasmothecium (chasmo = open – Greek) as an open state of the cycle [51] (fig 4). The cleistothecia develop from August to September in response to seasonal changes (photoperiod, temperature) [70]. Mating of *P. aphanis* requires a close proximity on the tissue surface to a suitable mate in order to develop cleistothecia [55]. The matured chasmothecium contains many asci which in turn contain eight ascospores (Figure 4) [70], four ascogonium (female) and four antheridium (male) [51], [55]. Under optimal temperatures the chasmothecium releases the ascospores, which are dispersed locally via the wind [57], [70]. After identifying a viable host, the ascospores germinate within 4 – 6 hours, followed by the production of mycelium, which lead to the formation of haustoria and development of conidial chain extensions [70]. The mycelium can be observed under the microscope 48 hours after the initial infection and conidiophores with conidia observed after 96 hours. [51]. During development, conidia cells extend upward on the chain, with the oldest being exposed at the top [51]. One week after inoculation the full characteristics of *P. aphanis* infection can be observed by the naked eye [51]. The ascospores that overwinter on the host are released at the beginning of spring and are considered to be the source of primary inoculum in the strawberry plants for the season [57]. Environmental factors have a large impact on *P. aphanis* reproduction cycles (both teleomorph and anamorph phases) [55]. During infection, cleistothecia development is stimulated by changes in the seasonal conditions and usually initiated in autumn [57]. The optimal temperature for successful initiation of chasmothecium

development has been reported to be 13 °C [55]. Conidium growth is temperature sensitive requiring optimal temperature and humidity to develop with temperatures between 15 to 25 °C and a humidity range of 75 - 98% [68], [71].

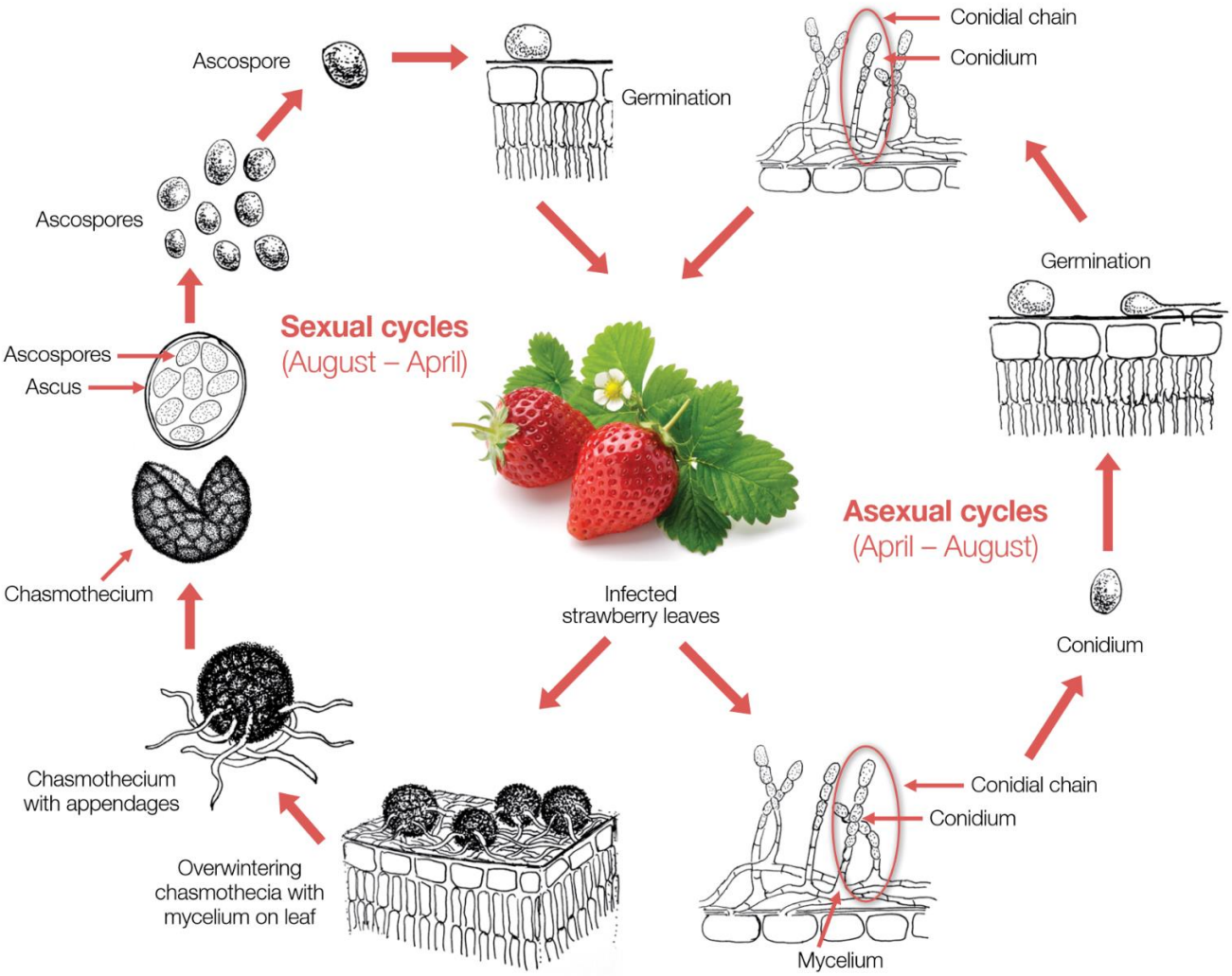

Figure 4. Life cycle of *Podosphaera aphanis*. Diagram depicts the holomorph cycles of *Podosphaera aphanis* infection – Hall *et al.* (2017) [57]

1.3.4 Haustoria

The white powdery covering commonly found on the surface of the leaves infected with *P. aphanis* consists of mycelia and hyphae [72]. The hyphal branching is suggested to aid the pathogen by an increased colony surface area, leading to an increase in nutrient uptake [72]. The hyphal branching assists hyphal fusion, which facilitates the exchange of nutrients and signals during mycelium development [72]. The haustoria construct a germ tube-like structure

that penetrates the epidermal layer and host cell between 24 to 36 hours after inoculation depending on cultivar susceptibility [51], [73]. The fungal haustoria functions to extract and transport essential nutrients and water from the host cell [73]. Laboratory studies in tissue culture plants have not successfully identified the haustoria *in vitro*, considered to be due to them being unable to develop in laboratory conditions. This may be due to the artificial environment affecting nutrient uptake and disrupting normal cellular signaling to initiate haustoria development [73].

1.3.5 Ontogenic resistance

It has been suggested that ontogenic resistance (age-related resistance) can suppress *P. aphanis* infection. A study by Asalf *et al.* (2016), investigated several cultivars to determine the development of *P. aphanis* infection. The findings revealed that older leaves exhibit less *P. aphanis* infection and that there was a clear absence of secondary hyphal growth. Results on young leaves showed that 50% of conidia were found to germinate and penetrate the epidermal layer, with functioning haustoria observed. Once the younger leaves completed unfurling, the presence of hyphal branching, haustoria development and sporulation was found to be noticeably reduced [65], [74], [75]. It has been suggested that the ontogenic leaf resistance is due to the thickening of cuticles in mature leaves, alongside the development of phenolic compounds such as salicylic acid, which inhibits pathogens [74]. Developing fruit also shows ontogenic resistance approximately 10 to 15 days after flowering, during the white phase of fruit development [65]. A similar result was observed in a glasshouse study detailing resistance at the pink stage of fruit growth [59]. However, the achenes in some of these cultivars still remain susceptible to *P. aphanis* infection [74]. In conclusion, these studies identify ontogenic resistance, stating young developing strawberry tissue being more susceptible to *P. aphanis* infection compared to older plant tissue [74], [75].

1.3.6 Powdery Mildew Control

Control of PM disease is mitigated by the application of stringent crop management practices and fungicide sprays. The fungicide sprays are introduced at regular intervals with chemicals such as myclobutanyl or demethylation-inhibiting fungicide (DMI) [59]. However, fungicides can only be applied a couple of times a year in order to slow down the possibility of resistance. DMI fungicides are the most effective and commonly used fungicide for PM control in commercial fields [76]. However, over time, the PM fungus has developed resistance to these fungicides, such as DMIs, whereby mutations in the *CY51* gene impacts the efficiency of the binding affinity of the DMI's to the target gene [76].

1.4 Genome Wide Association Studies (GWAS)

1.4.1 Advances in research

The domestication of crops and selective breeding has had a huge impact on genetic diversity in today's crops [77]. Advances in crop breeding technologies have been helped by the advancement of sequencing technologies. The development of more sophisticated genomic tools has been developed to allow an increasing number of markers to be screened and an increase in the number of individuals to be sequenced. The information collected can identify genetic associations with a desired trait by analysis using both, phenotypic and the genotypic information [79]. This has greatly aided breeders by providing a method to produce genotypic profiles for each individual plant, thus assisting with selection decisions [78].

Figure 5. Genome wide association study (GWAS) field experiment with 331 strawberry genotypes and 1655 individual plants (2021) (this study) Source S. C. Lynn (author)

1.4.2 Phenotypic data

Phenotypic data for disease resistance is obtained by scoring the severity of infection in individual plants using a five-point scale (Figure 6). For strawberry PM, the one to five-point scale developed by Simpson (1987) is the common scoring system used to identify the stage of the disease progression [66], [83], [84]:

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

The scoring is normally performed two to three times in a particular time frame e.g. before fruiting and twice in a season (e.g. May and August). The data collected are then used to calculate the area under the curve calculation for each genotype within the study (see section on statistical modeling) [66], [84].

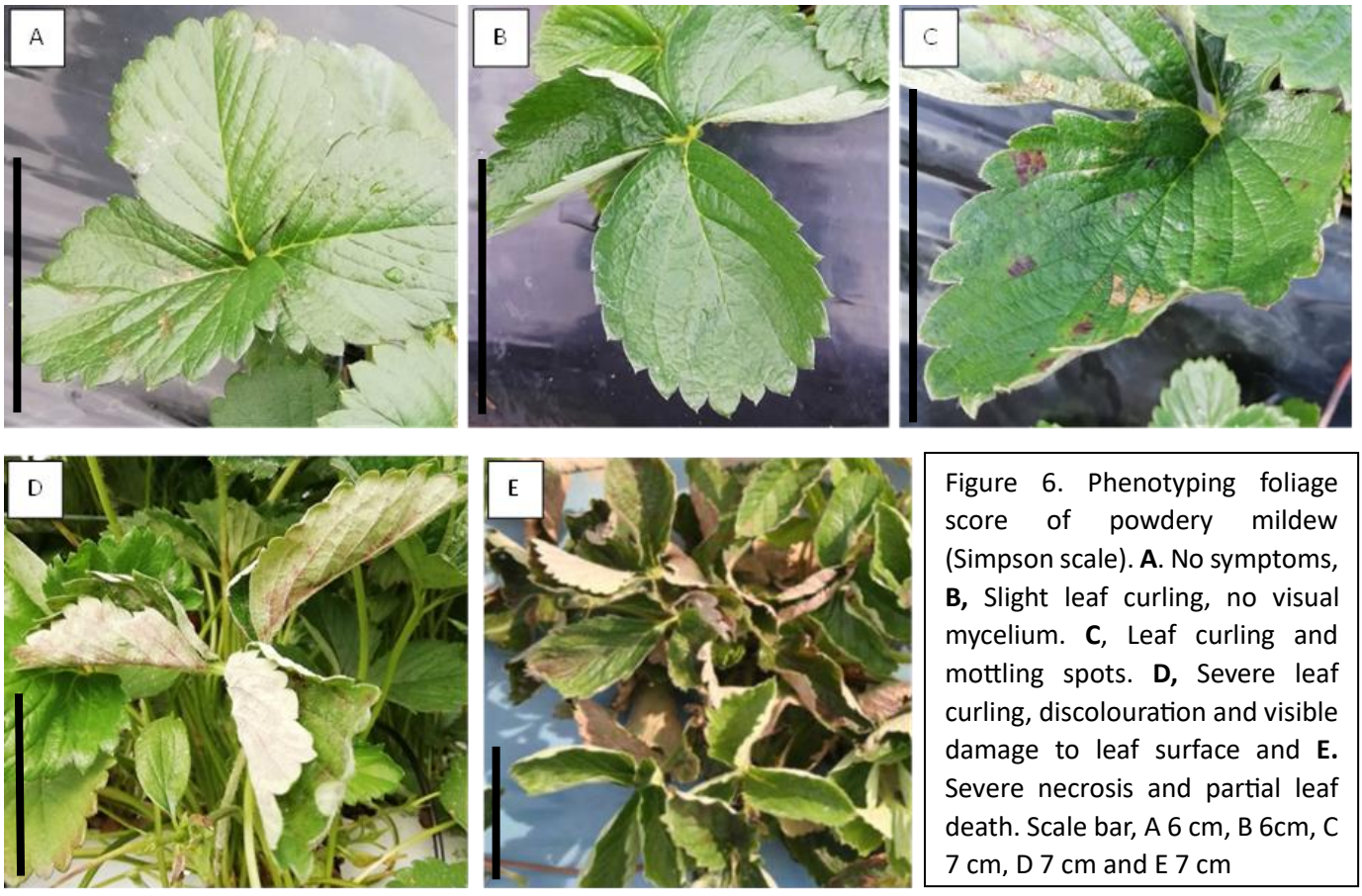

Figure 6. Phenotyping foliage score of powdery mildew (Simpson scale). **A**. No symptoms, **B,** Slight leaf curling, no visual mycelium. **C**, Leaf curling and mottling spots. **D,** Severe leaf curling, discolouration and visible damage to leaf surface and **E.** Severe necrosis and partial leaf death. Scale bar, A 6 cm, B 6cm, C 7 cm, D 7 cm and E 7 cm

1.4.3 Genome Mapping

For integrated genomic studies, analysis can be applied across the whole genome to identify regions of DNA associated with traits of interest. This can be achieved by testing the association between molecular markers and the trait. The molecular markers are then mapped onto the genome to detect specific regions that are predicted to contain genes or genetic elements controlling the trait. When dissecting more complex traits, two mapping methods are commonly applied – linkage mapping and genome wide association study (GWAS). The first method of linkage mapping localizes quantitative loci intervals, relying on the artificial linkage generated through genetic recombination whilst constructing the mapping population [86], [87]. The second method, GWAS (also known as linkage disequilibrium (LD)), is effective for identifying specific traits or a collection of traits such as candidate gene mapping. A GWAS looks at polymorphisms in selected genes involved in controlling phenotypic variation for specific traits. The application of the GWAS acts to survey genetic variation across the whole genome by testing the association of traits with hundreds of thousands of molecular markers that are distributed across the genome in order to identify variations of association for complex traits [86].

1.4.4 Quantitative trait loci

The response to disease in plants is controlled by either a single gene or may be associated with multiple alleles referred to as quantitative trait loci (QTLs) [88]. QTLs are identified through correlating the association between the phenotypes and genotypes of individuals within a population. Genotypes are described using molecular markers such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphisms (AFLP) or direct single nucleotide polymorphisms (SNP) assays [89], [90]. Different markers are selected for specific requirements; for example, AFLPs are chosen when there is no previous knowledge of the DNA sequence [86]. Many researchers have utilized SNPs for markers as they are easy to identify, cost-effective and the most abundant form of genetic variation [86]. SNPs are single base-pair changes in the DNA sequence that occur at a high frequency in the genome [91]. The microarray-based genotyping technology can be used to detect SNPs by the hybridization of the DNA of selected individuals to oligonucleotides spotted on to a SNP chip [66], [77]. The use of SNP chips in human and crop studies enables direct scanning of allelic variation across the genome covering thousands of SNPs in a short time and has the highest resolution for mapping QTLs [86], [92]. SNP chips can contain thousands of markers such as the Affymetrix Istraw 90 Axiom array, which contains approximately 90,000 potential molecular markers [66]. By using software such as Crosslink (designed for octoploids) the data can be used to produce

linkage maps [66]. The quality of the linkage map developed is influenced by the genetic marker coverage and the number of individuals included in the study [93].

1.4.5 Strawberry linkage mapping and GWAS

Recombination events that occur naturally contribute to new genetic variation within accessions. Favourable genetic combinations can be conserved through natural selection, resulting in different phenotypes within the population. These novel traits can be of great importance in agriculture advancements. Such recombination events can be utilized to facilitate our understanding and exploitation of the genetic components for desirable traits such as biotic, abiotic or fruit quality in order to enhance crop breeding [94]. Since the turn of the century, agriculture studies have utilized linkage mapping to identify quantitative trait loci (QTL) associated with phenotypic variations [95]. The advances in genetic mapping in crops have provided greater accuracy and precision to identifying QTL. In strawberry, Shulaev *et al.* (2011) were the first to generate a comprehensive map of the diploid *Fragaria vesca* accession 'Hawaii 4' [96]. Recently, in 2019 the octoploid reference genome was generated for the cultivar 'Camarosa' [80].

Linkage mapping has been employed across various crops, including Fragaria, in order to identify QTL. This method relies on bi-parental or multi-parental populations with contrasting phenotypes to facilitate identification of the genetic alleles associated with the phenotypic trait [1]. Through the identification of markers distributed across the genome, in combination with phenotypic differences observed within the population, statistical analyses enables the detection of trait associations [101]. Linkage mapping has led to the identification of significant loci and the elucidation of pathways involved with plant growth and development, notably in the studies of Arabidopsis and *Fragaria vesca* [102], [98]. However, the limitation of this method is associated with low resolution detection due to limited recombination events occurring in a single cross, potentially failing to reflect the genetic diversity present in a larger accession pool [95], [103].

Genome wide associations studies (GWAS) have emerged as a powerful tool since the early 2000, facilitating the identification of quantitative trait nucleotides (QTN) in human disease traits to aid medical advancement. Similar to linkage mapping, GWAS focuses on mapping associations between traits and genetic markers, with the added capacity to narrow in on the variant alleles involved [95], [101]. This method can increase the resolution of the mapping region, and thus reduce the research time required to find the causative genetic component. Association mapping requires a greater number of genetic markers as discovery of associations requires the existence of natural linkage between the genetic marker and the causative allele. GWAS requires thousands of SNP's across the genome for robust distribution

of markers linked with a well-annotated reference genome [101]. Its higher resolution capacity is attributed to the use of a larger diverse population, encompassing accessions with extensive number of recombination events occurring naturally or through extensive breeding. GWAS is suitable for mapping QTN that can be linked to complex traits, providing precise identification of genetic variation in the genome, enabling the localization of genes associated with the controlling phenotypic variation [101], [107]. The ability to identify genes associated with traits facilitates the identification of gene networks underlying complex traits [108]. Although GWAS is a powerful tool, its limitation is detecting rare alleles, as they can be concealed in a larger population.

An important aspect to consider when designing QTL studies is the impact of genetic x environmental (GxE) interactions [106], [109], [110]. Similar to human GWAS, studies should encompass multiple phenotyping events to achieve a comprehensive understanding of the molecular processes involved in strawberry disease and fruit traits. Genotypes exhibiting favourable traits may exhibit variations in phenotypes across different environmental conditions [111], [112]. Calenge *et al.* 2006 observed unstable/transient QTL following apple populations over several years, suggesting the transient nature of resistance genes influenced by environmental changes [104]. This was also noted in studies on strawberry by Cockerton *et al.* (2018) across different phenotyping events and Sargent *et al.* (2015) in glasshouse versus field conditions [113], [114]. Lewers *et al.* (2019) reported epistatic interactions in strawberry, with the presence of a suppressor loci present in the first year and absent in the succeeding years of the study [115]. Furthermore, when assessing the disease, it is important to consider not only environmental changes but also the specific race of pathogen prevalent to a particular region. The pathogen race may vary in pathogenicity across different regions and therefore influence the cultivar responses [114].

Powdery Mildew studies

The majority of studies investigating PM disease resistance in fruit crops have primarily been performed through linkage mapping, resulting in successful identification of gene resistance in several crops [25], [116], [117]. Notably, Calenge and Durel (2006) conducted research on apple powdery mildew *P. leucotricha*, identifying resistant molecular markers Pl-1 to Pl-10 conferring PM resistance [104]. Similarly, Karn *et al.* (2021) identified the REN11 locus, conferring strong and stable resistance to powdery mildew in grapevines over multiple years [118]. A major QTL on melon determining the response to *Podosphaera xanthii* has been identified - Pm-R, which suggested to contain the dominant gene from the parental resistant line [119]. A few studies of linkage mapping have been conducted on PM resistance markers in strawberry [113], [114], [120], [121]. Sargent *et al.* (2019) identified three significant QTL in

Norway across two locations, with one QTL identified in the glasshouse and two different QTL identified in the field. Similarly, a study based on multiple phenotypic assessments, conducted across the UK and Spain identified six stable QTL [113]. However, in a subsequent validation population the alleles were found to be poorly associated with resistance suggesting that the linkage between markers and causative gene is not retained over the wider population. Furthermore, Davik and Honne (2005) identified the genetic variance was contributed by additive effects, indicating the presence of an additive component in PM strawberry resistance [122]. The polygenic nature of powdery mildew in strawberry, previously suggested by Davik,, is now widely recognized [113], [114], [123], [124].

A small number of GWAS have been conducted focusing on disease resistance in strawberries, though a few studies have emerged with notable findings. Pincot *et al.* (2018) discovered a dominant QTL *FW1* linked with resistance to *Fusarium* wilt associated with a 85% phenotypic effect [109], [125]. Additionally, Anciro *et al.* (2018) identified a QTL, *FaRCg1*, conferring resistance to *Colletotrichum gloeosporioides* [126]. Nelson *et al.* (2021) identified three significant resistance QTN associated with Charcoal rot (*Macrophomina phaseolina*), with one QTN accounting for 44% of the phenotypic variation observed [127]. Subsequent research revealed the quantitative nature of *Macrophomina* resistance, suggesting that five resistance loci be stacked to provide successful resistance [128]. In the context of PM in strawberries, GWAS studies have been very limited. Only two such studies have specifically focused on PM resistance in strawberries. The first study by Tapia *et al.* (2021) involved sixty cultivars with the sole focus on identifying MLO genes in *Fragaria vesca* and *Fragaria* × *ananassa*. The second study by Cockerton *et al.* in 2018 performed a preliminary GWAS for powdery mildew resistance and identified one significant QTN, located on chromosome 6C [113]. The success of the 2018 GWAS, particularly with a small population of 75 accessions, suggested a wider pool of accessions could provide additional QTN to enable stacking of genes for a more durable resistance. More significantly, if performed over the course of two years the analysis would account for GxE interactions and thereby increase the identification of stable QTN.

Fruit Yield Studies

The ability to capture beneficial characteristics in strawberry breeding lines could lead to greater productivity and improved quality, thus resulting in increased economic benefits for the grower [129]. The method of linkage mapping has been effective in determining genetic components associated with flowering time and plant development pathways in the model species Arabidopsis, as well as abiotic and yield in barley [82], [94]. Yield consist of multiply components including seed number, weight, as well as other factors such as biomass,

resistance and plant architecture [130]. In the endeavor to improve yield, it is widely postulated that fruit quality and yield are quantitative traits, with traits being controlled by multiple genes or quantitative loci [106]. These quantitative traits have been observed in several studies of rosaceous species such as maturity date in peach and fruit size in pear [131], [132]. It is also suggested that quantitative trait loci can cluster in a single loci and that others could be masked by pleiotropic effects [131].

The primary approach to investigating strawberry yield has involved linkage mapping with bi- and multi- parental populations used to map loci of interest [133]. Numerous studies have focused on fruit quality and yield encompassing aspects such as shape, firmness, sugars and classification [105], [106], [134]. A study by Gaston *et al.* (2021) on early flowering within *Fragaria x ananassa* 'Sveva, mediated by Flowering Locus T (*FT*), found an interaction between *FvFT1* and *FvFT2*, with *FveFT3* inducing branching [135]. However, further investigation is required in different cultivars to determine the function of *FT* in octoploid strawberry. In another study it has been observed that the induction of the synthesis of the plant hormone, gibberellin, inhibits runner formation, consequently increasing flowering and yield number [136], [137]. Although other studies have detected potential regulatory and developmental genes in Fragaria there has been no definitive identification of causal gene(s) linked to flowering phenotypes [138], [139]. Flowering is regulated by complex and refined signaling pathways of multiple genes stimulated by environmental factors under desirable conditions [140], [141]. Further investigation is needed to better understand flowering, especially the genetic mechanism controlling variance between SD and LD flowering strawberries.

There have been many GWAS studies performed in agricultural crops including a study on days of flowering and flowering time in soybean. This study in soyabean demonstrated the effectiveness of conducting a GWAS for detecting complex components involved with these traits [142]. However, few GWAS studies have been conducted for fruit crops. Some investigations have been with peach, grapes, strawberry and apples, with an emphasis on fruit quality such as flavour, colour and firmness [101], [141], [143]. In strawberries, Wada *et al*. (2020) identified 166 QTL for fruit quality traits, such as fruit weight and firmness. The majority of the QTL identified were for fruit firmness trait, with genes associated with flanking markers [143].

As GWAS is a powerful method for detecting genetic components associated with quantitative traits, flower and fruit number in strawberry would be ideal traits to investigate. The results could provide evidence to increase strawberry number per plant produced without requiring additional production such as land or maintenance to achieve the increase.

1.4.6 Statistical modeling

With the advancement of genotyping platforms and improvements in statistical methods, high quality association mapping in strawberry can now be successfully achieved [93]. There are a variety of different statistical methods that can be applied to provide a more accurate and specific mapping.

QTL mapping

Disease phenotyping can be calculated by area under the disease progression curve (AUDPC). This method can be used to predict phenotypic scores for downstream QTL analysis [93]. The phenotypes used for QTL detection can be either endpoint, mean susceptibility scores or AUDPC. To understand environmental influences, genotypic and environment interactions impact on disease severity will be assessed, across phenotyping events using a two way ANOVA [84]. SNPs can be used to estimate genome-wide linkage disequilibrium (LD) using a customized R package such as Gblup to determine dominance and recessive patterns [144], [145]. Many researchers employ software designed especially for GWAS such as Haploview to view LD accompanied with PLINK. The PLINK tool is designed to calculate association results and is designed with automated GWAS quality controls and analysis tools. The R package involves functions for graphical diagnostic methods [145].

Spatial modeling

The statistical modeling for spatial variation in a plot can be used to control for uneven inoculation throughout the experiment. That is the possibility of high inoculation load possibly due to highly susceptible neighboring plots. Although randomized block design with replicates can provide some help with this problem, accounting for auto-spatial correlations can improve cultivar predictions. To account for these errors, Gilmour *et al.* (1997) established a more progressive modelling strategy to identify plot errors to facilitate correcting the problem. The modeling takes into account the correlations between neighboring plots and trends (linear and cubic) across the experimental fields [22], [84], [146].

Statistical corrections

GWAS analysis requires robust statistical model to ensure errors or false positives are taken into account [82]. To achieve the appropriate significant statistical power in a GWAS, a sufficient sample size is essential. Since many traits can be polygenic, and thus a single allele provides only a small effect size, a larger sample size is required to improve the statistical power to identify significant associations [82]. The most common method of genotyping to conduct is a crop GWAS through the use of single nucleotide polymorphisms (SNP) which

allow the scanning of the genome for variant alleles associated with the desired trait. Statistical association tests can then be applied to identify regions associated with variant alleles linked to the trait. To reduce the influence of low frequency alleles, minor alleles that represent less than 5% of the genotypes would be removed as well as any missing SNPs in more than 50 % of the population [147]. To account for bias resulting from population stratification, principle components analysis is applied to address false positives and false negatives [148]. Since a GWAS involves multiple testing, a Bonferroni correction would be employed to adjust the probability value (*p* value) threshold, correcting for false positives [149].

1.4.7 Candidate gene

Candidate genes associated with powdery mildew disease resistance can be identified in the genetic region obtained from the GWAS analysis. Screening gene models for motives can be performed following established resistance gene analog (RGAs) pipelines, looking at NBS, TM-CC proteins as well as membrane associated RLPs, RLKs collectively referred to as RGA families. These pipelines can be used to reveal disease related genes in conjunction with browser extensible data (BED tools) software to enable the comparison, manipulation and annotation of genomic features in browser extensible data (BED), general feature format (GFF) and blast tools [66], [150], [151]. R genes found within a 100 bp of the marker are considered to be a candidate gene associated with the SNP [84].

1.4.8 Genomic selection

Genomic Selection (GS) has been used to significantly improve genetic gains for animals and crop breeding. GS incorporates phenotyping information and genome wide markers to measure the effect of locus within the genome upon target traits. This information is used as a training set to model and predict the trait value of an individual using its DNA markers without phenotypic information. The larger the phenotypic data for the training set, the more stringent the analysis [153]. GS is superior to marker assisted selection (MAS), for improvement of quantitative traits, since GS uses all the markers identified across the genome. In addition, MAS has not demonstrated effectiveness on complex traits, whereas GS can exploits these markers to capture genetic variation among polygenic traits, measuring correlation among phenotypic predicted values [101]. Implementing GS can significantly improve accuracy in breeding, assisting the identification of providing favorable parents more quickly than traditional selection methods [156]. This method has successfully been incorporated in crops such as rice, maize and barley to assist the development of disease resistance [154], [155]. In a study with barley, a comparison of traditional phenotyping selection and GS found the results to be highly comparable [155]. Gezan *et al.* (2012) and Pinot *et al.* (2020) provided evidence for the benefits associated with the application of genomic prediction in strawberry

[156], [157]. This has also been observed in the strawberry breeding programme at the University of Florida, over the course of five season, resulting in a reduction of cost and time for selection of elite lines [153], [156]. The GS method is a valuable strategy for improving genetic gain and could be of substantial benefit for improving PM resistance and yield traits, as it would enable breeders to improved selection accuracy with a reduction of time and cost [152].

1.5 RNA sequencing

Another powerful method for investigating genetic complexity is RNA sequencing (RNA seq). Using advances of the next-generation sequencing (NGS), high throughput RNA sequencing provides information on the transcriptome association with a genome. This method was developed in the mid-2000s and is now considered a valuable tool for molecular biology [158]. RNA seq involves identifying differentially expressed genes (DEGs) involved in traits of interest, such as disease or development [159]. The application of this powerful tool is useful for exploring aspects of the plant development or stress response not previously discovered [160]. The method of RNA sequencing involves extracting RNA from tissue. The single strand mRNA is then enriched and converted into complementary DNA (cDNA), followed by the preparation of adaptor ligand library. The library is then run on a high throughput NGS platform, and the output reads are then aligned to the transcriptome. The statistical model then identifies significant DEGs [158]. RNA seq can provide a transcriptional map of the expression profiles involved with the trait or disease (Figure 7) [160], [161].

RNA seq provides quantitative measurements of expressed genes that can be used to identify expression changes related to trait of interest [160]. Many crop studies, such as those on apple, potato and rice, have incorporated this method for understanding genetic changes in the transcriptome in order to produce an enhanced crops [162], [163], [164]. Studies aimed at improving commercial strawberries have employed RNA seq to understand the complex nature of growth, development and disease resistance. Furthermore, with the addition of the *F* × *ananassa* reference genome in 2019, the accuracy of determining the complex genetic components underlying traits has greatly improved [80].

Recent studies on the complex mechanisms of disease have significantly improved our understanding of the host-pathogen interactions. RNA seq enables the identification of genes that change in expression due to interactions with the pathogen, as well as pinpointing signaling pathways involved in defence response [165]. A few studies have been performed with strawberry transcriptome analysis to analyse host response to pathogens. Strawberry foliage RNA analysis discovered changes in gene expression when plants were exposed to *Xanthomonas fragariae*, where the host was observed to adapt to the immune response, as

the infection progressed [166]. A study on strawberry fruit highlighted that immature strawberry fruit were resistant to *Botrytis cinerea*, unlike mature fruit. The authors identified that the DEGs involved in immature fruit disease response were associated with cell wall biosynthesis, which strengthens the cell walls in white fruit against attack [167]. Similarly, Lee *et al*. (2021) investigated differences in resistance between cultivars 'Sunnyberry' and 'Kingsberry' fruit inoculated with the pathogen *B. cinerea,* finding an increase in lignin (abundant in cell walls) in the more resistant fruit 'Sunnyberry' [168]. However, no studies have conducted a comparative analysis on both fruit and foliage in any crop pathosystem. A comparative tissue analysis would enhance our understanding of the tissue specific mechanisms in disease progression and identify potential universal resistance genes.

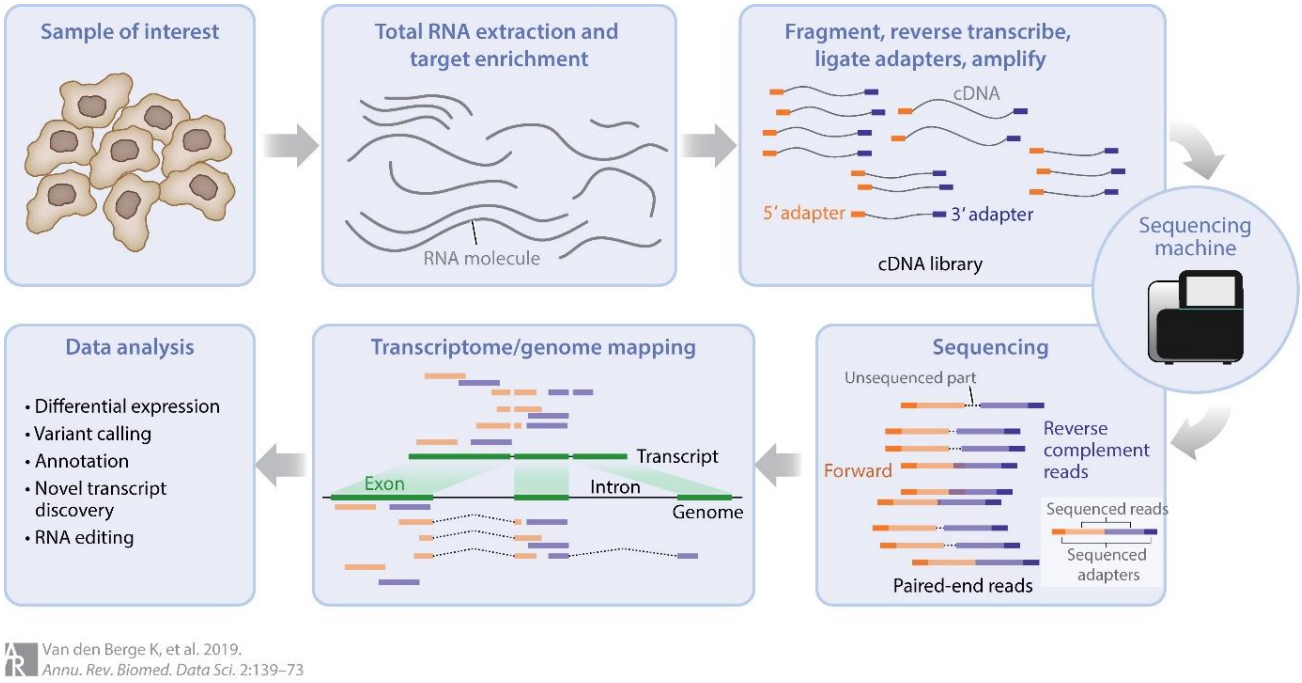

Figure 7. Methodology of RNA sequencing. The sample of interest is enriched and converted to complementary DNA library. The library is run on a high throughput next generation platform. Reads are mapped to a transcriptome for computational analysis of differentially expressed genes. Diagram from Van den Berge *et al.* (2019) [115].

1.6 Gene Editing

For centuries the breeding of crops has been employed to improve desirable traits for improved harvests. Traditional plant breeding techniques involve many rounds of hybridization and selection in order to obtain plants with the desired genetic combination [169], [170]. Since the 1930's, crop breeding has used mutagenesis techniques to introduce new traits in to the germline in order to discover novel traits for future breeding, using chemical and/or biological methods such as gamma radiation and transposon insertions [169], [171]. Although this has led to more traits for use in breeding, mutagenesis methods have their drawbacks such as the generation of random mutations, they are costly and the screening processes are complex and laborious. In nature random genetic mutations can produce point mutations, deletions, rearrangements and gene duplications [89]. These natural mutations have led to the production of over 3000 crop varieties and the method is still widely used today for traits such as flower colour in Chrysanthemums [169]. With the rise in global food consumption due to a growing population, climate change and loss of arable land, the demand for improved crops has increased, with a particular requirement to develop traits such as disease resistance, higher yields, stress and drought tolerance [89], [172].Since the 1990's the development and advancement of gene editing has transformed the progression of genetic breeding. The value of genetic editing is that it can be applied to crops where a known causative genetic component has been identified. Genetic editing of genomes consists of modifications at specific sites via targeted mutagenesis or site-directed insertion/deletion/substitution [89], [173].

Modern gene editing focuses on a specific target locus to modify the genome in a precise and accurate manner. CRISPR-Cas systems represented a groundbreaking discovery in molecular biology. CRISPR-Cas was originally discovered in bacteria as an adaptive defence system protecting the host from plasmid or viral infection [176]. The discovery of CRISPR and sequenced-specific nucleases (SSNs) such as Cas9 has enabled precise targeting of specific regions in the DNA for editing, either to knockout or overexpress the genes [176], [177]. In parallel, the method of host-induced gene silencing (HIGS) utilizes Interference RNA (RNAi) as another powerful tool for genetic modification. RNAi, is an intrinsic component of plant defence mechanism against pathogens, which leverages the endogenous RNAi pathway to induce target gene silencing. Typically involving the introduction of hairpin RNA construct that disrupt gene expression. The RNAi method has been extensively employed in research to elucidate gene function by silencing candidate genes, offering the ability to discern gene function [178]. Studies employing both these methods have achieved knocked out genes or deleted promoters to enhance crop traits, such as improved rice yield or promoter disruption conferring to disease resistance [179]. Additionally, researchers utilize genetic engineering

approaches to introduce exogenous genes for overexpression, aiming to unravel gene function and regulatory mechanisms [180]. Insertion of the desired gene can be subcloned and transformed into the host plant to identify gene function, such as overexpression of flower promotor to determine flowering regulation in strawberry or to improve abiotic stress tolerance by enhancing stomatal closure in Arabidopsis [135], [181]. The validation of candidate gene function through genetic manipulation techniques facilitates the development of molecular markers for breeding programs. This provides advancements in molecular breeding, resulting in a reduced cost and time for establishing elite lines.

1.7 Thesis aim

This project investigated powdery mildew resistance and flower/fruit number in strawberry. Here, three experimental studies are presented with the aim to determine potential genetic markers to advance strawberry breeding: The investigation into powdery mildew resistance was performed through two approaches to provide a comprehensive evaluation. Firstly, a large Genome Wide Association Study (GWAS) was conducted with 331 individual accessions, over a two-year period. This was conducted to obtain a robust dataset for identifying stable significant resistance quantitative trait nucleotides (QTN) associated with PM. Secondly, RNA sequencing was performed to comprehensively quantify and compare the transcriptome of different tissues in the presence and absence of PM. Applying this method presents insights into the molecular mechanisms underlying tissue specific resistance and potentially to identify universal candidate resistance genes. The RNA sequencing experiment also provides further insight into the mechanisms of host-pathogen interactions and important regulatory pathways. Lastly, a GWAS was conducted to establish a robust method for identifying novel genes that are associated with flower/fruit number. The overall aim in this thesis was to identify significant QTN and genes relevant for practical application in the future advancement of strawberry breeding.

1.8 Thesis Objectives

Genome association study investigating powdery mildew resistance in strawberry

- Phenotype PM disease symptoms in strawberry octoploid plants
- Genotype strawberry population –
    - extract DNA for all cultivars
    - quantify SNP markers with Affymetrix Axiom strawberry genotyping array.
- Statistical analysis of phenotype
    - Calculate Area Under the Disease Curve (AUDPC)
    - Calculate Spatial analysis
    - Calculate Best Linear Unbiased Estimate (BLUE).
- Genome wide association study
- Identify genes neighbouring QTN associated with resistance to PM

RNA Sequencing for tissue specific powdery mildew resistance in octoploid strawberry

- Extract RNA from foliage and fruit tissue from two selected strawberry cultivars infected and non-infected with powdery mildew for Illumina sequencing
- Identify differentially expressed genes (DEGs) associated with PM across different tissue types using integrated differential expression and pathways analysis (iDEP) platform.
- Investigate differentially expressed genes functions by utilizing biological databases.

Genome association study investigating flower and fruit number loci in strawberry

- Phenotype flowers and fruit number in strawberry octoploid plants
- Genotype strawberry population –
    - extract DNA for all cultivars
    - quantify SNP markers with Affymetrix Axiom strawberry genotyping array.
- Statistical analysis of phenotype
    - Calculate Spatial analysis
    - Calculate Best Linear Unbiased Estimate (BLUE).
- Genome wide association study
- Identify genes neighbouring QTN associated with flower and fruit number

The chapters written in this thesis are presented as research articles authored by Samantha C Lynn.

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Chapter 2

Materials and Methods

2.1 Genome Wide Association Study (GWAS)

A Genome wide association study (GWAS) was conducted to investigate the qualitative trait nucleotides (QTN) associated with powdery mildew (PM) resistance and flower/fruit number. The GWAS experiments characterizing both PM resistance and flower/fruit number were performed in one field trial plot, with plants remaining in the ground for a two-year duration of the investigation.

2.1.1 Plant Material

The plants for the GWAS trial were selected from commercially important stock, new breeding lines and early ancestry genotypes. All accessions were obtained from the NIAB (NIAB, East Malling, Kent, UK) (Accessions included were for the advancement of elite breeding lines) (see Appendix for selection of cultivars). A broad range of cultivars were selected which included a mixture of everbearing and June bearing cultivars. Additionally, included for a complementary pool of accessions were a selection of varieties documented with powdery mildew phenotypes (as identified in house) highly susceptible varieties – 'Hapil', 'Vibrant' and 'Flair', as well as more resistant cultivars 'Buddy', 'Portola' and 'San Andreas'. The total number of varieties assembled for the trial was 331 different cultivars. In April 2020, these selected cultivars were transferred to a single polytunnel for propagation. In July 2020, six clonal replicates of each plant were extracted and transferred to the glasshouse.

2.1.2 Propagation of misted tips

In the glasshouse, replicates stolons were pinned in 9 cm pots containing compost for six weeks with misting to enable root development. The replicates were maintained in a heated compartment at 25 ^{o}C with 16hr/8 hr day/night cycle. The initial humidity was set at 100% for 2 weeks, then reduced to 80% for two weeks and set at 60 % for the final two weeks with addition of fertigation (NPK, 4:1:2, 15 g/m^2/week).

2.1.3 Field Experiment

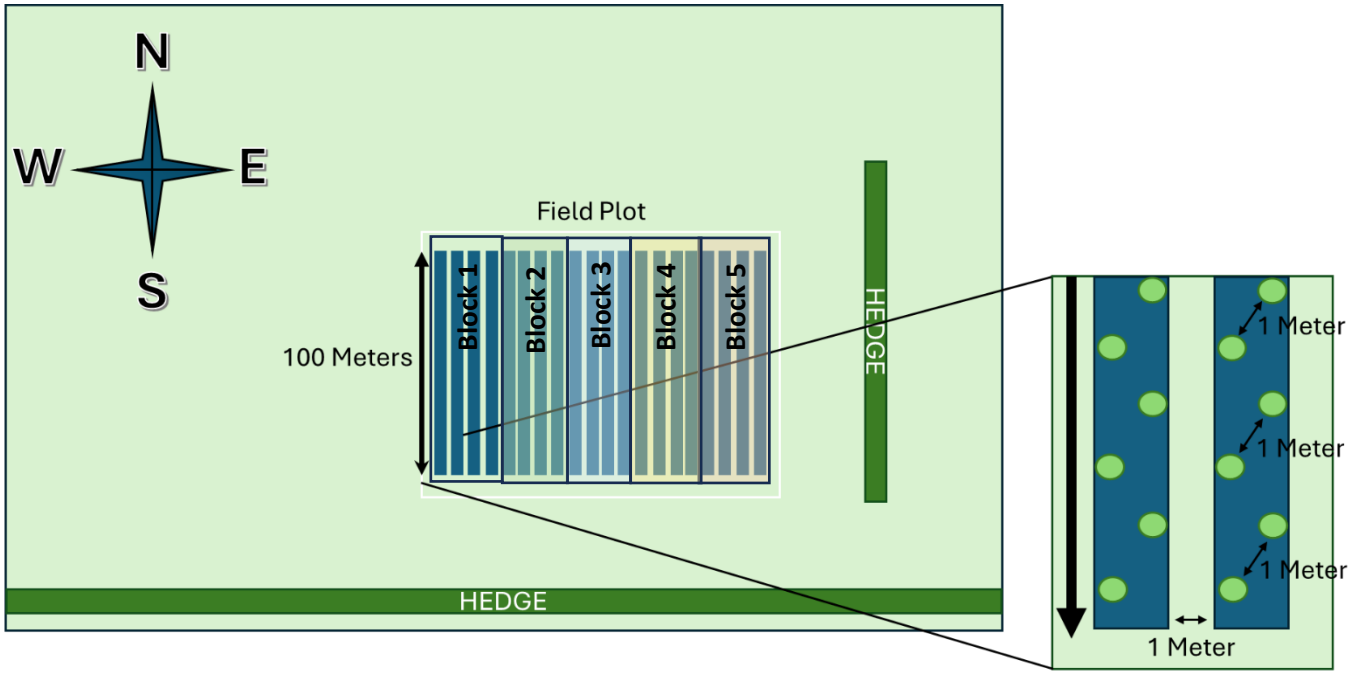

Figure 1. Genome Wide Association Study field plot diagram. Position of plants (green circles) and blocks in the field trial with 100 meter length per row, 1 meter between rows and 1 meter between cultivars. On a North – South trajectory in a five-block randomized design with one clonal replicate present per block for accessions.

The field was prepared eight weeks in advance by fumigating for the control of soil borne disease and pest larvae. The raised beds were covered with a polythene mulch covering with a trickle fertigation (NPK, 22:4:22 at 25 kg ha-1). The replicates were planted in August 2020 in a randomized block design in five blocks, each block contained one cultivar replicate for all accessions (Figure 1). The planting configuration featured rows with a length of 100 m and a 1 m inter-plant spacing, with a North to South orientation, located at NIAB, East Malling, Kent (51˚17'20.1"N 0˚27'11.0"E). A natural infection of *Podosphaera aphanis* was established within the field. Maintenance of the plants consisted of trimming each season in March and October to remove dead leaves and runners, supplemented with two mid-season maintenance events, targeting runner removal. For the duration of the trial no fungicides were administered to the GWAS field plot.

No other diseases were detected throughout the duration of the trial. Low level pests were observed during the trial. Pests reported were Aphids (*Aphis* spp.), Thrips (*Frankliniellas* spp.), Fruit fly (*Drosophila* spp.) and Tarnished plant bug/Capsids (*Lygus rugulipennis/ Lygocoris pabulinus).* To reduce fruit fly infestation, ripe strawberry fruit was continually removed and disposed of as per site procedure. Note: it was hypothesized that low-level pests were observed in the plot due to the lack of pesticides application, resulting in a high level of

predatory insects present in the trial such as Hoverflies (*Syrphids*), Lacewings (*Chrysopidae* spp.) and Parasitic wasps (*Aphidius* spp., *Brachonids*) were observed.

Results for method 2.1 are found in Chapter 3 and 5.

2.2 Powdery Mildew Phenotyping

2.2.1 Strawberry Foliage Phenotyping

Disease severity for powdery mildew foliage assessments was initially phenotyped after planting in October 2020 and subsequently each month from June to September for 2021 and 2022. The trial was performed over the course of two years with the same plants remaining in the field plot. Phenotypic scoring of foliage for powdery mildew disease was based on the Simpson *et al.* five point scale [1] (Figure 2). Periodic visual confirmation of PM infection was conducted by assessing the presence of mycelia and discoloration on the foliage throughout the study to clarify positive symptoms. However, these observations were not scored due to time limitations associated with assessing every individual as part of the phenotyping scoring event.

Five-point scale:

1. No symptoms
2. Slight leaf curling
3. Leaf curling and mottling
4. Severe leaf curling, discolouration and visible leaf damage
5. Severe necrosis and partial leaf death

Figure 2. Strawberry plant powdery mildew symptoms scale for strawberry foliage:

A. 1 - No symptoms observed,

B. 2 - Slight leaf curling,

C. 3 - Leaf curling and mottling,

D. 4 - Severe leaf curling, discoloration and visible leaf damage

E. 5 - Severe necrosis and partial leaf death.

2.2.2 Strawberry Fruit Phenotype

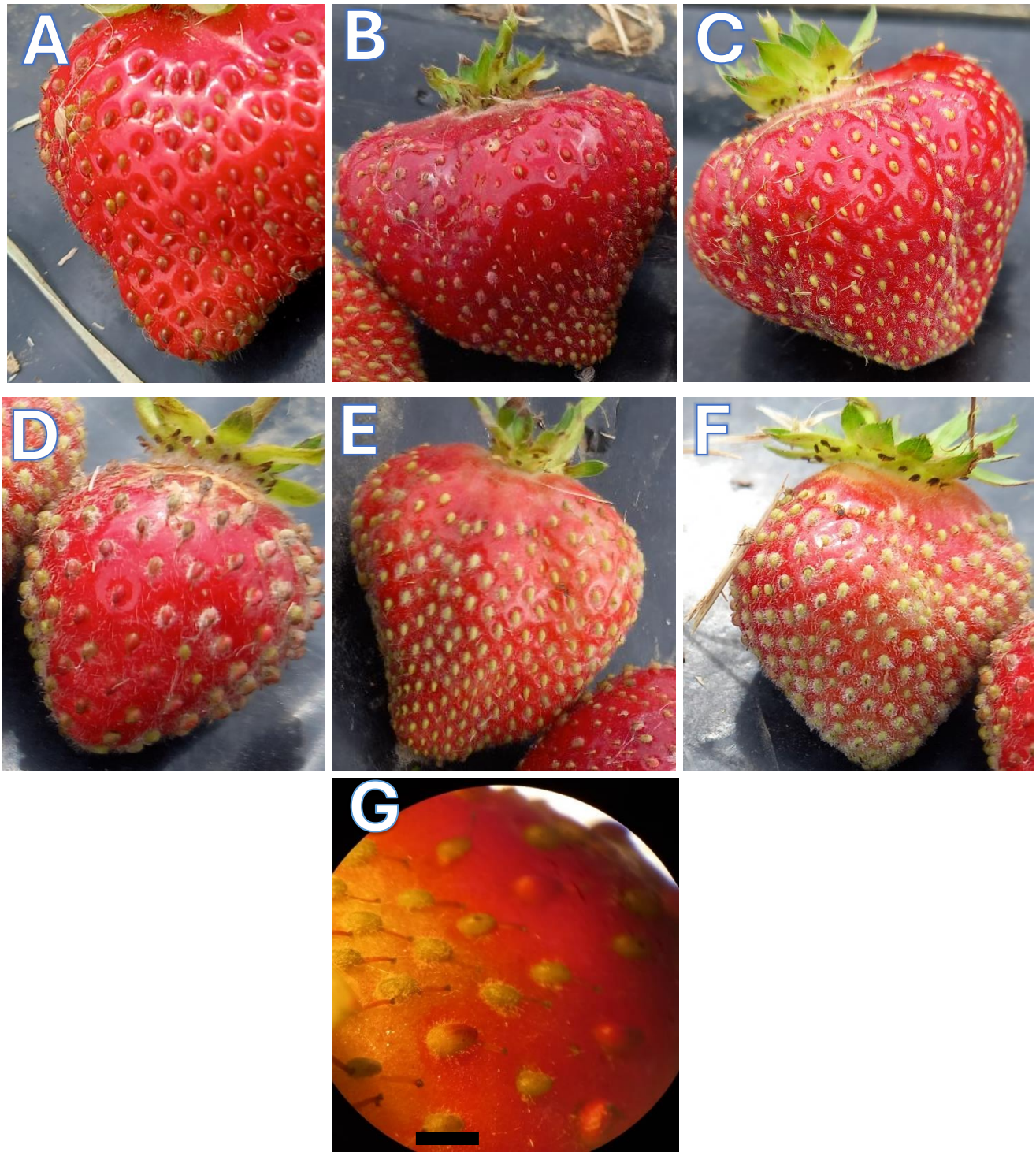

Figure 3. Strawberry fruit powdery mildew symptoms percentage scores scale. A. 0 = No superficial mycelium on fruit surface, B. 1 = < 10 % covered with mycelium, C. 10-25 % covered with mycelium, D. 25-50 % covered with mycelium, E. 50-75 % covered with mycelium and F. 5 = 75-100 % covered with mycelium and G. Microscope image of <10 % with scale bar of 2 mm.

The first year was not assessed in order to leave the June bearers required chilling period for induction of flowers. Assessment of the strawberry fruits commenced in August 2022. Up to five fruits from each plant were scored for disease symptoms. The scoring system was based on a modified protocol from Palmer *et al.* 2007 [2]. Strawberry Phenotyping scoring scale: 0. No superficial mycelium on fruit surface, 1. < 10 % of the fruit surface covered with mycelium, 2. 10-25 % of the fruit surface covered with mycelium, 3. 25-50 % of the fruit surface covered with mycelium, 4. 50-75 % of the fruit surface covered with mycelium and 5. 75-100 % of the fruit surface covered with mycelium (Figure 3).

To ensure a full comprehensive disease visualization, each fruit was assessed using a x30 jeweller's loupe to confirm PM infection. Notably, scores below 50 % were observed with the mycelia primarily within the achene pits (Figure 3).

Results for method 2.2 are found in Chapter 3.

2.3 Genotype collection

All GWAS strawberry cultivars subject to statistical assessment were genotyped, amounting to a total of 331 cultivars. Tissue samples from young unfolding leaves of vegetative cultivars were collected, freeze dried and stored at room temperature. Extraction of genomic DNA was performed using the Qiagen DNeasy plant mini kit (Qiagen Ltd., UK) following the manufacturer's instructions. Dried tissue was ground either by pestle and mortar or TissueLyser. Subsequently, DNA samples were then quantified with the Qubit 2.0 Fluorometer (Therma Fisher Scientific, Inc, USA) and stored at -20 ºC until required. The genotyping of DNA samples was carried out on the Afffymetrix Istraw90 Axiom Array (i90k) or Istraw35 (i35k) [3] to achieve genome wide coverage of single nucleotide polymorphisms (SNPs) for the allo-octoploid cultivated strawberry *Fragaria* × *ananassa*. The Istraw90 comprised of approximately 90,000 genetic markers [3]. The linkage map utilized was generated using five diploid biparental mapping populations for a fully comprehensive octoploid linkage map [Vickerstaff *et al.*, unpublished]. The pseud-octoploid linkage map was employed to define SNP marker location. Markers were assigned on to chromosomes denoted 1-7, sub-genomes were assigned A-D as described by Davik *et al*. (2015). The genomic positions of SNPs were defined using *Fragaria vesca* genome V2.0.

Results for method 2.3 are found in Chapter 3 and 5.

2.4 Powdery Mildew Statistics

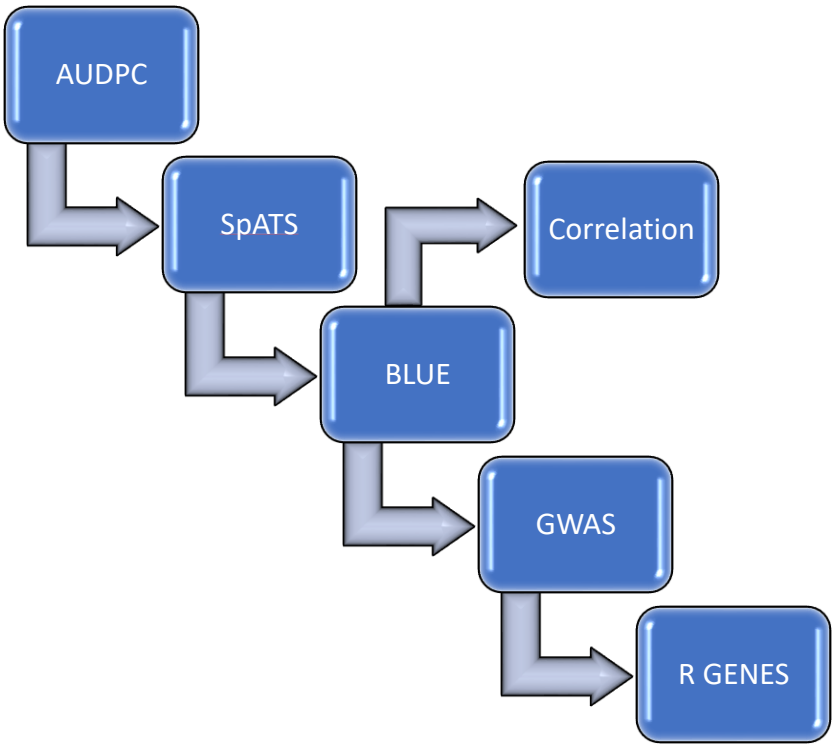

Figure 4 PM phenotype and genotype statistical analysis flow chart. The flow chart specifies the steps employed for comprehensive statistical analysis for the identification of quantitative trait nucleotides and resistance genes (R genes) associated with powdery mildew resistance. AUDPC – area under the disease progression curve, SpATS – spatial analysis, BLUE – best linear unbiased estimate, GWAS – genome wide association study.

Area Under the Disease Progression Curve (AUDPC)

In order to establish variations in differing disease levels of cultivars and accommodate the quantitative resistance present within strawberry, it is necessary to measure the disease progression over time. Assessment of disease symptoms at different scoring events can determine the extent of the disease progression observed based on incidence and severity [4]. The disease intensity over time was calculated in this experiment using the area under the disease progression curve (AUDPC) calculation. The scores collected over a two-year period (eight scoring events) of foliage assessment were analysed and scores for 2021 and 2022

independently calculated. The AUDPC was performed using the R package 'agricolae' [5] and calculated as follows:

$$AUDPC = \left\{ \sum_{i=1}^{n-1} \left[\frac{y_{i+1} + y_i}{2} \right] * [X_{i+1} - X_i] \right\}$$

Where *y* is the mildew severity score, for score *i*, *X* represents the time in months and *n* is the number of scoring events. Relative AUDPC (rAUDPC) was calculated by dividing the AUDPC value by the number of phenotyping events.

SpATS

Within a field plot, spatial variation can arise due to microenvironments, impacting the expression of traits under study due to plant location or environmental movement [6]. Although measures were implemented to potentially overcome these variations such as standardizing plant distance and implementation of a randomized block design, to provide a more robust analysis to account for spatial variation in the field, a spatial model was employed. The method in this experiment selected the two-dimensional smooth surface model, incorporating a Penalised splines approach to correct environmental variation of phenotypes across the field trial (SpATS package) [6]. Broad sense heritability (H^2) was applied to calculate the percentage of phenotype variance associated with the genetic variance, defined as $H^2 = V_G/V_P$. The genetic associations for H^2 were calculated using the SpATS package [6].

Best Linear Unbiased Estimates (BLUE)

A best linear unbiased estimates (BLUE) linear regression model was used to calculate overall phenotypes for each genotype. Here, the BLUEs were generated using the R package 'lme4' through a mixed linear effect model where genotype was specified as a fixed effect and block a random effect [7]. BLUEs values were utilized to provide an overall disease score for each genotype; the individual genotype scores were then employed for the GWAS analysis.

GWAS

A Genome Wide Association Study (GWAS) analysis was conducted applying the BLUE foliar disease scores for 2021, 2022 as well as the fruit scores in 2022, across 331 different genotypes. The GWAS analysis was conducted with the phenotype and genotype data using PLINK, with the detailed procedure outlined on GitHub [8], [9]. SNP filtering involved removing minor allele representing less than 5 % of the genotypes. Additionally, any SNP missing in more than 50 % of the population was excluded from the analysis. Principle component co-variants were employed to adjust the analysis for population stratification. A Manhattan plot

was generated using the 'CMplot' R package to visualize GWAS results, with Bonferroni corrected *p*-value ($p < 3.423 \times 10^{-6}$) plotted across the octoploid strawberry chromosomes.

Focal SNP

From the generated SNPs, the most significant focal SNP was identified for each chromosome. In accordance with related GWAS studies [10], [11], [12], disease related genes within a 100 Kbp range of the focal SNPs were identified using browser extensible data software (BED tools) [13]. The bedtools consisted of a script to identify resistance genes from the annotated *F. vesca* genome. The resistance genes identified were: Nucleotide Binding Site (NBS), Receptor Like Kinase (RLK), Mildew Loci O (MLO), Trans Membrane Coiled-Coiled (TMCC) and Receptor Like Protein (RLP) [14]. The molecular and biological functions of the genes underlying the quantitative trait nucleotide (QTN) were characterised using Genome Data base for Rosaceae (GDR), EMBL European Bioinformatics Institute 'InterProScan' tool, Pathogen Receptor Genes data base (PRGdb), Uniport tools and NCBI BLAST alignment tool 'BLASTn' [15], [16], [17], [18], [19] (Figure 4).

Correlation analysis

The linear relationship between PM foliage and fruit were tested through a correlation analysis using the BLUEs scores values. The correlation matrices were created using the 'corrplot' R package with Spearman's correlation to visualize the genotypic and individual correlations between phenotypic foliage scores and fruit scores.

Results for method 2.4 are found in Chapter 3.

2.5 Flower/fruit Number Phenotyping

The assessment of flower/fruit number was performed in mid-May 2022, during cooler temperatures before the establishment of powdery mildew infection. The evaluation of strawberry fruit was conducted in 2022 at a single phenotyping event. The flower/fruit number count was carried out on 328 cultivars, comprising of 244 June bearers and 84 everbearers, within the GWAS field plot. The flowers, buds and fruits were measured through visual counting and recorded (Figure 5). This assessment was conducted across all five randomized blocks. In total, 1640 individuals were counted. A team of five were involved with the assessment, over the duration of five days, to collect the complete data set.

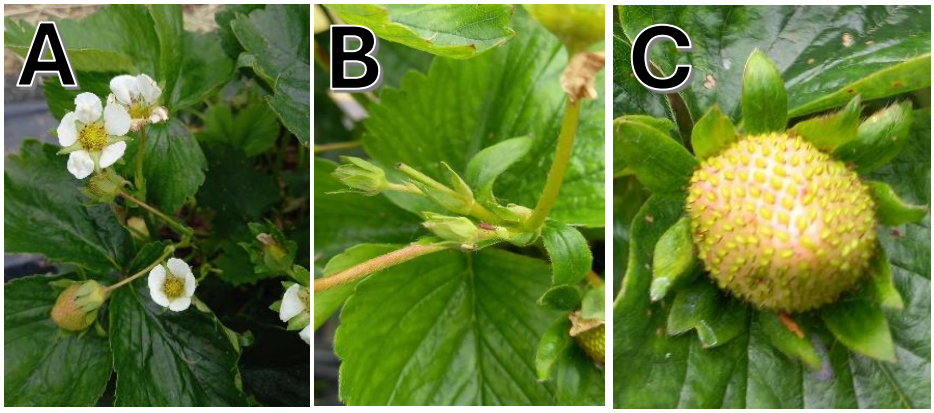

Figure 5. The quantification of strawberry physiology for flower/fruit number phenotyping, A. Flowers, B. buds and C. strawberry fruit.

Results for method 2.5 are found in Chapter 5.

2.6 Flower and fruit number statistical analysis

The strawberry flower and fruit number scores were generated through weighed averages taken across the five pseudo-replicate fruit disease score assessments. As detailed in 2.4.1, these scores were applied to the SpATS and BLUES package, followed with the GWAS analysis (Figure 6). QTN were investigated through GDR to identify genes related to flower and fruit number.

Results for method 2.6 are found in Chapter 5.

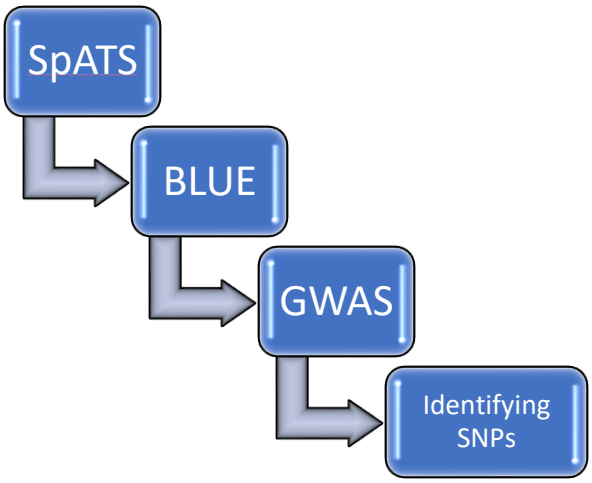

Figure 6. Flower and fruit number statistical analysis flow chart. The flow chart indicates the steps applied for complete statistical analysis to obtain flower and fruit number SNP markers. The markers were then used to identify associated genes with the flowering/fruit number trait. SpATS – Spatial analysis, BLUE – Best linear unbiased estimate, GWAS – Genome wide association study and SNP – single nucleotide polymorphisms.

2.7 Genomic selection

The extensive GWAS data collected in this experiment was utilized as a training set for genomic selection (GS) to predict the efficacy of application of GS for PM resistance and flower/fruit number. The potential of genomic selection for use in genetic informed breeding was calculated using the ridge regression best linear unbiased prediction “rrBLUP” R package to estimate the effect of markers on disease score [20]. GWAS marker data and phenotype data were split into a training sample of 60 % of the population and a test sample of 40 %. The phenotype of the test sample was predicted and then compared to the actual phenotype values in order to assess the predictive accuracy. The model was run with 100 permutations; for each iteration a random selection of genotypes were allocated to either the training or test data set [21], [22]. Computations above 30 % were denoted as a good trait for future GS application in strawberry breeding.

Results for method 2.7 are found in Chapter 3 and 5.

2.8 RNA Sequencing

RNA sequencing was performed to isolate differentially expressed genes (DEGs) associated with powdery mildew infection. The DEGs provide significant candidate genes for resistance against PM and a more in-depth knowledge of the pathways involved.

2.8.1 Plant material

Fragaria × *ananassa* cultivars were 'Hapil' (with susceptible foliage and fruit considered moderately resistant, based on in house data) and 'E10' (with susceptible fruit and resistant foliage). Plants were housed in a split plot design arrangement in a polytunnel at NIAB, East Malling (GPS co-ordinates - 51.291586, 0.447843). Plants were grown in 1 meter coir bags with 8 plants per bag and fertigation supplied, NPK 12:12:36 at 1 g l^{-1}. The first plot arrangement included both cultivars and allowed for natural PM infection. The second plot had fungicides applied based on weekly rapid disease assessment of the managed plots as well as disease forecasts and forecast weather conditions to prevent PM infection. Several fungicides were applied throughout the season based on standard operating procedures for PM infection at NIAB. No other diseases were observed for the strawberry plants in the polytunnel for the duration of the trial. Plant material from foliage and strawberry fruit tissue was harvested from biological replicates, taken from different plants. Five replicates were taken from infected and clean plots, except for "old foliage" treatments with four foliage replicates taken (Table 1). The plant tissue sampled comprised young leaves (expanded but folded), mature leaves (fully expanded) and whole ripe fruit. PM Infected tissue was taken with 30 % mycelium coverage and was observed on the surface tissue (Figure 7). Both uninfected and infected samples were harvested, flash frozen in liquid nitrogen and stored at -80 °C until RNA extraction.

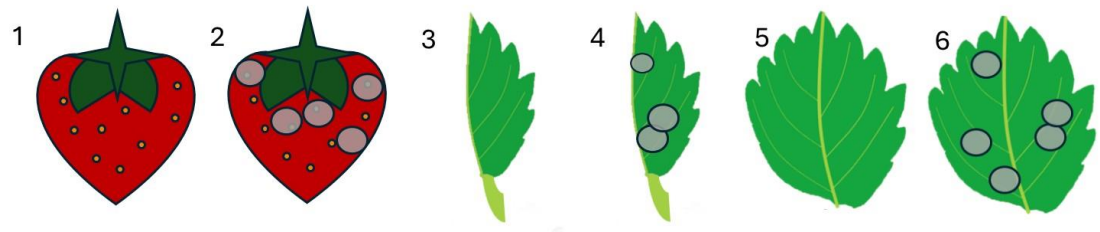

Figure 7. Illustration of Strawberry tissue samples harvested for non- infected and infected with powdery mildew (PM). 1. Strawberry fruit non – infected, 2. Strawberry fruit with >30 % PM infection, 3. Strawberry young foliage non-infected, 4. Strawberry young foliage with >30 % PM infection, 5. Strawberry old foliage non-infected and 6. Strawberry old foliage >30 % PM infection

2.8.2 RNA extraction

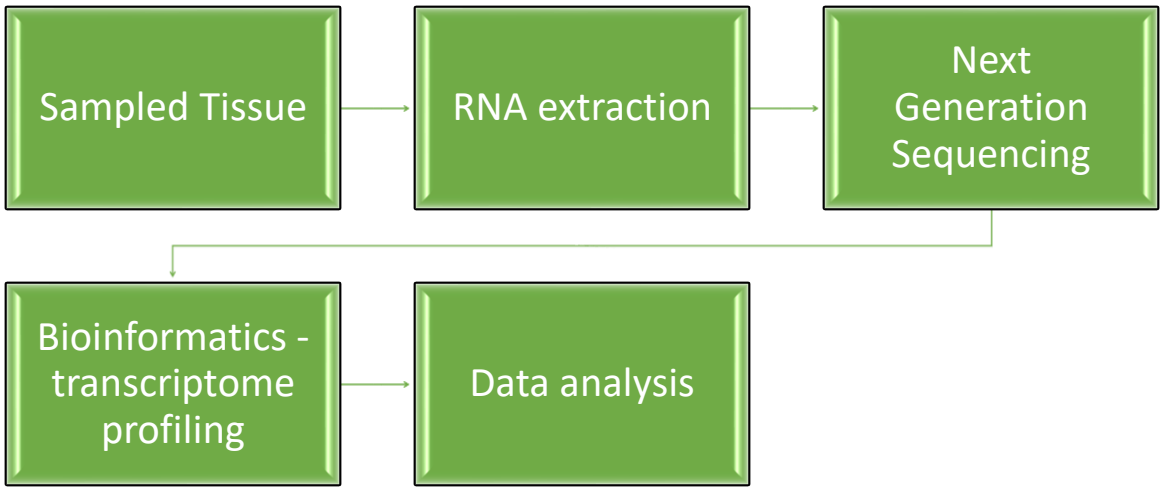

Figure 8. Flow diagram for RNA sequencing. The sample tissue collected, and RNA extracted. The RNA sent for Next Generation Sequencing. Data from Next Generation Sequencing (NGS) is processed to bioinformatic analysis for transcriptome profiling. Data is analysed downstream to determine gene expression and function.

The tissue samples were ground with a pestle and mortar, whilst continually supplied with liquid nitrogen to prevent samples from thawing. The RNA was extracted using the Qiagen RNAeasy plant kit (Qiagen, UK), performed as per manufacturers specifications. RLC lysis buffer was chosen for all samples due to higher yield and sample purity. RNA Sequencing was conducted by Novogene transcriptome sequencing. Stringent sample requirements included a concentration of >20 ng/µl, RNA Integrity number (RIN) above 8, optical density ratios above 2 (260/280 and OD 260/230) for successful sequencing outcomes. Prior to dispatch, samples specifications were verified using a spectrophotometer (Nanodrop™) and fluorimeter (Qubit, Invitrogen). RNA degradation was assessed using Agilent RNA ScreenTape system (2200 Tapestation, Agilent, Germany). A total of 48 samples were sent for sequencing at Novagene for poly A enrichment mRNA library preparation and sequencing (Table 1). Subsequent, sequencing was performed on an Illumina Novaseq 6000 sequencing system employing paired end 150 bp chemistry (Figure 8). Hapil fruit was not analysed as the level of resistance was not determined.

Table 1. Tissue samples for cultivars ‘Hapil’ and ‘E10’ collected. Samples of infected and non-infected tissue were extracted and prepared for RNA sequencing.

Cultivar	Tissue	Treatment	Biological replicates
E10	Young foliage	Infected	5
E10	Young foliage	Non-Infected	5
E10	Fruit	Infected	5
E10	Fruit	Non-Infected	5
Hapil	Young Foliage	Infected	5
Hapil	Young Foliage	Non-Infected	5
Hapil	Old Foliage	Infected	4
Hapil	Old Foliage	Non-Infected	4
Hapil	Fruit	Infected	5
Hapil	Fruit	Non-Infected	5

2.8.3 RNA analysis

The received data output contained raw data (G), raw reads, Q20 and Q30 Phred quality scores indicating the probability of base calling error and GC content. The Raw reads were in FASTQ format and were trimmed with a multithreaded command line tool Trimmomatic [23]. This tool was utilized to remove adapters, reads containing poly-N and low-quality reads from the raw data to produce clean reads. Filtering with the Pred quality scores and trimming yielded high-quality reads for analysis. The RNA-seq reads were then individually aligned against the predicted gene model *Fragaria* × *ananassa* ‘Camarosa’ genome, downloaded from the Genome Database for Rosaceae (GDR) [19]. Mapping of the RNA seq reads to the genome was performed using the program, ‘Salmon’. The Salmon model incorporates a dual phase inference algorithm as well as sample-specific bias. This model was favoured due to a greater sensitivity and lower false discovery rates. Additionally, the Salmon model allows for tracking the position and orientation of all mapped fragments, generating total aligned read counts per transcript [24].

Differentially expressed genes (DEGs)

The analysis of RNA sequencing data involved identifying differentially expressed genes across the treatment samples. Differentially expressed genes (DEGs) were identified using the integrated Differential Expression and Pathway analysis (iDEP) webserver (V1.1) [43]. Deseq 2 model based on read count data, were normalised within iDep using the DESeq2 EdgeR. The DESeq 2 package identifies and corrects for estimated dispersion and fold change providing more accuracy for quantitative analysis [25]. Principle component analysis (PCA) was utilized to cluster read-count data across samples, visually assess consistency of biological replicates in the dataset. Subsequently, DESeq2 was further employed within iDEP to identify upregulated and downregulated genes with a threshold of 2 log fold change and a false discovery rate (FDR) of 0.05 between treatments. Volcano plots were generated to represent the significant differentially expressed upregulated and downregulated genes associated with PM resistance measured by log2 fold change against the adjusted *p* value (log10 padj). Heatmaps were generated with iDEP using the "DESeq2" to visualize the expression changes in tissue types across samples with and without infection (Figure 9) [44,45].

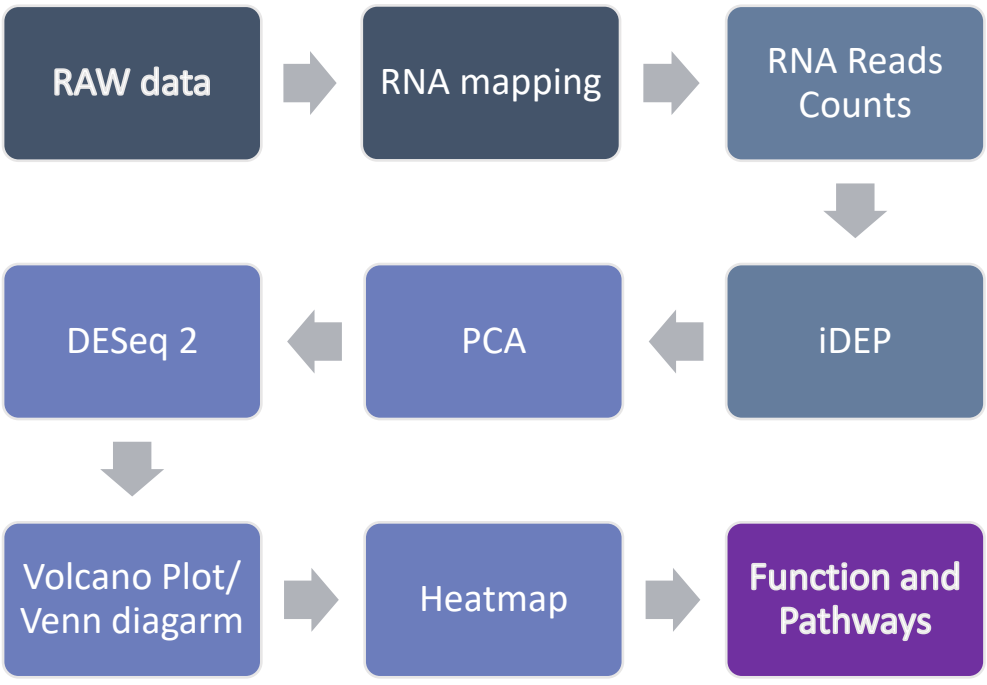

Figure 9. Raw data flow chart for differentially expressed genes (DEGs). The RNA sequence raw data obtained through sequencing through the process using iDEP to gain DEGs. DEG function was identified with Genome Database for Rosaceae and pathways analysis with STRING.

To explore the transcriptional response elicited by PM infection caused by *P. aphanis,* an assessment was conducted across three different resistance types: cultivar resistance, tissue resistance and ontogenic resistance (Table 2). The experimental design employed an innovative approach aiming to analyse cultivar resistance within different tissues. This methodology involved a comprehensive comparison of all tissues to elucidate pivotal DEGs associated with PM resistance. The primary objective was to examine foliage expression differences between infected and uninfected samples across the two different cultivars (‘E10’ young leaf versus ‘Hapil’ young leaf), where ‘E10’ has relatively resistant foliage and ‘Hapil’ foliage is susceptible. Tissue resistance was evaluated through comparing expression differences between infected and uninfected samples across ‘E10’ resistant foliage and susceptible fruit ('E10’ young leaf versus ‘E10’ fruit). Ontogenic resistance was assessed through comparing expression differences between infected and uninfected samples across old and young foliage (‘Hapil’ old leaf versus ‘Hapil’ young leaf). Subsequently, the identified DEGs in each comparative analysis were collated to ascertain DEGs involved in pairwise interactions and subsequently combined for all three resistance types.

Table 2. Experiment parameters with different tissue types.

Experiment parameters	**Experiment name**
‘Hapil’ Young foliage vs ‘E10’ Young foliage	Cultivar resistance
‘E10’ fruit vs ‘E10’ Young foliage	Tissue resistance
‘Hapil’ Old foliage vs ‘Hapil’ Young foliage	Ontogenic resistance

Significant DEGs identified with DESeq2 were initially annotated with strawberry gene nomenclature, which was converted to gene names through the GDR database. GDR provided the gene name, location in the genome and Gene Ontology terms (GO) [19], [26]. The identified genes were then subjected to gene interaction analysis, conducted by using the STRING database (v12.0). The STRING database collates functional interaction data from various research literature and sources, thereby providing pseudo-linkages to genes associated with pathways. In order to generate functional gene association networks for *Fragaria*, *Arabidopsis thaliana* gene orthologues of the significant DEGs were identified [27]. STRING was utilized to determine functionality and pathways identified from significant DEGs in the RNAseq. Investigating the nodes was used to determine the resistance biological processes to PM in different tissue types. In the generated STRING networks, interconnect genes are represented as nodes (circles) and functional interactions as edges (lines), the

edges, indicating interactions with other nodes. In this experiment nodes are colour coded to denote various biological processes as recognized with GO terms (Table 2) [27]. In this experiment the green nodes are representative of genes involved with the defense pathway. Yellow is representative of the regulation of defense pathway. Red is representative of the response to stress pathway and was chosen as this would likely be involved with pathogen infection albeit if the defense pathway has not yet been established for this disease. The purple node is representative of the response to abiotic stress, elected due to reports of genes associated with abiotic stress later discovered to play key roles in pathogen responses (Table 3) [28], [29].

Table 3. Assigned node pathways. Node pathways indicated through different colours identified using Gene Ontology terms.

Node symbol	Node Colour	Pathway	GO term
	Green node	Defence response	GO:0006952
	Yellow node	Regulation of defence	GO:0031347
	Red node	Response to stress	GO:0006950
	Purple node	Response to abiotic stress	GO:0050896

The identity of gene function was determined via NCBI and published research [18]. Furthermore, various transcription factors identified in this experiment were verified using the Plant Transcription Factor Database to ensure accurate identification [30].

Results for method 2.8 are found in Chapter 4.

Appendix

Supplementary Table S1. Commercial cultivars. Cultivars included in the GWAS experiments for powdery mildew and flower/fruit number.

June bearer	Everbearer
VIBRANT	DIAMANTE
ELSANTA	ALBION
CAMBRIDGE FAVORITE	MARA DES BOIS
SONATA	SELVA
FENELLA	BOLERO
FLAIR	PORTOLA
REDGUANTLET	SAN ANDREAS
ELSANTA	BUDDY
CAMBRIDGE FAVORITE	CALYPSO
HAPIL	

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Chapter 3

Genetic loci associated with tissue specific resistance to powdery mildew in octoploid strawberry (*Fragaria* × *ananassa*)

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Abstract

Powdery mildew is one of the most problematic diseases in strawberry production. To date, few commercial strawberry cultivars are deemed to have complete resistance and as such, an extensive spray program must be implemented to control the pathogen. A large-scale field experiment was used to determine the powdery mildew resistance status of leaf and fruit tissues across a diverse panel of strawberry genotypes. Broad-sense heritability values obtained were 0.83 in 2021 and 0.87 for 2022 for foliar assessments, indicating that there was a large genetic component controlling the level of PM disease observed. In total, six stable Quantitative Trait Nucleotides (QTNs) associated with PM resistance, with one highly noteworthy QTN exhibited a 61% effect on resistance. To date, breeding of robust PM resistance in strawberry has been impeded by the quantitative nature of the trait and the resulting lack of genetic resources. These results address this shortfall, through providing the community with multiple genetic markers and putative resistance genes for application in future resistance breeding, implementation of which could deliver a natural resistance strategy to combatting PM.

Introduction

Strawberry powdery mildew is a widespread, ubiquitous disease caused by the fungus *Podosphaera aphanis* (formerly *Sphaerotheca macularis f. sp. fragariae*). Uncontrolled epidemics can lead to complete crop abandonment and substantial economic losses for producers [1]. *P. aphanis* is an obligate, biotrophic fungus, from the *Erysiphaceae* family that relies solely on its host for survival [2]. *Erysiphaceae sp*. infect a wide range of eudicot hosts and upon establishment, form white powdery mycelia structures consisting of branched, tubular filaments (hyphae) covering all above ground plant tissues (leaves, fruit, stolons and flowers) [3], [4]. The lifecycle of *P. aphanis* undergoes both a sexual and asexual stages. The fungus overwinters as mycelium and produces sexual fruiting structures called chasmothecia on dormant strawberry plants [5], [6]. The chasmothecia appendages intertwine with hyphae anchoring the fruiting body to the surface of the host [5]. In the spring, the ascocarp separates

from its host, releasing ascospores for dispersal by the air current or free water [7]. After landing on a suitable host, the ascospores germinate and penetrate through the plant cell wall before inducing the production of specialised plant-fungal cell interaction structures called haustoria. The fungus then generates aerial conidiophores, which release asexual conidia to enable secondary infection of the host and surrounding plants [8], [9].

P. aphanis undergoes rapid asexual reproduction during the summer and autumn months, with optimum temperatures for infection ranging between 15-25 °C, where humidity levels are above 75 %RH [8]. In infected strawberry leaves, fungal mycelia typically develop first on the underside (abaxial) of the leaf, before spreading to the upper side (adaxial) causing the leaves to curl inwards [7], [10]. Powdery mildew foliage infection can lead to a reduction in photosynthesis and therefore a lowering of CO_2 assimilation and a decrease in transpiration, leading to induced cell death [10], [11], [12]. The pathogen also impacts yield directly through infection of strawberry reproductive tissue, it can restrict growth and cause fruit to become misshapen and even terminate fruit. As such, even with control measures in place, PM infections can lead to unmarketable fruit and can result in up to 70% annual yield loss [8], [10], [13].

Prevention of PM epidemics can be achieved by the application of chemical pesticides such as quinoxyfen, myclobutanil, or demethylation-inhibiting (DMI) fungicides [15–17]. To reduce the risk of fungicide resistance evolution, UK regulations have been put in place to restrict the number of permitted fungicide applications per year for each active [14]. Despite these restrictions, *P. aphanis* has developed resistance to multiple sterol demethylase inhibitors [15]. With the emergence of fungicide resistance, there is a greater need to reduce reliance on fungicide management practices through utilising non-chemical disease control strategies. Harnessing natural genetic sources of PM resistance in strawberry germplasm stands to provide an environmentally favorable disease control strategy. Indeed, there is a great need for germplasm resistance improvement, particularly where everbearing varieties are cropped throughout the growing season leading to prolonged disease exposure. Ultimately, there is a clear requirement for robust and effective disease control strategies as the majority of commercial cultivars still require chemical sprays for PM control [14], indicating a need for alternative control strategies.

Disease resistance in plants can be controlled by either a single genetic factor (monogenic) or multiple genetic elements (polygenic) [16]. Although monogenic resistance has been introgressed into other crops, there have been many reports of major resistance gene breakdown over time, due to a single locus imposing a high selection pressure on the pathogen [17], [18], [19]. Studies in other pathogen systems have identification major effect

resistant genes associated with PM resistance, thus enabling subsequent exploitation in breeding programmes. Examples of such genes include *Pmr* (Powdery mildew resistance) in cherry, *MLO* (mildew resistant loci O) in barley and *Edr* (Enhanced disease resistance) in wheat [10], [20], [21]. In addition**,** previous studies have shown that PM resistance in hop and apple are controlled by single major resistance genes [19], [22]**.** Current commercial cultivars are susceptible to PM, with a only a few varieties that are deemed to be moderately resistant [10]. However, no major gene or single locus has been found to endow resistance to strawberry PM. Moreover, this trait is believed to be a highly polygenic trait controlled by many small effect alleles [23], [24]**.** Indeed, selecting for polygenic resistance, combining or stacking several resistance genes in a single cultivar has been shown to enhance the durability of the disease resistance in comparison to utilisation of a single gene resistance approach [26], [27].

One of the core breeding objectives for temperate strawberry breeding programmes is the development of PM disease resistant varieties. Understanding the genetic components that are responsible for disease resistance is required to select the best genetic informed breeding strategy to achieve resistance longevity [17], [19]. For example, marker assisted breeding can be employed to assist capture of monogenic disease resistance controlled by a single resistance gene, such as *Rvi6* for apple scab resistance and *Fw1* for Fusarium resistance in strawberry [28], [29] In this study, we seek to generate resources that will allow us to investigate this polygenic resistance trait and capture the genetic elements associated with disease resistance for use in strawberry breeding programmes. To achieve this, we characterise the genetic elements associated with strawberry powdery mildew resistance through a Genome Wide Association Study and investigate the efficacy of genomic prediction.

Methods

Experimental design

The association panel contained a total of 331 strawberry genotypes, including breeding lines and varieties of commercial importance. The population contained a mixture of June bearers and everbearers. All plant material was housed in a polytunnel before clonal propagation. Five replicate clonal daughter plants were collected and propagated as misted tips in 9 cm pots containing compost in a heated glasshouse compartment (25 °C, 16 hr/8 hr day/night cycle), humidity was set at 100 %RH for 2 wks, 80 %RH for 2 wks and 60 %RH 2 wks. In August 2020, plants were transferred into fumigated polythene raised beds (row length 100 m; space between rows 1 m; spacing between plants 1 m; rows ran from North to South) in an open field at NIAB, East Malling, Kent (51˚17’20.1”N 0˚27’11.0”E); five replicate plants were assessed per genotype. Plants were arranged in a randomized block design, each block

contained a single replicate plant per genotype [23]. Underground irrigation was provided, and no fungicides were applied to allow a natural PM infection to establish.

Phenotyping

Foliar disease symptom scores were assessed on a monthly basis from June to October for 2021 and 2022 using the five-point scale [30]. The symptom scoring system was 1. No symptoms, 2. Slight leaf curling, 3. Leaf curling and mottling, 4. Severe leaf curling, reddening and visible damage to lower leaf surface and 5. Severe necrosis and some leaf death (Figure 1) [30]. Strawberry fruits were assessed in August 2022. Up to five fruits from each plant were scored for disease symptoms. The scoring system was based on a modified protocol from Palmer *et al.* 2007 [31]: to ensure full visualization of the disease, the fruit was assessed using a x30 jeweller's loupe. The symptom scoring system was 0. No superficial mycelium on fruit surface, 1. < 10 % of the fruit surface covered with mycelium, 2. 10-25 % of the fruit surface covered with mycelium, 3. 25-50 % of the fruit surface covered with mycelium, 4. 50-75 % of the fruit surface covered with mycelium and 5. 75-100 % of the fruit surface covered with mycelium.

Figure 1. Phenotyping scores 1. No symptoms, 2. mild symptoms – upward curling of leaves, 3. Medium symptoms – further upward curling, 4. Severe curling, reddening and leaf damage, 5. Severe necrosis and leaf death [32]. Scale bar 6 cm.

Genotyping

Genomic DNA was extracted from newly formed strawberry leaves using the Qiagen DNAeasy plant mini extraction kit (Qiagen Ltd., UK) to manufacturer's specifications. Genotyping was performed for 331 accessions using the Affymetrix Istraw90 Axiom array (i90k) [32] or the Istraw 35 384HT Axiom Array [33]. The consensus linkage map denoted 28 groups classified with 1 to 7 representing chromosome number and A to D representing sub-genome group. Genomic positions of SNPs were defined using the *Fragaria vesca* genome v2.0 [34], with physical positioning of each maker corresponding to a 'pseudo-octoploid' chromosomes mapping for *Fragaria* × *ananassa* [35].

Statistical Analysis

Scores for the two years of foliage assessment were analysed independently for 2021 and 2022. The Area Under the Disease Progression Curve (AUDPC) was calculated across the

foliage disease symptom scoring events. The AUDPC was performed using the R package 'agricolae' [36] and calculated as follows:

$$AUDPC = \left\{ \sum_{i=1}^{n-1} \left[\frac{y_{i+1} + y_i}{2} \right] * [X_{i+1} - X_i] \right\}$$

Where *y* is the mildew severity score, for score *I*, *X* represents the time in months and *n* is the number of scoring events. Relative AUDPC (rAUDPC) was calculated by dividing the AUDPC value by the number of phenotyping events. To generate an overall fruit disease score per plant, weighted averages were taken across the five pseudoreplicate fruit disease score assessments. Spatial modelling was used to correct for environmental variation across the field trial. Autospatial correlation analysis was performed in R by applying Moran's I test [23], [37]. Disease scores were corrected for spatial heterogeneity across individual plants, using penalized splines (SpATS package) [38]. Broad sense heritability (H^2) for genetic associations was calculated using SpATS [39]. Best Linear Unbiased Estimates (BLUE) were generated using R package 'lme4' through a mixed linear effect model where genotype was specified as a fixed effect and block a random effect [40]. BLUEs were used as an overall disease score for each genotype; these genotype scores were used for downstream genetic analysis.

Genetic Analysis

A Genome Wide Association Study (GWAS) analysis was conducted using BLUE foliar disease scores for 2021 and 2022 and BLUE fruit disease scores in 2022 across 331 different genotypes. The GWAS analysis was conducted using PLINK as detailed on github [41], [42]. SNPs were filtered to remove those where the minor allele was represented in less than 5 % of the genotypes. Any SNP that was missing in greater than 50 % of the population was removed from the analysis. The analysis was adjusted using principal component co-variates to account for population stratification. A Manhattan plot was produced using the 'cMplot' R package to visualize GWAS Bonferroni corrected *p*-value ($p < 3.423 \times 10^{-6}$) results across the octoploid strawberry chromosomes. The correlation matrices were created using the 'corrplot' R package with Spearman correlation matrix to visualize the genotypic and individual correlations between phenotypic foliage scores and fruit scores.

Identification of candidate resistance genes

The most significant focal SNP was identified for each region of interest. Disease related genes within 100 Kbp of focal SNPs were identified using browser extensible data software (BED tools) [43]. Resistance genes identified from the annotated *F. vesca* genome were Nucleotide Binding Site (NBS), Receptor Like Kinase (RLK), Mildew Loci O (MLO), Trans Membrane Coiled-Coiled (TMCC) and Receptor Like Protein (RLP) [44]. Genes underlying

the identified QTN were characterised for molecular and biological functions using Genome Data base for Rosaceae (GDR), EMBL European Bioinformatics Institute 'InterProScan' tool, Pathogen Receptor Genes data base (PRGdb), Uniport tools and NCBI BLAST alignment tool 'BLASTn' [45], [46], [47], [48], [49].

Genomic selection

The potential of genomic selection for use in genetic informed breeding was calculated using the ridge regression best linear unbiased prediction "rrBLUP" R package to estimate the effect of markers on disease score [50]. GWAS marker data and phenotype data were split into a training sample of 60 % of the population and a test sample of 40 %. The phenotype of the test sample was predicted and then compared to the actual phenotype values in order to assess the predictive accuracy. The model was run with 100 permutations; for each iteration a random selection of genotypes were allocated to either the training or test data set [51], [52].

For full methods refer to Chapter 2: 2.1–- 2.4 and 2.7.

Results

Disease variance over the years

Powdery mildew disease symptoms were assessed across a replicated field trial of 331 strawberry genotypes in 2021 (assessment of foliage only) and 2022 (assessment of both fruit and foliage). Overall PM disease severity was observed to be higher in 2021 in comparison to 2022, with a higher variability observed across the field in 2022 (S1). This was also reflected in the spatial analysis, which shows a comparatively consistent level of disease incidence throughout the field in 2021 compared to 2022 (Figure 2, S2). Broad-sense heritability scores were 0.83 for 2021 and 0.87 for 2022; these show that a large proportion of the observed variation in infection levels was caused by genetic factors. However, PM fruit infection showed a lower broad-sense heritability score of 0.53 indicating that environmental factors have a greater impact on disease incidence in fruit. There were generally lower levels of infection observed on the fruit in comparison to foliage.

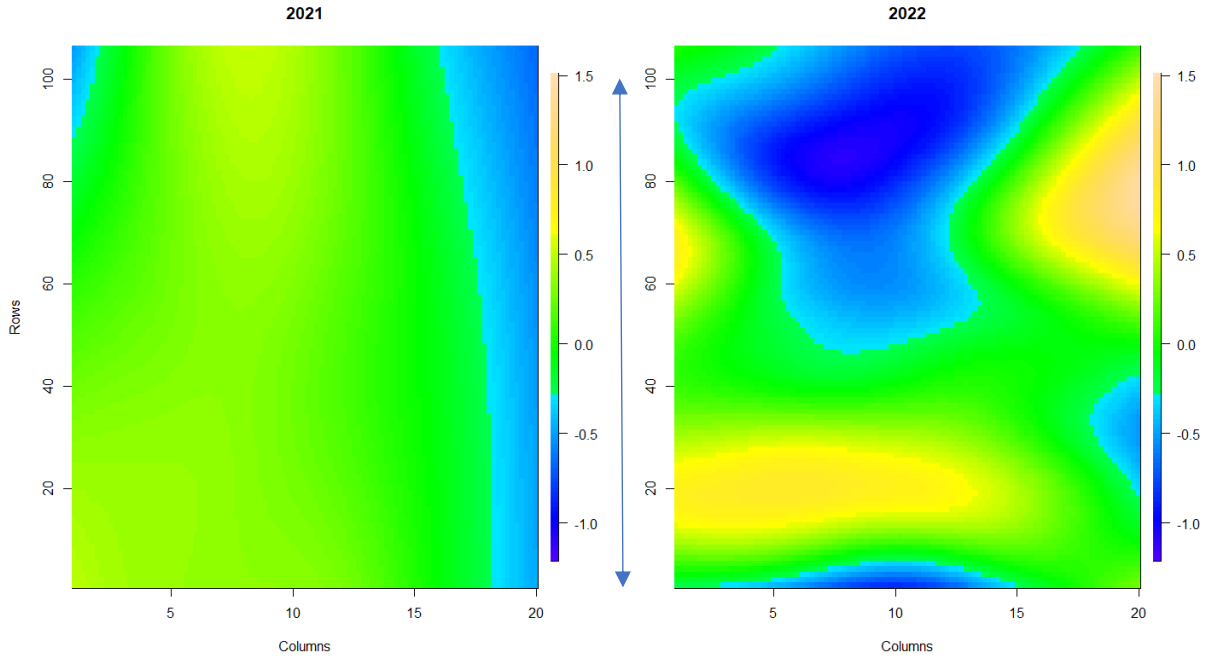


Figure 2: Spatial trends of foliar PM disease symptoms across strawberry plants in the field plot. The scale bar indicates the relative level of disease incidence yellow – 1.5 – Blue -1.0. Columns denote each raised bed. Rows denote the position of each plant along the raised bed. Arrow denotes 100 meters. 1a shows spatial trend for 2021, 1b shows the spatial trend for 2022.

Stable and transient QTNs identified

Multiple significant SNP associations were identified on the majority of chromosomes (Figure 3) for both 2021 and 2022 foliar disease assessments. SNPs identified above the significant threshold (p=0.05) in both years were located on chromosomes 3D, 4A, 5A, 5C, 6A and 7D. The most significant single locus was located on chromosome 6C was a transient QTN FaRPa6Cb associated with a 78.7 % increase in resistance. The most significant stable QTN was on Chromosome 7D FaRPa7Dab with 61 % effect on resistance. Multiple Quantitative Trait Nucleotides (QTNs) were identified as significantly associated with disease resistance in 2021 and 2022. By contrast, no significant QTNs were identified in association with fruit disease resistance (Figure S3). For each QTN, resistance genes were identified within 100 Kbp of the focal SNPs (Table 1). GWAS analysis led to the identification of six stable QTNs (*FaRPa1Bab, FaRPa3Dab, FaRPa4Bab, FaRPa5Aab, FaRPa7Aab and FaRPa7Dab*) associated with PM resistance over both years. The focal SNP representing *FaRPa3Dab* was associated with both RLK (Receptor like kinase) and RLP (Receptor light protein) resistance genes and the focal SNP representing *FaRPa7Dab* was associated with a RLK and NBS (Nuclear binding site) resistance gene. *FaRPa5Aab* was associated with RLK and TMCC (transmembrane coiled coil) resistance genes, which encodes a mitogen-activated protein kinase kinase kinase 7-like (MAPKKK) and is involved with ATP binding and protein phosphorylation for signal transduction. The *FaRPa3Dab* located inside a gene which functions as pattern recognition receptors (PRR), which recognises the presence of pathogens and initiates PAMP triggered immunity. *FaRPa7Dab* was associated with RLK, TM (transmembrane) domains with C terminal LRR (Leucine Rich Repeat) and NBS, TIR (Toll/Interleukin-1). One gene associated with *FaRPa7Dab* was identified as a putative plant disease resistance gene, encoding a TMV resistant protein that contains a TIR domain, P-loop containing nucleoside triphosphate hydrolase, which is involved with ATP binding, signal transduction and defence response to disease and stress.

In addition, twenty-six transient QTNs were identified, in either 2021 or 2022. This included the focal SNP Affx-88876085 representing *FaRPa6Cb* (6C), that was strongly associated with resistance in 2022 showing a 78.7% increase in resistance in the presence of the resistance allele. The genes associated with transient QTN *FaRPa6Cb* encode RLK and RLP proteins.

Table 1 Significant focal Single Nucleotide Polymorphism (SNP). SNPs associated with strawberry PM resistance after GWAS analysis for 2021 and 2022 foliar assessment and effect size for each focal. Quantitative trait loci (QTN) name with linkage group and position found. Gene No. indicates the number of resistant genes within 100 kb of the focal SNP. Type of gene illustrates the resistance gene identification by flanking molecular marker: RLK – Receptor Like Kinase Alleles (RLK), Receptor Like Protein (RLP), Transmembrane coiled coil (TMCC), Mildew loci O (MLO), Nuclear Binding Site (NBS). Effect size indicates the magnitude of the resistance relationship of the QTN between the PM disease. Alleles indicates the number of genotype combinations present in the population. Model represents genetic control of alleles in presence to PM. Bold effect shows focal single nucleotide polymorphism representing stable QTN identified in both 2021 and 2022, as well as effect sizes scoring over 35%.

2021

QTN Name	Linkage group	Position (Mb)	Focal SNP	Type of Gene	Effect size	Gene No	Model	Alleles
FaRPa1Aa	1A	2.0	Affx-88810185	RLP, RLK	17.2	2	Dominant	3
FaRPa1Bab	1B	14.8	**Affx-88817415**	NBS, TMCC	20.2	2	Additive	3
FaRPa2Da	2D	14.5	Affx-88822125	TMCC	16.9	1	Additive	3
FaRPa3Aa	3A	10.0	Affx-88843277	RLK	16.2	1	Additive	2
FaRPa3Ca	3C	8.4	Affx-88835462	RLK	13.8	3	Dominant	3
FaRPa3Dab	3D	14.6	**Affx-88838088**	RLK, RLP	**35.8**	2	Additive	2
FaRPa4Bab	4B	7.7	**Affx-88848257**	TMCC	**38.2**	1	Additive	3
FaRPa4Da	4D	16.3	Affx-88853237	RLK	11.4	1	Additive	3
FaRPa5Aab	5A	2.3	**Affx-88859881**	TMCC, RLK	25.5	3	Additive	3
FaRPa5Ba	5B	3.3	Affx-88860439	RLK, RLP	15.7	2	Additive	2
FaRPa5Ca	5C	12.8	Affx-88865131	TMCC	**39.2**	1	Additive	3

FaRPa6Aa	6A	6.6	Affx-88876363	RLK, RLP	**36.6**	4	Additive	3
FaRPa6Ba	6B	6.5	Affx-88876423	TMCC, RLK, RLP	17.8	5	Additive	3
FaRPa6Ca	6C	32.0	Affx-88880233	RLK, TMCC	21.3	2	Additive	3
FaRPa6Da	6D	38.1	Affx-88890456	RLK, TMCC, NBS	**35.8**	4	Additive	3
FaRPa7Aab	7A	12.9	**Affx-88892535**	RLP, TMCC	24.1	2	Additive	3
FaRPa7Ba	7B	12.0	Affx-88892929	TMCC	12.6	1	Dominant	3
FaRPa7Ca	7C	9.4	Affx-88896002	RLK	10.6	1	Hetero dominant	3
FaRPa7Dab	7D	19.8	**Affx-88899847**	RLK, NBS	**36.9**	5	Additive	3

2022

QTN name	Linkage group	Position (Mb)	Closest SNP	Type of Gene	Effect size (%)	Gene No	Model	Alleles
FaRPa1Ab	1A	14.8	Affx-88817415	NBS, TMCC	26.7	2	Additive	3
FaRPa1Bab	1B	14.8	**Affx-88817415**	NBS, TMCC	26.7	2	Additive	3
FaRPa1Cb	1C	8.8	Affx-88902877	RLK	29.6	1	Additive	3
FaRPa3Ab	3A	30.9	Affx-88843644	RLP, NBS, RPL	**48.1**	8	Additive	3
FaRPa3Bb	3B	9.5	Affx-88843060	MLO	27.2	16	no minor homozygote	2
FaRPa3Dab	3D	14.6	**Affx-88838088**	RLK, RLP	**48.9**	2	Additive	2
FaRPa4Bab	4B	7.7	**Affx-88848257**	TMCC	**38.2**	1	Additive	3
FaRPa4Db	4D	22.2	Affx-88854014	RLK	27.7	1	Dominant	3

FaRPa5Aab	5A	2.3	**Affx-88859881**	TMCC, RLK	**31.9**	3	Additive	3
FaRPa5Bb	5B	15.0	Affx-88866774	RLK	**50.5**	1	Additive	2
FaRPa5Cb	5C	10.5	Affx-88863794	RLP	**50.4**	1	Additive	3
FaRPa6Ab	6A	33.7	Affx-88888706	NBS	**40.4**	3	Additive	3
FaRPa6Bb	6B	6.6	Affx-88876401	RLK, RLP	22.6	4	Dominant	3
FaRPa6Cb	6C	7.0	Affx-88876085	RLK, RLP	**78.7**	4	Additive	3
FaRPa6Db	6D	39.6	Affx-88904022	-	**--**	-	--	-
FaRPa7Aab	7A	12.9	**Affx-88892535**	RLP, TMCC	28	2	Dominant	3
FaRPa7Bb	7B	15.4	Affx-88897245	RLK, NBS	6.33	2	Hetero recessive	3
FaRPa7Cb	7C	13.6	Affx-88892283	RLK	**61.1**	1	Additive	3
FaRPa7Dab	7D	19.8	**Affx-88899847**	RLK, NBS	**60.5**	5	Additive	3

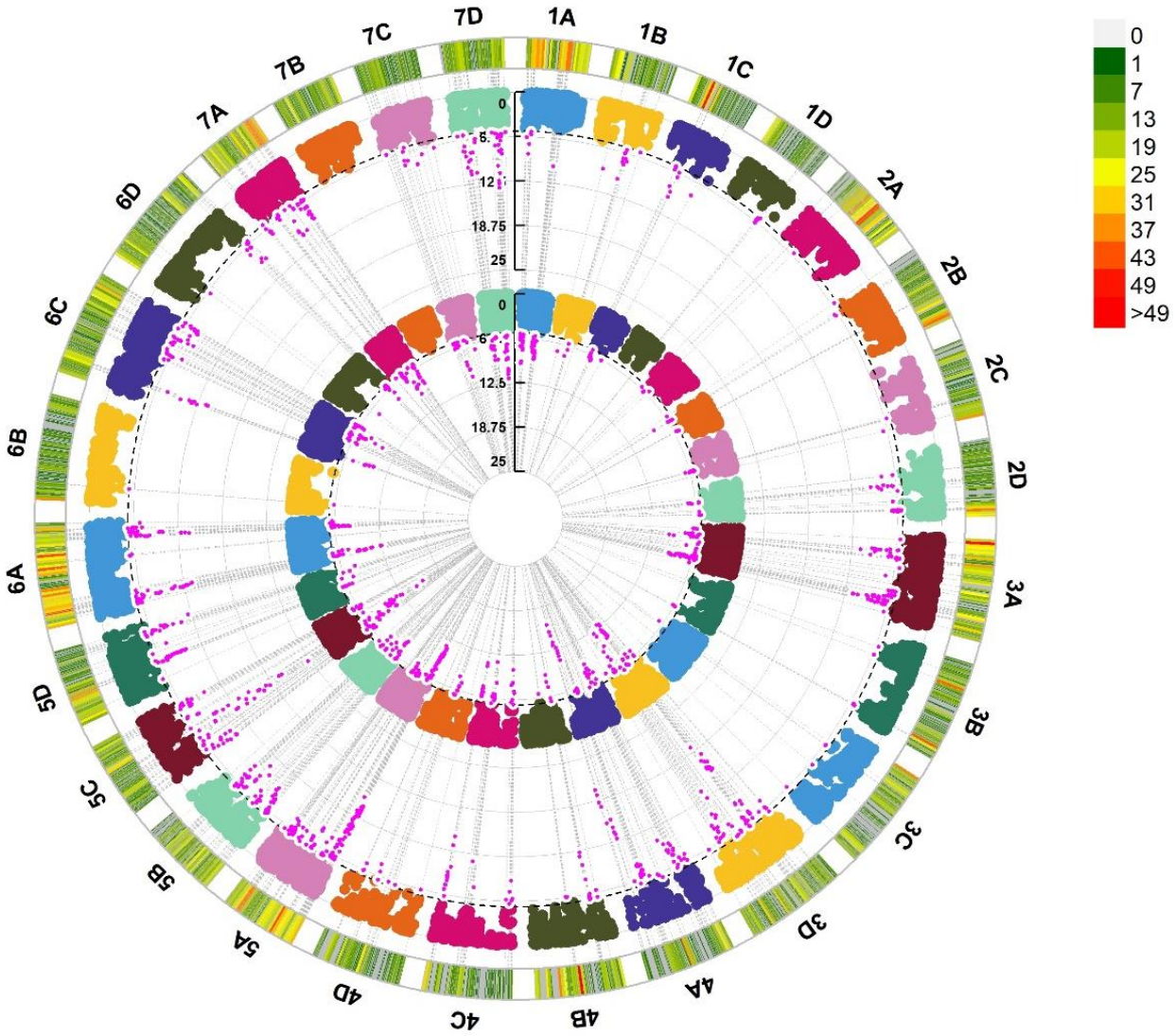


Figure 3: Manhattan plot of SNP markers across the 28 linkage groups of *Fragaria* × *ananassa* illustrating the relative association of SNPs with PM foliar disease symptom expression. Points represent markers. Pink points represent markers that fall above the -$\log^{10}(p)$ significance threshold represented by the black dotted line. The inner circle represents SNPs associated with foliar PM disease symptoms in 2022, middle circle in 2021. The outer circle represents the density of SNPs present on each chromosome within a 1 Mb window, with reference to the key coded from 0 to >49 SNPs.

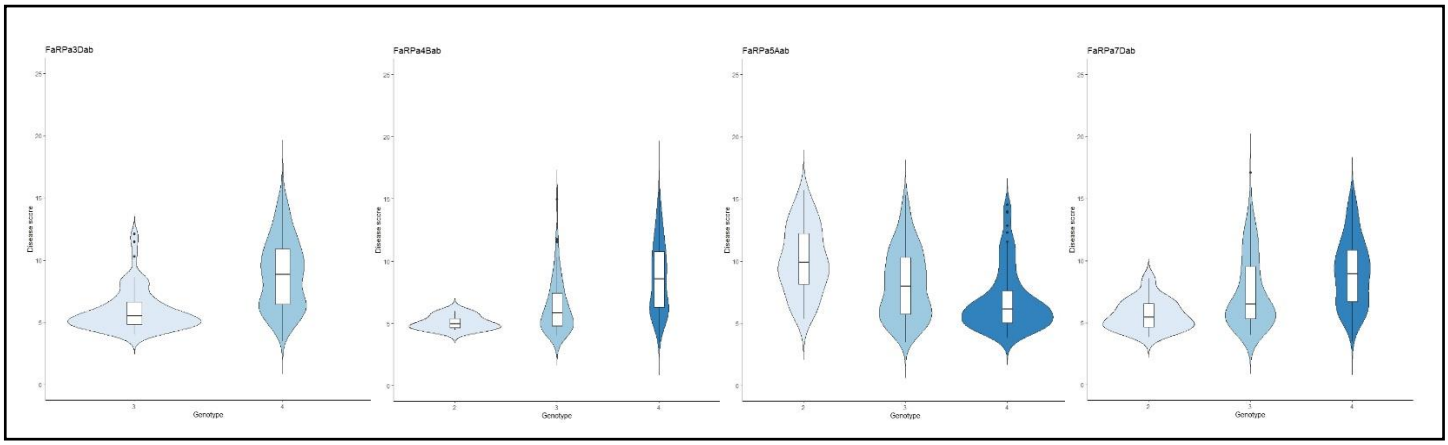


Figure 4: Boxplot of foliage PM disease score for each genotype of stable QTNs associated with a large effect size. a), *FaRPa3Dab* on chromosome 3D, b) *FaRPa4Bab* on chromosome 4B c) *FaRPa5Aab* on chromosome 5A and d) *FaRPa7Dab.* All QTN show additive alleles.

The Manhattan plot shows nine of the QTN's extend above a threshold $-\log^{10}(p)$ for both years – on chromosomes 3A, 3D, 4A, 5A, 5C, 6A, 6C, 7A and 7D providing nine highly significant QTNs for use in future breeding programmes for PM resistance. Peaks on chromosomes 3D, 4A and 5C substantially exceed the significance threshold.

In 2022, stable QTN FaRPa7Dab was associated a with the highest effect size of 61%, with *FaRPa3Dab,* FaRPa4Bab and FaRPa5Aab were associated with effect sizes of 49 %, 38 % and 32 %, respectively. All four of the stable QTNs with the highest effect size were identified in both foliage phenotype events, display additive genetic components (Figure 4).

The relationship between disease scoring events shows a significant positive correlation between 2021 and 2022 foliage phenotyping events (p<0.001) for both the genotypic and individual correlations (Figure 5). The foliage and fruit infection levels from 2022 showed a weak positive correlation (p<0.05) when paired measurements were taken from the same plants. By contrast, the fruit and foliage disease assessments for 2022 did not demonstrate a significant genotypic correlation.

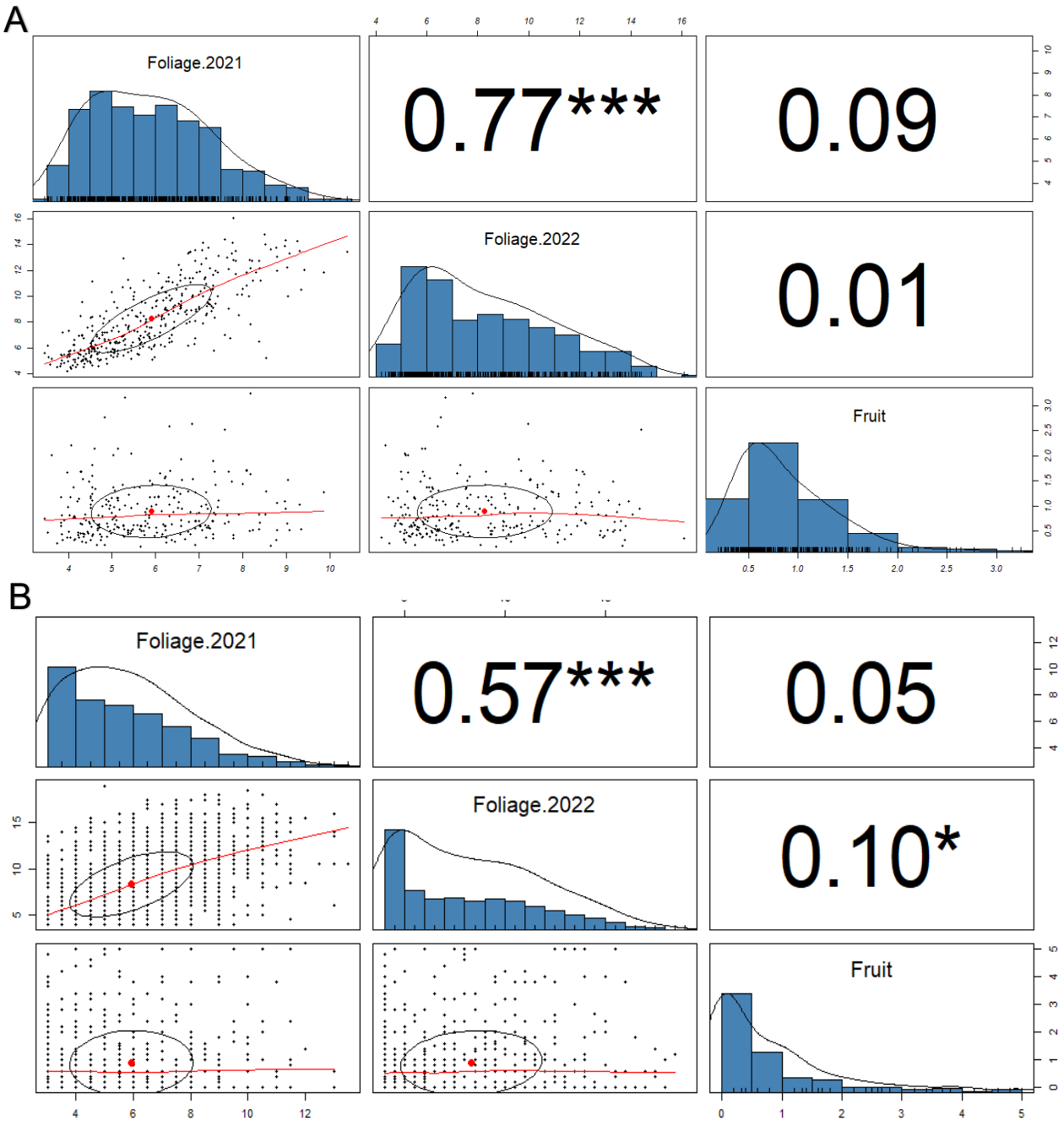


Figure 5: Spearman correlation matrix for PM phenotype data for foliage phenotypes for 2021 and 2022 and fruit scores 2022. A) genotype correlation and b) individual plant correlation. Significance (*p*) values are denoted by red stars: * < 0.05, ** <0.01, ***<0.001, numbers are Spearman correlation coefficients (r values).

Genomic selection for the 2021 and 2022 foliage indicated a predictive accuracy of 0.57, and 0.5 and predictive ability of 0.47 and 0.44, respectively. However, the fruit predictive accuracy score was very low at only 0.035 with a predictive ability of 0.018. These values indicate the relative potential of increasing cultivar resistance through genomic selection in the study population.

Discussion

The majority of commercial strawberries worldwide are susceptible to PM infection [10]. Resistance to this disease is a complex trait, typically controlled in a polygenic fashion, rather than through large effect major genes [53]. A GWAS was used to identify allelic variants associated with PM resistance across a range of diverse germplasm. Here we identify for the first time, a large number of alleles associated with disease resistance to strawberry PM that are relevant across diverse genetic material. Critically, using a GWAS approach allowed the identification of genetic markers that have linkage with the causative alleles across the wider germplasm. This benefit is not typically shared by the markers identified in linkage mapping studies (which artificially generate linkage between markers and the causal genetic element). Ultimately, this retained linkage means that the alleles identified using a GWAS are directly useful for molecular assisted plant breeding [84]. Moreover, the high level of resistance associated with some of the stable QTN, means that this data contains a valuable set of markers, which could be exploited for the generation of disease resistant varieties.

In total, six stable QTNs were identified across both years, two of which displayed substantial effect on resistance shown by *FaRPa7Dab* (61 %) *and FaRPa3Dab* (49 %), with the remaining QTNs were associated with effects above 20 %. The *FaRPa7Dab* allele was associated with a 61 % effect size and was close to a disease resistant gene with a TIR domain, which guards the plant by recognising a-virulence pathogen proteins before triggering the plant's defence response [54], [55]. The majority of the QTNs in this study showed quantitative resistance to PM with additive genetic components. In alignment with other studies, our findings suggest that several additive components are required to achieve PM resistance and thus accumulation of multiple resistance genes should be adopted as a breeding approach to develop resistance in strawberry cultivars [23], [56], [57].

Several QTN were only identified in one of the assessment years. In 2021, thirteen transient QTNs associated with foliar disease resistance were identified, in addition to thirteen different transient QTNs identified for 2022. The observation of transient QTN supports the hypothesis that resistance genes may have an environmental and/or race specific response to powdery mildew infection [23]. A transient QTN *FaRPa6Db* found in the 2022 analysis corresponds to a PM QTL identified by bi parental investigation [23]. The QTN *FaRPa6Db* (Affx-88904022) was positioned at the same location: 38.9 Mb on chromosome 6D with three neighbouring RLK resistance genes. This resistance QTL was identified in the 'Red Gauntlet' cultivar in four separate phenotyping events [23]. As such, it is evident that this locus plays a role in strawberry PM resistance and the mechanism of resistance in this area should be investigated in the future.

The QTN associated with powdery mildew disease resistance were in close proximity to a variety of putative disease resistance genes. The R genes identified in this study include the RLK and RLP genes known to be involved with plant disease resistance; these genes play a large role in activating a plant immune response through pathogen detection [58] [59], [60]. For instance, a RLK has been identified as responsible for non-host complete resistance in barley to the wheat adapted form of powdery mildew (*Blumeria graminis f.sp. tritici*) [61]. Furthermore, several resistance genes containing NBS-LRR domains were identified in this study. NBS-LRR proteins are intracellular immune receptors that can lead to plant cell death through the hypersensitive response; these receptors act as an “on / off” switch and negatively regulate resistance through degradation in response to pathogen effector detection [62], [63]. NBS-LRR have been found to provide protection against powdery mildew in grape vine, common bean, and wheat [64], [65], [66]. For instance, two QTNs were found adjacent to resistance genes involved in the plant stress response pathways. The first, *FaRPa5Aab*, was close to a MAPKKK gene involved in cascading a general stress signalling response [67], and the second, an *FaRPa6Ab* allele, was close to a receptor-like protein G-type lectin S-receptor-like serine/threonine-protein kinase that is involved in mediating the abiotic stress response to changing environments [68], [69]. As multiple candidate resistance genes have been detected in this study, future work should look to determine the function of these candidate genes and ultimately stack validated resistance genes into a single cultivar. This strategy may prove more successful than a single gene strategy, particularly when combining resistance genes representing different pathogen defence mechanisms, as this has been shown to provide more robust resistance and increase the longevity of protection against infection [62]. Future breeding strategies could focus on validating the function of these candidate genes and subsequently stacking validated R genes into a single cultivar. Combining R genes representing different pathogen defence mechanisms, could provide a more robust resistance and longevity of protection to PM infection.

A susceptibility gene Mildew Loci 0 (MLO) was associated with QTN *FaRPa3Bb*. Disruption of MLO genes can lead to a loss of host recognition and result in resistance to PM. Many MLO genes have been identified in a variety of crops such as rice, maize and strawberry [73], [74]. The QTN *FaRPa3Bb* was associated with an MLO gene identified on chromosome 3B (mrna31264.1-v1.0-hybrid) and corresponds with the presence of the *FvMLO16 gene* reported by Pessina *et al.* (2014) and Jambagi and Dunwell (2017) and Cockerton *et al.* (2019) in *F. vesca*. Cockerton *et al.* reported the MLO homolog gene associated marker on the strawberry chromosome 3D that was found associated with Verticillium resistance [75]. A sequence analysis of the *Fragaria vesca FvMLO16* indicated the gene had three orthologs (resulting from the truncation or extension of the protein sequences) consequently generating a more

diverse protein; future investigations should take into account the additional diversity that may be present in *F. ananassa* [76].

Only one accession in the field trial was observed to be completely resistant across both years, a related octoploid species *F. virginiana.* The fact that *F. virginiana* is highly resistant is not surprising, as many wild Fragaria species are known to be resistant to PM as noted previously [3]. Additionally, the cultivars 'Selva' and 'EE64 were seen to exhibit a high level of disease resistance across both years of assessment. This finding compares with previous reports of 'Selva's high susceptibility [66].

The mildew resistance of foliage was shown to be under strong genetic control with high broad-sense heritability values of over 83 % for both years of assessment. These findings correspond with those of Nelson *et al.* (1995) but were higher than reported by Tapia *et al.* (2022), Davik and Honne (2005) who observed a more moderate level of heritability [77], [78], [79]. Heritability values depend upon the variation that is present within the study material being used; as such, it is clear that our study population contains a relatively large proportion of genetic variation, which can be selected upon by a breeder. Researchers have suggested that a high infection level is key to achieve uniform inoculation and thus reduce possible disease scoring errors and achieve an accurate assessment of phenotype [79]. A strong correlation was observed between foliage disease scores in 2021 and 2022 (Figure 2), adding to the evidence that there was a strong genetic component controlling PM disease resistance. Slight variations in cultivar disease resistance over the two years could be accounted for by variation in the level of disease pressure as postulated by Nelson *et al.* (1996) [80]. Such discrepancy may have resulted from the unprecedented long duration of heat with temperatures over 27 ^{o}C in 2022 with a maximum of 38 ^{o}C in August 2022, compared to 2021, when the weather was more favourable for PM, thus leading to a higher infection pressure [81], [82], [83]. In contrast, the strawberry fruit PM resistance heritability was moderate showing at 53 % of variation could be accounted for by genetic components. Heritability scores reflect the level of phenotypic variation present within the population: our results show that there were high levels of resistance observed in the strawberry fruit. This low level of phenotypic variation could account for the low level of heritability and lack of ability to discern genetic regions associated with the trait.

Foliage disease phenotypes illustrated low genetic correlation with fruit disease phenotypes for both years, suggesting that two different genetic mechanisms may control disease resistance in the leaves and fruit. Differences were also observed in heritability between foliage and fruit and lack of QTN associated with fruit resistance leads to the hypothesis that strawberry PM resistance is tissue specific. Future work should look to discern tissue specific

disease resistance in order to enable selection for both fruit and foliage resistance. However, strawberry fruit generally exhibited a high level of disease resistance; therefore introducing durable foliage resistance alone, could be sufficient to provide a more stable and desirable crop [10].

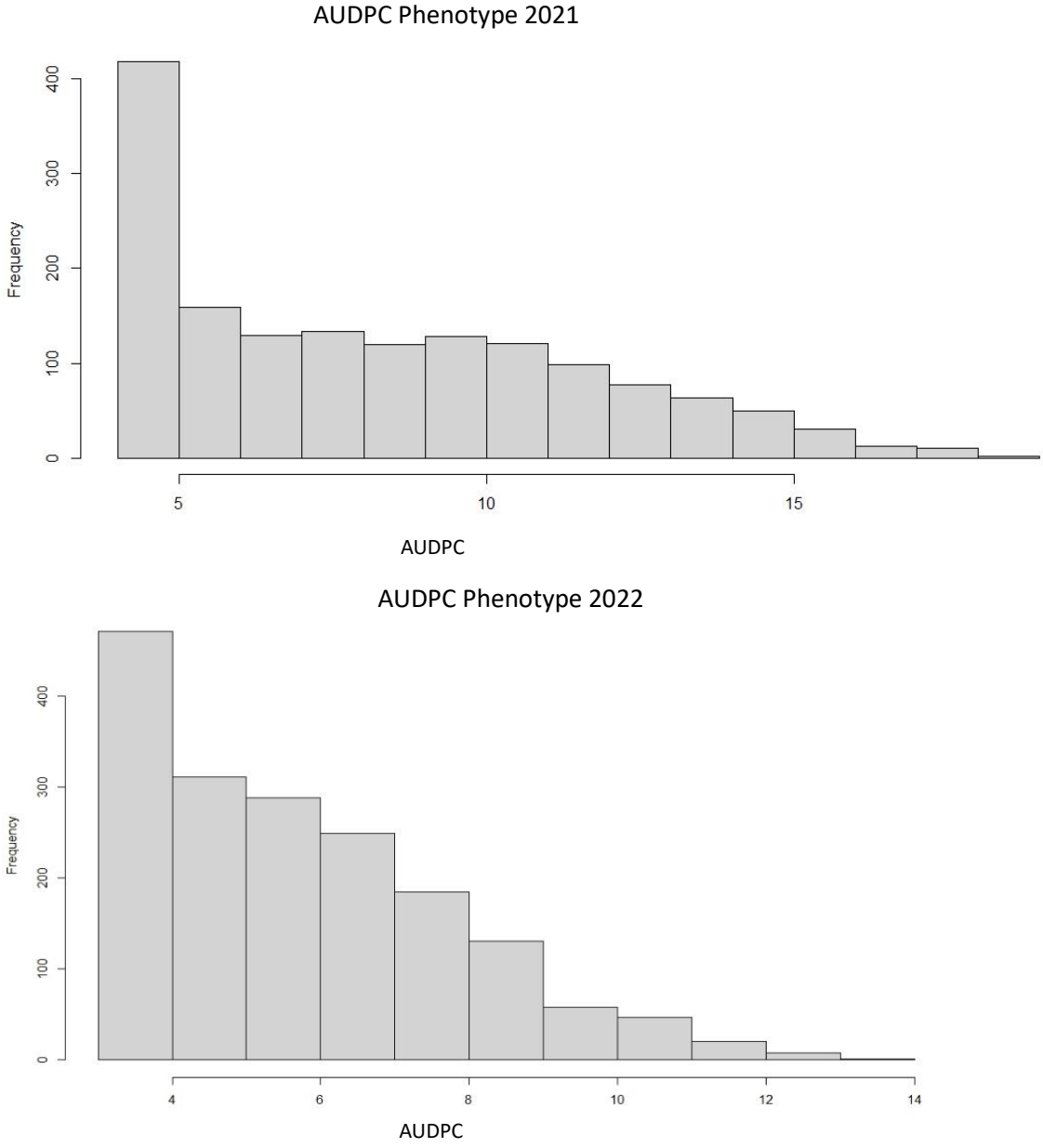
A genomic prediction model was used to calculate the predictive accuracy associated with the use of all genetic markers to improve strawberry PM resistance. Our results indicate a high potential of genomic selection for increasing cultivar foliar but not fruit PM resistance, with the ability to capture over 44% of the observed variation in the disease.

Strawberry PM resistance breeding is complicated as natural resistance is typically incomplete and polygenic in nature; however, capture and exploitation of polygenic resistance has been shown to have more durability in the field [17]. A multiple gene resistance strategy avoids the complications associated with single gene resistance. Specifically, single gene resistance can breakdown over multiple generations and induce the pathogen to evolve resistance [76]. Understanding the genetic components involved in disease resistance is an important part of informing genetic guided improvement to achieve resilient strawberry cultivars [17].

Conclusion

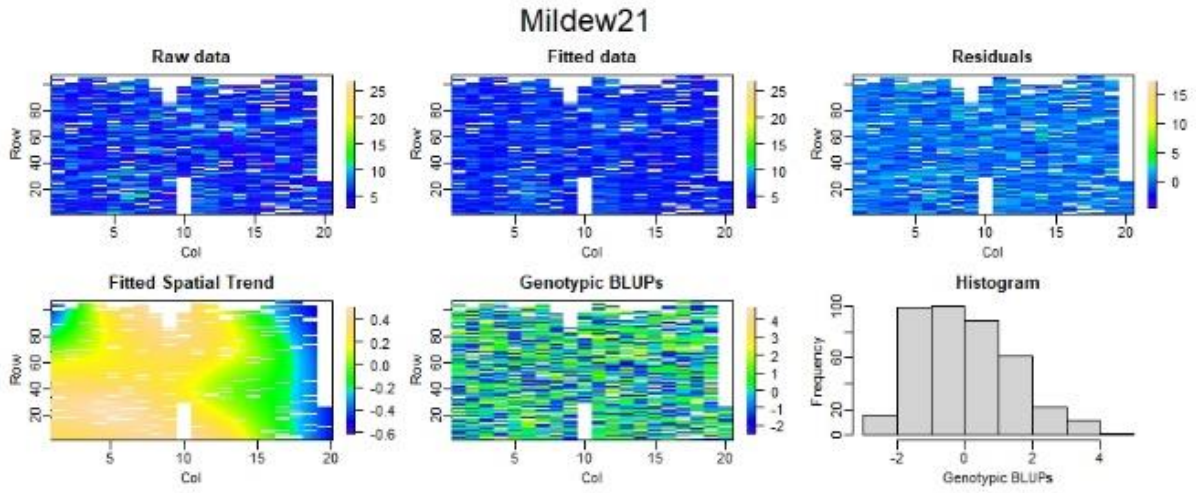
We have identified multiple genetic loci associated with strawberry powdery mildew disease resistance. Most importantly, the association between the identified markers and the causative alleles is maintained across the population. As such, this data will allow marker assisted breeding to be incorporated into strawberry breeding programmes to develop elite varieties with durable disease resistance. Moreover, we have confirmed that a genomic selection approach can be used to capture over 44% of the genetic variation associated with foliage resistance present in the population. As there was no genetic correlation between fruit and foliar symptoms and there were no QTN associated with fruit disease resistance, our results lead us to hypothesise that fruit and foliage mildew resistance is mediated by a different genetic mechanism of defense.

Supplementary information



Supplementary Figure S1. Frequency distribution for area under the disease curve (AUDPC) for foliage scores in 2021 and in 2022

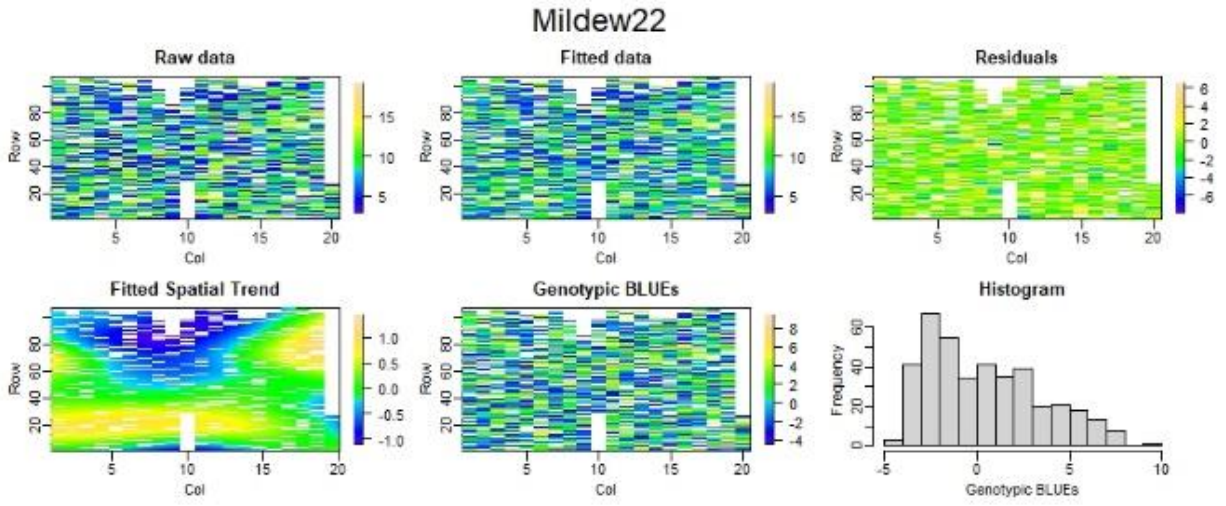
2021




```
Spatial analysis of trials with splines

 Response:                audpc
 Genotypes (as random):   x
 Spatial:                 ~SAP(Col, Row, nseg = c(10, 20))
 Random:                  ~R + C
```

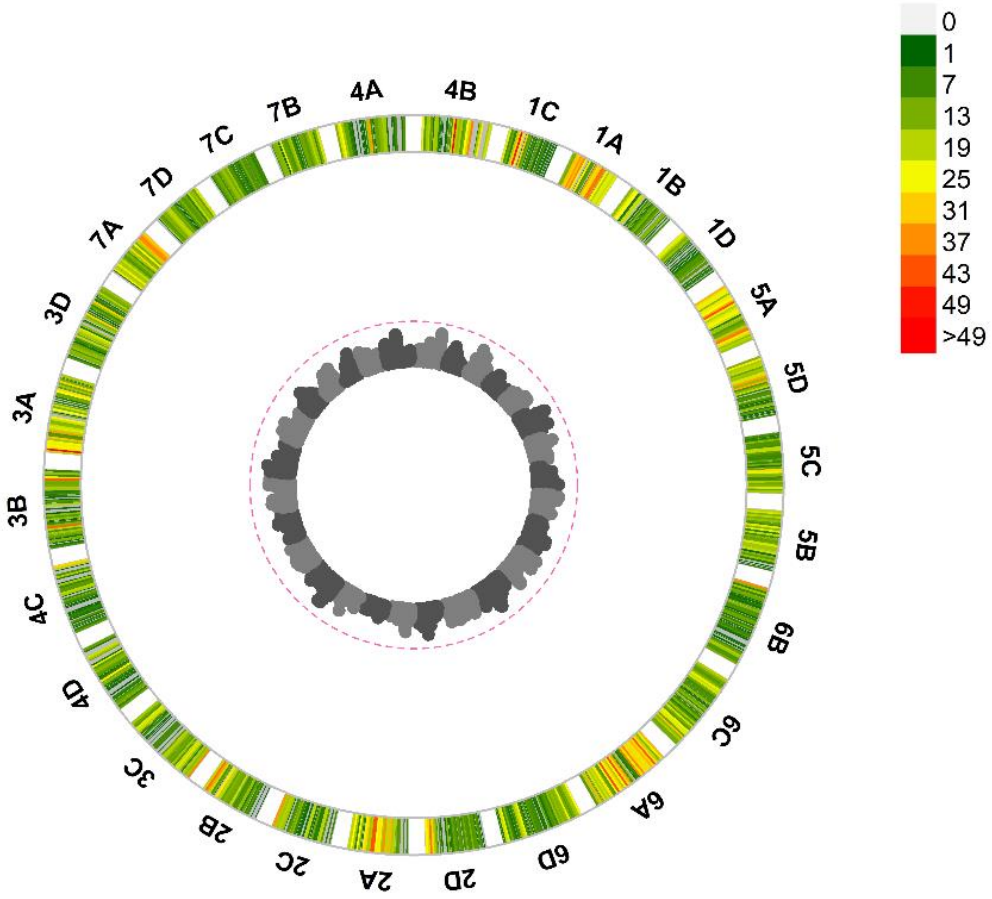
2022




```
Spatial analysis of trials with splines

 Response:                audpc
 Genotypes (as fixed):    x
 Spatial:                 ~SAP(Col, Row, nseg = c(10, 20))
 Random:                  ~R + C
```

Supplementary Figure S2 – Raw foliage spatial analysis for foliage in 2021 and 2022. Columns denote each raised bed and rows denote the position of each plant. Scale represents the level of disease score.



Supplementary Figure S3 – Manhattan plot of SNP markers across the 28 linkage groups of *Fragaria* × *ananassa* illustrating the relative association of SNPs with PM fruit disease symptom expression. Points represent markers. Pink points represent markers that fall above the-log10(*p*) significance threshold represented by the black dotted line. The inner circle represents SNPs associations with fruit PM disease symptoms. The outer circle represents the density of SNPs present on each chromosome within a 1 Mb window, the key represents the number of SNPs segregating from 0 to >49.

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Chapter 4

CCR4 associated factor 1 homolog 11 (*CAF1-11)* is linked to cultivar, tissue, and ontogenic resistance to powdery mildew in strawberry

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Abstract

Epidemics of *Podosphaera aphanis* infection during strawberry cultivation can lead to severe yield losses due to unmarketable fruit. This study involved a naturally infected replicated trial of two cultivars 'Hapil' and 'E10' with contrasting levels of fruit and foliage resistance. The experimental design enabled the use of transcript analysis to investigate cultivar, tissue, and ontogenic resistance. Cultivar, tissue and ontogenic specific resistance differences were described through the identification of differentially expressed genes (DEGs). DEGs that were common across two or three resistance types have provided a narrow list of candidate resistance genes for future breeding. Overall, 2692 DEGs directly related to pathogen resistance were determined across the three experiments (24 % of the total DEGs). Several pathogenesis related transcription factor groups were identified to be upregulated during infection of all resistant material as well as multiple other genes groups known to be involved with pathogenesis. The most noticeable resistance gene, found to be differentially expressed in all resistant material types, was carbon catabolite repressor protein 4 (CCR4) associated factor 1 homolog 11 (*CAF1-11*). *CAF1* is part of the CCR4 complex, an enzymatic complex involved in the de-adenylation of mRNA. This finding may be exploited to generate strawberry cultivars with resistance to PM infection.

Introduction

Powdery mildew (PM) disease infects close to 10,000 species of angiosperms including many economically important crops such as grapes, apples and grains [1], [2]. The disease is caused by a collection of different obligate biotrophic fungal species that are part of the *Erysiphaceae* family [3]. The fungal species causing PM are highly diverse, with a great degree of host specificity exhibited by each species and a life cycle that has synchronized to the biological clock of the host plant [3]. The release of the conidia occurs during daylight, when temperature and humidity are at their optimal range for the PM establishment. A correlation has been noted between dispersal of conidia and nearby infected host tissue. The average conidium dispersal rate is approximately 38 progeny conidia release in a span of four

days [4]. Upon germination, the fungal conidium forms an appressorium, that uses turgor pressure to penetrate the plant cell wall, allowing hyphae to access host nutrients After infection, the fungi form the characteristic white mycelium structures covering the above ground plant tissue [3], [5]. A fungal infection of *P. aphanis*, usually starts on the underside of newly developing leaves (abaxial) ultimately leading to leaf necrosis on mature leaves [6]. Studies into oncogenic or age-related resistance in crops have shown that young foliage and berries are more susceptible to *P. aphanis* infections [7], [8], [9]. Various reasons have been postulated as to the cause of ontogenic resistance, including higher levels of cutin and salicylic acid; however the identity of the causative mechanism is still to be ascertained [8], [9]. PM infection on the foliage causes a reduction in photosynthesis and thus lower carbon assimilation, ultimately leading to a reduction in crop yield, with severe infections leading to plant death [2], [10], [11]. Infection of reproductive tissue can compromise yields through the reduction of pollen production and restriction of pseudocarp expansion, leading to misshapen fruit, hardening and even complete termination of fruit development [2], [12].

Transcriptome sequencing uses high throughput next generation sequencing and can be used to provide a comprehensive understanding of gene expression during plant-pathogen interactions [13], [14]. In this study, sequenced RNA with 3' poly-A tail of mRNA was performed to focus the investigation on the coding RNA molecules [14]. Many transcriptome analyses have been conducted on *Fragaria spp.* But these have primarily investigated the role of transcripts in fruit development/ripening [13], [15], [16], [17]. One subsequent PM transcriptome analysis in strawberry fruit investigated different disease stages of infection in the fruit. The results revealed the defense response involved phenols as well as the production of reactive oxygen species. In addition, the authors observed an upregulation of chitinase that may be used by the host to degrade the PM cell walls [18]. Another study focused on PM infecting the achene, their findings identified DEGs involved with ethylene and auxin metabolism, with ethylene response factors (ERFs) playing a key role in the resistance pathway [19]. Similar findings were observed with foliage by Feng *et al*. (2020) who investigated the Japanese strawberry variety 'Beni Hoppe', and reported salicylic acid/jasmonic acid crosstalk involved in the resistance to the infection [20]. This corresponds to the emerging knowledge relating to host- pathogen interactions, showing a positive and negative regulation system involving the salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) pathways. This crosstalk between the pathways proves essential for the hosts defence against pathogen infection [21]. Additionally, Feng *et al*. (2020) reported pathogenesis-related (PR) genes and transcription factors involved with phytohormone signaling [20]. Comparably, Jambagi *et al.* (2015) studied foliage PM in *F. vesca* accessions 'Hawaii 4' and 'Yellow Wonder'

and reported several transcription factors and resistance genes involved with the infection of powdery mildew[22]

However, to date, no studies have explored the gene expression patterns associated with tissue specificity, cultivar resistance nor ontogenic resistance in octoploid strawberry. Additionally, no crop studies have conducted a combined analysis, looking for universal key genes associated with pathogen disease. The information gained from this unique tissue specific comparative study could ascertain for the first time key universal gene targets for the development of resistance in future strawberry varieties. In this study two octoploid cultivars were selected, ‘Hapil’ with susceptible foliage and ‘E10’ with resistant foliage and susceptible fruit. Gene expression profiles were generated through RNA seq to provide a comprehensive picture of infection response to PM. This was achieved through quantifying the transcriptional changes between *P. aphanis* infected and uninfected foliage and fruit, mature and young foliage and resistant and susceptible foliage. In addition, conducting a comparison across resistance types allows the identification of genes associated with an overlapping immune response to PM.

Methods

Plant material and RNA extraction

Fragaria × *ananassa* tissue was harvested from replicate experimental plants arranged in a split plot randomized design in a polytunnel at NIAB, East Malling (GPS co-ordinates - 51.291586, 0.447843). A natural infection of PM was allowed to establish on infected plants. Plants were grown in 1 meter coir bags with 8 plants per bag. Fertigation was supplied, NPK 12:12:36 at 1 g l^{-1}. Cultivars were 'Hapil' (with susceptible foliage) and 'E10' (with susceptible fruit and resistant foliage). Samples were biological replicates taken from different plants, five replicates for each treatment, except for "old foliage" treatments with four replicates (Supp. Table 1). The plant tissue sampled comprised young leaves (expanded but folded), mature leaves (fully expanded) and whole ripe fruit. Both infected (where mycelium was observed to cover at least 30 % of the surface) and uninfected samples were harvested, flash frozen in liquid nitrogen and stored at -80 °C until RNA extraction. Tissue samples were ground in a pestle and mortar under liquid nitrogen; RNA extraction was conducted using the RNAeasy plant kit (Qiagen, Hilden Germany) and performed as specified by the manufacturer's protocol using the RLC lysis buffer. To ensure the samples met the quantity and quality thresholds required for sequencing, samples were checked using a spectrophotometer (Nanodrop™ and Qubit). The RNA integrity Number was assessed to check for degradation using Agilent RNA ScreenTape System on the 2200 Tapestation (Agilent Technologies, Germany). The 38 samples were submitted to Novogene for poly A enrichment mRNA library preparation and sequencing was performed on an Illumina NovoSeq 6000 sequencing system using paired end 150 bp chemistry. Sequencing data output from Novogene comprised of Raw data (G), Raw reads, Q20 and Q30 Phred quality scores indicating the probability of base calling error and GC content (S1 table).

RNA analysis

Raw RNA-Seq data were trimmed to remove sequencing adapters and low-quality data using Trimmomatic a read trimming tool. Following this, trimmed RNA-Seq reads for each sample were aligned against predicted gene models from the *Fragaria* × *ananassa* 'Camarosa' genome (Version v1.0 a1), downloaded from the Genome Database for Rosaceae (GDR) [4,9]. Alignment was performed using the pseudoalignment programme Salmon, returning total aligned read counts per transcript. Differentially expressed genes (DEGs) were identified using the integrated Differential Expression and Pathway analysis (iDEP) webserver (V1.1) [43].

Read count data were normalised within iDep using the DESeq2 EdgeR transformation (Table S2). Following this, DESeq2 was further used within iDEP to identify upregulated and downregulated genes with a threshold of 2 log fold change and a false discovery rate (FDR) of 0.05 between treatments. The Volcano plots were generated to represent the significant differentially expressed upregulated and downregulated genes associated with PM resistance measured by log2 fold change against the adjusted *p* value (log10 padj). Heatmaps were generated with iDEP using the “DESeq2” to visualize the expression changes in tissue types across samples with and without infection (Figure S2) [44,45].

Transcriptional response to infection with *P. aphanis* was assessed across three resistance types: cultivar resistance, tissue resistance and ontogenic resistance. Cultivar resistance was assessed through comparing foliage expression differences between infected and uninfected samples across the two different cultivars (‘E10’ young leaf versus ‘Hapil’ young leaf), where ‘E10’ has relatively resistant foliage and ‘Hapil’ foliage is susceptible. Tissue resistance was assessed through comparing expression differences between infected and uninfected samples across ‘E10’ resistant foliage and susceptible fruit ('E10’ young leaf versus ‘E10’ fruit). Ontogenic resistance was assessed through comparing expression differences between infected and uninfected samples across old and young foliage (‘Hapil’ old leaf versus ‘Hapil’ new leaf). The function of DEGs associated with each resistance type (tissue specific, ontogenic and cultivar) were investigated separately. Subsequently, overlapping DEGs from each experiment were identified and the functions of DEGs present in all three resistance types and involved in each pairwise interaction were investigated. The functions of significant DEGs were identified using GDR and Gene Ontology (GO) [23], [24]. Identified differentially expressed genes were used for gene interaction analysis, conducted using the STRING database (v12.0). To generate functional gene association networks for *F. ananassa*, *Arabidopsis thaliana* gene orthologues were identified (Figure S3) [25]. Functional interactions were then established through node interactions described through known scientific literature between proteins, based on differentially expressed genes in the RNAseq. Nodes (circles) represent proteins (circles) and functional interactions by edges (lines). Colored nodes represent biological processes (Gene ontology) – Green node: defence response (GO:0006952), yellow node: regulation of defence (GO:0031347), red node: response to stress (GO:0006950) and purple node: response to abiotic stress (GO:0050896) [25]. BLAST alignments were conducted in GDR and NCBI using ‘Nucleotide BLAST’ [23], [26]. Transcription factors were verified using the Plant Transcription Factor Database [27]. CAF1 regular expression levels (no infection) were determined with Klepikova Arabidopsis (At5g22250) Atlas with TAIR database (Figure S4) [28].

For full methods refer to Chapter 2: 2.8

Results

DEG profile for response to PM infection

The transcriptome analysis of different tissue responses related to powdery mildew (PM) infection had a total of 533.5 G of RNA sequencing data for 38 biological samples generated in this study, with 108087 genes investigated across the data set. High value Q20 percentage scores (based on Phred value) were ≤ 96.8 % for all samples and effectiveness values (clean/raw reads) were ≤ 98.7 %, indicating the high quality of identified nucleobases.

Expression profiles of PM resistance

Differential gene expression (DEG) for cultivar resistance between infected and uninfected samples was compared between the foliage susceptible ‘Hapil’ and foliage resistant ‘E10’ cultivars. A total of 294 DEGs were identified in cultivar specific differences corresponding with infection in ‘E10’ resistant variety, with 201 upregulated and 93 down regulated genes (Figure 1A). Differential gene expression for tissue resistance between infected and uninfected samples was compared between ‘E10’ resistant foliage with ‘E10’ susceptible fruit. A total of 149 DEGs were identified, with 134 upregulated and 15 downregulated genes, corresponding to resistant foliage specific expression upon infection (Figure 1B). Differential gene expression for ontogenic resistance between infected and uninfected samples was compared between ‘Hapil’ old and new foliage. This showed a total of 2249 DEGs, with 1633 up regulated and 616 down regulated genes corresponding to resistant old foliage specific expression upon infection (Figure 1C).

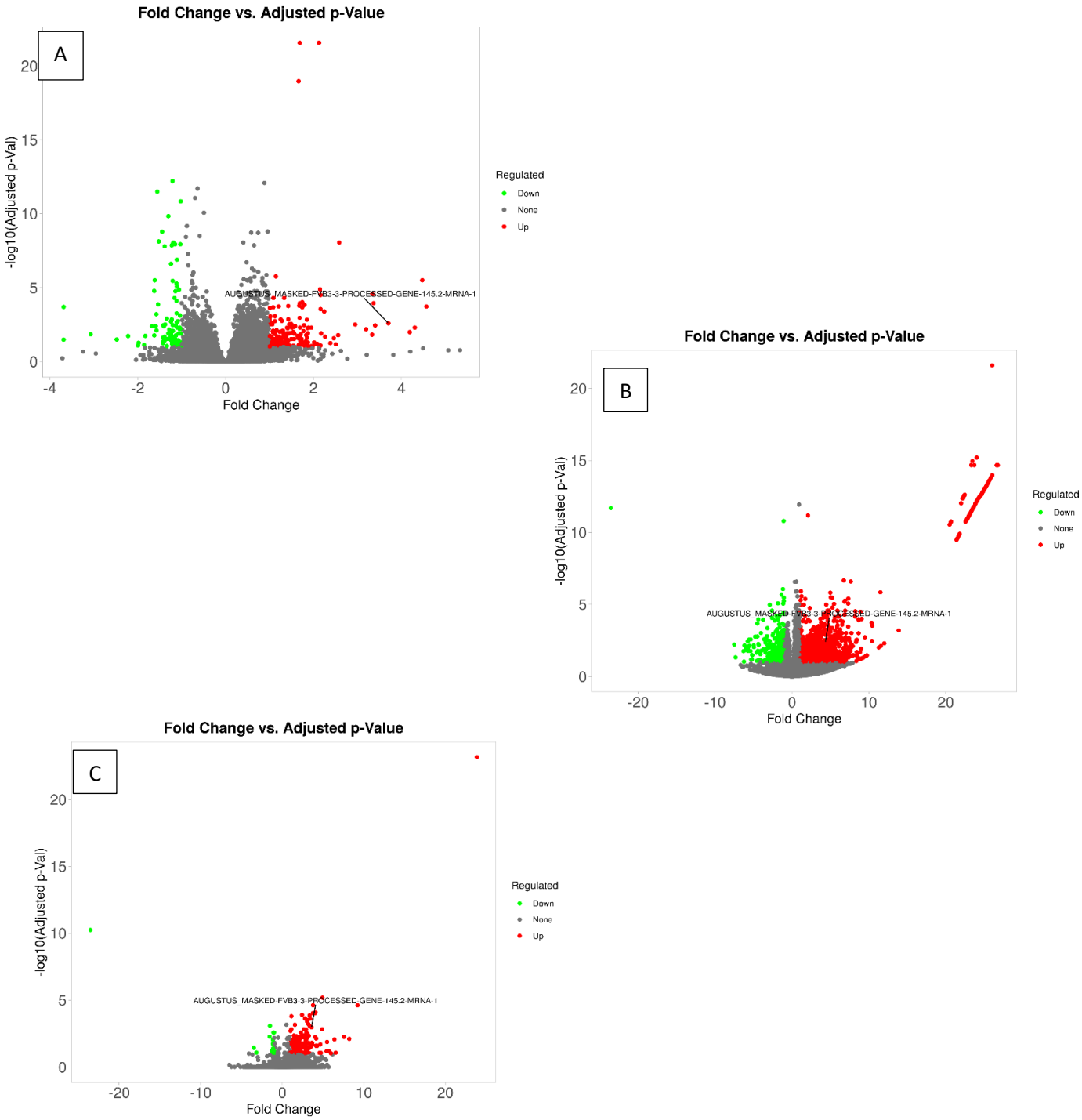


Figure 1. Volcano plot of differentially expressed genes (DEGs) in powdery mildew infection. A. Cultivar resistance, B. Tissue resistance, C. Ontogenic resistance. The CAF1-11 gene is denoted as AUGUSTUS_MASKED-FVB3-PROCESSED-GENE-145.2-MRNA-1. Scatter plot dots represent each gene, green dots indicate 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).

Expression profiles of comparative PM resistance

The number of DEGs that were associated with a resistant response in each experiment were assessed for commonality as illustrated in the Venn diagram (Figure 2). Evidence for overlapping DEGs between resistance types was established, with 67 DEGs coinciding across more than one experiment. The comparison between cultivar resistance and tissue resistance led to the identification of 25 common DEGs, the comparison between tissue resistance and ontogenic resistance established 6 common DEGs and in the comparison between cultivar and ontogenic resistance identified 34 common DEGs. One universal DEG was identified in all comparisons associated with resistance - CAF1-11 (Table 1).

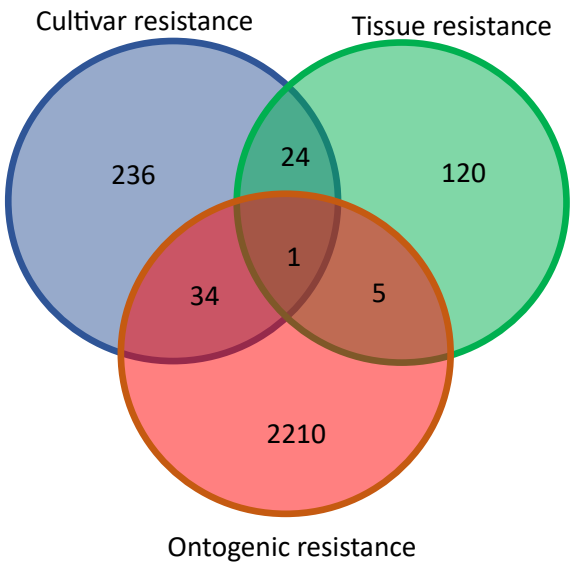


Figure 2. Venn diagram, illustrating the number of DEGs involved in strawberry powdery mildew resistance. Cultivar resistance denotes comparison between two cultivars (‘Hapil’ susceptible and ‘E10’ resistant foliage). Tissue resistance denotes comparison between ‘E10’ resistant foliage and susceptible fruit. Ontogenic resistance denotes comparison between ‘Hapil’ young susceptible foliage and old resistant foliage.

Table 1. Differentially expressed genes identified for all observed resistance in *Fragaria* × *ananassa*. Genes identified in each parameter of resistance. Gene ID determined by Genome database for Rosaceae and the genes biological involvement. DEGs in. bold font represents gene found in all resistance types.

Genes	Gene ID	Biological Involvement
Cultivar resistance		
CCR4-associated factor 1 homolog 11	**CAF1-11**	**Defense response**
Dehydration-responsive element-binding	DREB2C	Transcription factor
Myelocytomatosis oncogene 2	MYC2	Transcription factor
Dehydration-responsive element-binding	DREB1D	Transcription factor
NAM, ATAF and CUC	NAC072	Transcription factor
NAM, ATAF and CUC	NAC101	Transcription factor
Myeloblastosis 44	MYB44	Transcription factor
Exocyst complex component EXO70A1	EXO70H2	Cell wall thickening
Tissue resistance		
CCR4-associated factor 1 homolog 11	**CAF1-11**	**Defense response**
Ethylene-responsive transcription factor ERF109-	ERF109	Transcription factor
Ethylene-responsive transcription factor ERF017	ERF017	Transcription factor
Dehydration-responsive element-binding	DREB1D	Transcription factor
Myelocytomatosis oncogene 2	MYC2	Transcription factor
Mitogen-activated protein kinase kinase kinase	MPKKK19	Hormone signal transduction
RING-H2 finger ATL2	ATL2	Early defence signaling pathway
U-box domain-containing protein 21	PUB21	Ubiquitin ligase
Exocyst complex component EXO70A1	EXO70H2	Cell wall thickening
Ontogenic resistance		
CCR4-associated factor 1 homolog 11	**CAF1-11**	**Defense response**
Calmodulin-binding transcription activator 3	CAMTA3	Transcription factor
Heptapeptide WRKYGQK and Zing finger motif	WRKY47	Transcription factor
Heptapeptide WRKYGQK and Zing finger motif	WRKY33	Transcription factor
Heptapeptide WRKYGQK and Zing finger motif	WRKY53	Transcription factor
Heptapeptide WRKYGQK and Zing finger motif	WRKY70	Transcription factor
Heptapeptide WRKYGQK and Zing finger motif	WRKY72	Transcription factor
CBL-interacting protein kinase 2	CIPK2	Hormone signal transduction
Protopanaxadiol 6-hydroxylase	CYP716A1	Hormone signal transduction
Cytochrome P450 94C1	CYP94C1	Response to stress and wounding
Aminotransferase	ALD1	Systemic acquired resistance (SAR) response
Resistance Pseudomonas syringae pv. maculicola 1	RPM1	Disease resistance
Suppressor of BIR1-1	SOBIR1	Disease resistance
Pathogen related B1	PRB1	Disease resistance
Target of AvrB operation	TAO1	Disease resistance
Dominant suppressor of Camta3 number 1	DSC1	Disease resistance
Cyclic nucleotide gated channel 1	CNGC1	Ligated ion channel
Cultivar and Tissue resistance		
CCR4-associated factor 1 homolog 11	**CAF1-11**	**Defense response**
Dehydration-responsive element-binding protein	DREB1D	Transcription factor
Heat stress transcription factor B-2b	HSFB2B	Transcription factor
Late Elongated Hypocotyl	LHY	Transcription factor
Myelocytomatosis oncogene 2	MYC2	Transcription factor

Two-component response regulator-like APRR5	APRR5	Circadian biological events
UDP-glucuronate 4-epimerase 1	GAE1	Response to stress and defence
Plant uncoupling mitochondrial protein 4	PUMP4	Oxidative stress
Exocyst complex component EXO70A1	EXO70A1	Cell wall thickening
Tissue and Old resistance		
CCR4-associated factor 1 homolog 11	**CAF1-11**	**Defense response**
Heptapeptide WRKYGQK and Zing finger motif	WRKY53	Transcription factor
Ethylene-responsive transcription factor ERF105	ERF105	Transcription factor
Suppressor of BIR1-1	SOBIR1	Disease resistance
Dominant suppressor of Camta3 number 1	DSC1	Disease resistance
Cultivar and Ontogenic resistance		
CCR4-associated factor 1 homolog 11	**CAF1-11**	**Defense response**
LUX Arrhythmo	LUX	Transcription factor
Low-temperature-induced 65 kDa protein isoform X1	LTI65	Response to stress
Glycine-rich RNA-binding	RBG7	Response to stress
Ubiquitin-specific protease 13	UBP13	Hormone signal transduction
Transducin/WD40 repeat-like superfamily	DAW1	Hormone signal transduction
Lysine-specific demethylase	JMJD5	Response to wounding and ABA
Aquaporin PIP2-1	PIP2	Early immune response to PAMP
DNA mismatch repair MSH2	MSH2	DNA repair

DEGs related to PM cultivar resistance

Cultivar resistance was investigated through a comparison between the strawberry cultivars 'Hapil' and 'E10' infected and uninfected foliage. A total of 14 (out of a total of 294) DEGs that were related to defence genes significantly upregulated in 'E10' resistant foliage compared with susceptible 'Hapil' (Figure 3A). The genes that were highly expressed in the resistant cultivar 'E10' only and known to be involved in plant-pathogen defence were: Four transcription factors (TF) families NAC (NAM, ATAF and CUC), DREB (Dehydration responsive element), MYB (Myeloblastosis) and MYC (Myelocytomatosis Oncogene) (Table 1). These TFs are linked with the ABA, SA and JA pathways known to be involved with pathogen defence. Also found to be upregulated was *EXO70* (Exocyst subunit) involved with cell wall thickening and a gene involved with multiple processes including defence against bacteria *CAF1-11* (CCR4-associated factor 1 homolog 11)**.** Predicted functional interactions of DEGs identified in cultivar resistance. Five of the transcription factors interact with each other MYC2, MYB44, NAC072, DREB1D, DREB2C (ABA mediators), as well as with PYL6 an ABA receptor, suggesting a major immune response in 'E10' cultivar resistance involving the abscisic acid (ABA) pathway (Figure 4A highlighted in the dotted circle). CAF1-11 was shown to have a very high interaction score of 0.8, with gene encoding PUMP4 (plant uncoupling mitochondrial protein); whether the relationship is involved with pathogen defence, is yet to be ascertained.

DEGs associated with PM tissue resistance

Tissue resistance was investigated through comparison between resistant foliage 'E10' and susceptible fruit 'E10'. In total 31 DEGs (out of a total of 149) were identified related to defence genes that were upregulated in foliage compared to fruit (Figure 3B) (Table 1). Three transcription factors families were identified including DREB and MYC. Also upregulated were ERFs (Ethylene transcription factors), which belong to the AP2/ERF family. ERFs are mediators of the stress signal transduction pathway and act as activators in gene regulation, with expression initiated in the presence of stress factors. Additional DEGs included a gene encoding *ALT2* (RING-H2), proposed to be involved with early defence signaling pathway and *MPKKK19* (Mitogen-activated protein kinase kinase kinase), known to be involved with regulating signal transduction in growth, development and pathogen response. Predicted DEG interaction analysis indicated in tissue resistance were demonstrated to be highly interconnected. The ERFs play a large role in interacting with the plant defence and were linked with MYC2, WRKY, ALT2 and DREB suggesting a pathogen-collaborative response (Figure 4B highlighted in the dotted circle). The strength of interactions between this cluster ranged from moderate to very high (0.4-0.9), with MYC2 notably showing the strongest interaction of 0.89.

DEGs related with PM ontogenic resistance

Ontogenic resistance was investigated through comparison of resistant 'Hapil' old foliage and susceptible new foliage with and without PM infection. A total of 1391 (out of 2249) upregulated genes were associated with defence, a selection of genes that had a direct relation to resistance were identified (Table1) (Figure 3C). Six transcription factors were upregulated, one of which was a *CAMTA3* (Calmodulin-binding transcription activator 3), involved in mediating a stress response to pathogenic fungi and bacteria as well as leaf senescence. The remaining TFs were from the WRKY family, known for mediating defence against pathogens such as *Pseudomonas syringae* and *B. cinerea*. In addition, five disease resistant genes were upregulated including *TAO1* (Target of AvrB operation) known to be resistance to *P. syringae* and when in conjunction with *RPM1* (Resistance *Pseudomonas syringae pv maculicola* 1) provides full pathogen resistance. Also identified was the gene *DSC1* (Dominant suppressor of Camta3 number1) which is required to activate the hypersensitive response (HR). Ontogenic resistance DEG interactions analysis revealed numerous interconnections involved. Predicted interactions show a defence cluster linking cytochrome P450 (*CYP94B3* and *CYP74A*), WRKY, SOBIR1, TAO, RPM1, ALD1(Figure 4C highlighted in the dotted circle).

DEGs involved in cultivar and tissue PM response

Comparative analysis of putative resistance DEGs associated with both cultivar and tissue specific resistance revealed a total of 25 genes, with 17 up regulated and eight down regulated. Out of the 25, eight were found to be directly involved with plant defense (Table 1). Identified were five transcription factors including DREB. Also identified was *APPR5* (Two component response regulator) which is part of the E3 ubiquitin ligase Skp1/Cul1/F-box protein complex (SCF) complex and *GAE1* (UDP-glucuronate 4-epimerase 1) known to be involved in response to stress and defence against fungi. The interaction analysis showed that transcription factors – MYC2 and DREB1D were highly interconnected.

DEGs involved in tissue and ontogenic PM response

The tissue and ontogenic comparative analysis identified five DEGs involved in plant defence. Two of these were *WRKY53* and *ERF105* and two disease resistant genes *DSC1* and *SOBIR1* (Suppressor of BIR1) both involved with initiating hypersensitive response, leading to induced localized cell death. Interaction analysis also showed strong interactions between WRKY53 and SOBIR1 suggesting that they may play a significant role together involving the HR responses.

DEGs involved in cultivar and ontogenic PM response

The cultivar and ontogenic comparative analysis identified a total of 35 DEGs, 17 up regulated and 18 down regulated. Nine upregulated were determined to be related to pathogen resistance. Of these, only one TF was reported related to cultivar and ontogenic resistance, a putative transcription factor LUX (LUX Arryhthmo) known to activate *LHY.* Seven of the DEGs identified were associated with stress response including a gene *PIP2;1* (Aquaporin PIP2-1), found to be downregulated and are known to be involved with initiating an early immune response to PAMP. Another DEG identified *LTI65* (encodes a low-temperature-induced 65 kDa protein isoform X1) which is involved in response to stress and leaf senescence. Interaction of DEGs were found between RGB7, LUX, JMJD5 and UBP13, as well as downregulated LHY possible through interacting with LUX.

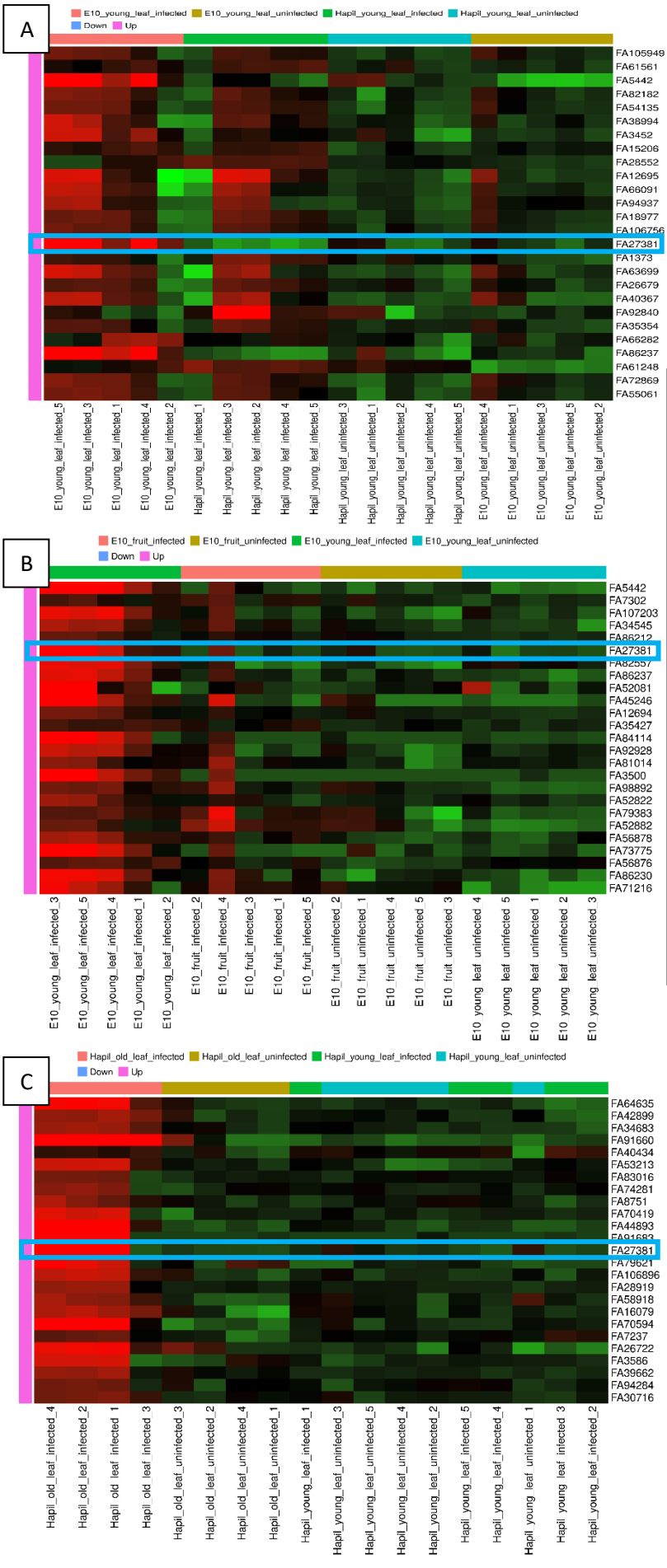


Figure 3. Differentially expressed genes (DEGs) between *P. aphanis* infected and uninfected tissue in strawberry plants. *Fa27381 – CAF1-11 gene* (blue box). A. Cultivar resistance expression profile of upregulated DEGS to differentially expressed genes (DEGs) compared with 'Hapil' and 'E10' foliage. B. Tissue resistance expression profile of upregulated DEGS (DEGs) compared with 'E10' foliage and fruit. C. Cultivar resistance expression profile of selection of upregulated DEGS compared with 'Hapil' old and young foliage. Horizontal rows represent genes, vertical columns represent samples. Expression levels are denoted by colours scale 4 (Red) to -4 (green).

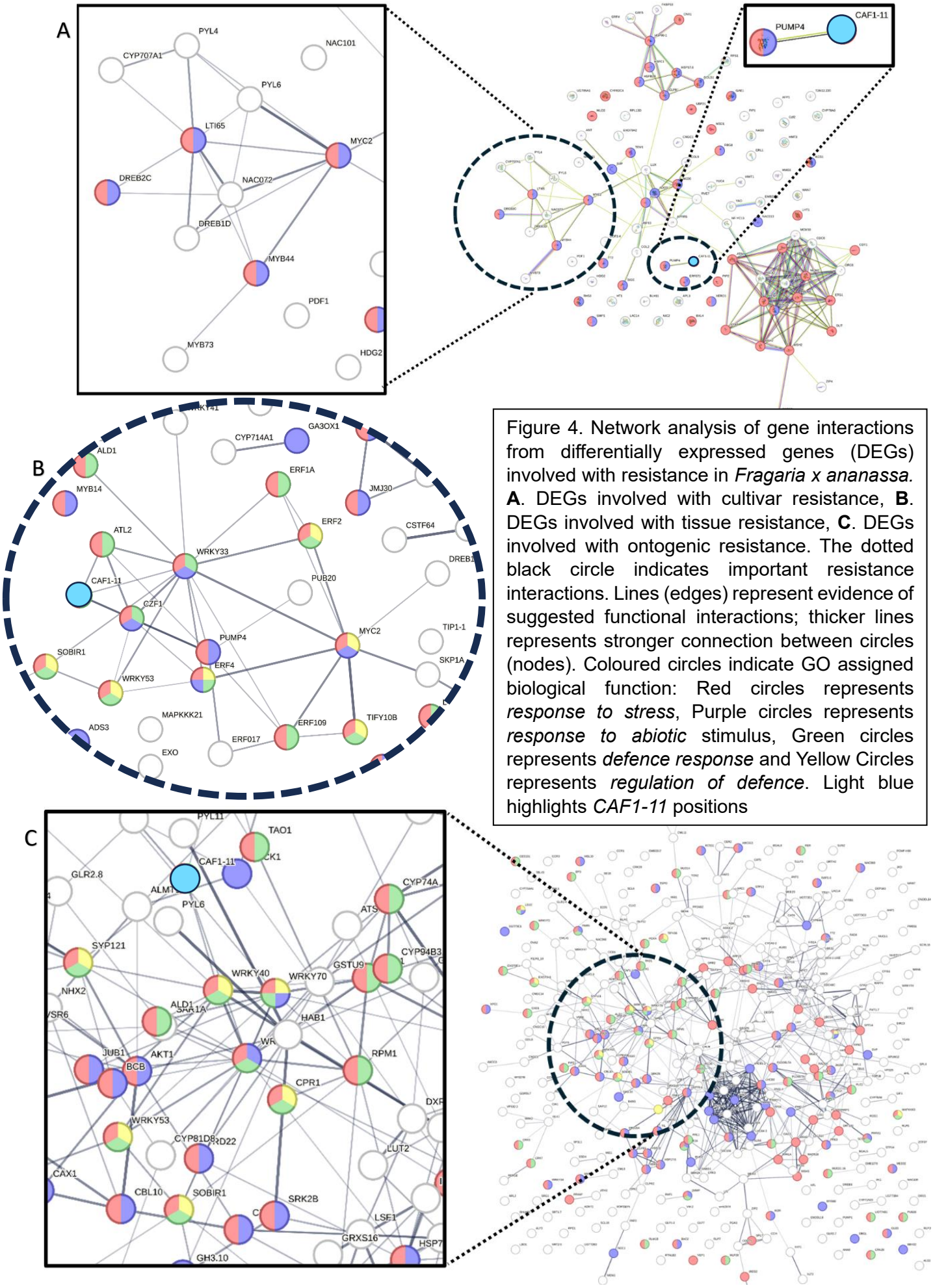


Figure 4. Network analysis of gene interactions from differentially expressed genes (DEGs) involved with resistance in *Fragaria x ananassa.* **A**. DEGs involved with cultivar resistance, **B**. DEGs involved with tissue resistance, **C**. DEGs involved with ontogenic resistance. The dotted black circle indicates important resistance interactions. Lines (edges) represent evidence of suggested functional interactions; thicker lines represents stronger connection between circles (nodes). Coloured circles indicate GO assigned biological function: Red circles represents *response to stress*, Purple circles represents *response to abiotic* stimulus, Green circles represents *defence response* and Yellow Circles represents *regulation of defence*. Light blue highlights *CAF1-11* positions

MLO

Across the cultivar, tissue and ontogenic resistance, four MLO (Mildew Loci O) differentially exressed genes were identified (*MLO3*, *MLO4*, *MLO6* and *MLO14*). *MLO3* and *MLO4* were observed down regulated in cultivar resistance and tissue resistance respectively. Notably, in ontogenic resistance, four MLO genes were upregulated (Table 2). An alignment with *F. vesca* MLOs showed that *FvMLO3*, *FvMLO4* and *FvMLO11* had the highest conservation across orthologs with *F.× ananassa*, at 99%, 97% and 100% respectively (Table 2). DEG interactions analysis identified MLO6 and MLO3 individually having a functional link with TET2 (tetraspanin-2) known to be involved with regulation of cell differentiation (Figure 5). MLO6 also interacted with PMI2-2 (Mannose-6-phosphate isomerase) and the transcription factor WRKY33. Furthermore, MLO14 was also found to interact with a calcium sensor CML11 (calmodulin-like 11). These interactions are noteworthy, as that may provide insight on individual MLOs mechanisms involved with strawberry PM infection.

Table 2. Identified differentially expressed *Fragaria x ananassa* MLO genes (*FaMLO*) in all observations of resistance. Gene name was identified from GDR and location of MLO on chromosome, *Fragaria vesca* orthologs (*FvMLO*) and Nucleotide BLAST % with *FaMLO*. Arrows represents up or down regulated.

MLO	**Resistance**	**GENE**	**Location**	***F. vesca* Orthologs**
FaMLO3	↓Cultivar	augustus_masked-Fvb6-1-processed-gene-258.4-mRNA-1	25.9	*FvMLO3* (99 %)
FaMLO3	↑Ontogenic	maker-Fvb6-4-augustus-gene-108.54-mRNA-1	10.9	*FvMLO3* (96 %)
FaMLO4	↓Tissue	maker-Fvb1-3-snap-gene-62.65-mRNA-1	6.25	*FvMLO4* (97 %)
FaMLO6	↑Ontogenic	maker-Fvb6-2-augustus-gene-179.25-mRNA-1	17.9	*FvMLO6* (94 %)
FaMLO6	↑Ontogenic	maker-Fvb6-4-augustus-gene-109.24-mRNA-1	10.9	*FvMLO6* (96%)
FaMLO14	↑Ontogenic	maker-Fvb3-3-augustus-gene-104.19-mRNA-1	10.4	*FvMLO11* (100 %)

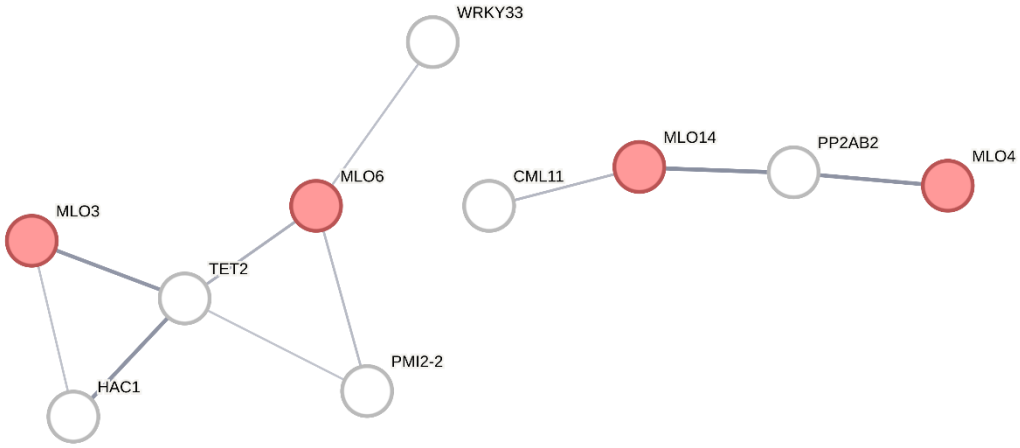


Figure 5. Network analysis of gene interactions identified of Mildew loci O genes. MLO interactions involved with other identified DEGs. Lines (edges) represent evidence of suggested functional links with MLO nodes (circles). Red circles indicate the biological association with a defence response.

DEG analysis

In total 2692 DEGs were identified across all resistance experiments with only one gene identified throughout all experiment parameters – CCR4-associated factor 1 homolog 11 (*CAF1-11*) which was identified in response to stress and defence response. Analysis in Arabidopsis (At5g22250) reports low levels for normal expression of *CAF1* in Arabidopsis in young and old uninfected foliage. In contrast expression levels are high in the presence of *Golovinomyces orontii* (powdery mildew) in *Cucurbitaceae* and *Brassicaceae* foliage (Figure S4) providing additional evidence of *CAF1-11* association in PM disease resistance.

Summary

In all the analysis, ten transcription factor families were identified including ERF, MYC, MYB and WRKY, which are known to be involved with pathogen related responses. Table 1 shows all genes associated with resistance identified for all experimental parameters. Analysis of cultivar and tissue resistance both identified transcription factors MYC and DREB to be associated with resistance. Ontogenic resistance, however, identified transcription factors from the WRKY family, genes encoding cytochrome P450 and several disease resistance genes, none of which were identified in the cultivar or tissue parameters. The overall analysis identified several DEGs involved with Phytohormone signaling in salicylic acid (SA), Abscisic Acid (ABA), Auxin (AUX), ethylene (ET) and jasmonic acid (JA) known to be essential for plant-pathogen response. The DEGs in cultivar resistance comprised a major pathway involving ABA, suggesting this may be the primary resistance response in young foliage. Notably, *CAF1-11* was the only DEG that was identified across all experiments (Figure 2).

Discussion

In this study a novel strategy was used to narrow down candidate resistance genes through RNA sequencing and comparison of DEGs across multiple resistance types. PM is a major global disease affecting above ground tissue. While PM disease in crops has been studied, less is known about the factors involved in tissue specific PM infection and the overlapping factors contributing to different types of resistance response. Here, RNA analysis was conducted to investigate similarities between the cultivar-specific, tissue-specific and ontogenic resistance responses during PM infection. In total, 2692 differentially expressed resistance genes were identified to be involved in PM infection. Many of these DEGs identified were associated with the hormone signaling pathways ABA, ET, SA and JA, associated with plant defence response. The SA pathway is activated by elevated levels of salicylic acid and is involved in the early recognition phytopathogens. As the pathogen infection progresses it switches to a necrotrophic phase, through the activation of transcription factors WRKY suppressing the SA pathway and initiating the JA pathway. Additionally, the ET pathway plays a key role in the responses to pathogen attack and these responses are activated by transcription factors ERFs [29].

Cultivar resistance

The gene expression profile of pathogen foliage resistance revealed 294 genes in the resistant cultivar ‘E10’. Fourteen genes were expressed in the resistant foliage ‘E10’, with four that were known to be involved in plant defence. Notably, the transcription factor *MYB44* was identified, that belongs to the *R2R3 MYB* family and is a component of the hormone signaling pathways known to mediate abiotic/biotic stress response, including the defence response against fungi [30], [31], [32]. A *R2R3 MYB* transcription factor was also previously identified in a genome-wide association study associated with PM resistance by Lynn *et al.* (2023) [unpublished]. In Arabidopsis, the *AtMYB44* gene is known to regulate WRKY70 which mediates the switch between JA and SA antagonistic pathways [30]. *MYB44* is involved with regulation of defence via Pathogen Associated Molecular Pattern (PAMP) triggered immunity (PTI) pathway for defence against bacterial and fungal pathogen infections such as *Pseudomonas syringae* in Arabidopsis and *Golovinomyces ambrosiae* in Cannabis [33]. [34]. DREB transcriptional factors have mostly been associated with abiotic stress such as salt, cold or drought stress; however, future studies should investigate their potential involvement in biotic responses as several were identified in response to strawberry PM [35]. Another gene that was up regulated in ‘E10’ foliage was exocyst complex component *EXO70A1*, which plays a role in mediating a response to EXO subunits involved with plant-microbe interactions through the hormone

system [36]. *EXO70* has been demonstrated to provide a barrier defence against biotrophic pathogens *Phytophthora infestans* [36], [37].

Transcription factor MYC2 regulates the signalling pathway in JA responses, in response to pathogen attack, MYC2 initiates the early immune response in the JA pathway [38]. MYC2 is also involved in crosstalk with different hormone signalling pathways, response to wounding and serves as a negative regulator of the SA pathway against bacterial infections [39]. In pathogen attacks, *MYC2* is activated when the Jasmonate Zim-Domain (JAZ) is repressed and initiates an early JA immune response [38]. Over expression of *MYC2* is associated with the triggering of hypersensitivity response to avirulent bacterial pathogens [40] Transcription factors NAC 072 and 101 were identified. Interaction analysis for NAC072 shows a functional interaction with MYB44, MYC2 and DREB1D. The NAC protein family regulates plant development and numerous abiotic and biotic stress related responses in plants [41]. In the presence of downy mildew, *NAC072* has been shown to increase levels of expression in response to *Plasmopara viticola*, promoting resistance through the downregulation of glyoxalase. [42]. Resistance in strawberry foliage shows *MYB44*, *MYC2* and NAC072 play key roles in PM resistance. Functional interactions between these transcriptional factors suggests 'E10' resistance to PM may be due to this combined immune response.

Tissue resistance response

Gene expression profiles for tissue specific response between 'E10' resistant foliage and susceptible fruit, identified 149 DEGs. Of the DEGs identified and known to be involved with resistance, all were upregulated in foliar tissue. Among the DEGs observed, Ethylene Response Factors (ERFs: 109 and 017), which were upregulated during infection in foliage. ERFs are known to be involved with plant defence regulating both JA and ET mediated defence genes [43]. The ERF/AP2 family as a whole is involved in mediating response to oxidative stress, salt stress and pathogen defence. ERFs have been found to be expressed as a defence response to pathogenic fungi such as in *Fusarium oxysporum* infection of Arabidopsis [37], [44], [45]. In particular, *ERF109* is known to respond directly to fungi and other transcription factors such as *WRKY40* and *ERF13* in Arabidopsis during stress [37] [46]. Similarly, *ERF017* is involved in responding to environmental stress, specifically lead stress and drought tolerance [47], [48]. [49]. Since two transcription factor ERFs were found it may suggest ET pathway is important for immune response in tissue resistance.

Another important gene that was found to be up regulated, was mitogen-activated protein kinase kinase kinase (*MPKKK 19*) known to be associated with abiotic and biotic stresses.

MAPKKKs are involved with various cellular responses including disease resistance such as activation of early immune response and defence to TMV defence in *N. benthamiana* [50], [51]. Additionally, the RING-H2 finger protein (*ATL2*) was upregulated in foliage, and known to be part of the Ubl pathway involved with early stages of plant defence signalling [52]. Although there is no report of ATL2 involvement with a particular pathogen, other genes encoding RING finger proteins have been associated with defence signalling such as *CaRING* against *Xanthomonas campestris* in pepper and *OsBBI1* against *M. oryzae* in rice [53]. The gene interactions for tissue resistance illustrated there were strong interactions between, ERFs, CAF1-11 and MYC2.

Ontogenic resistance response

The differential expression profile observed in ontogenic resistance was ten times higher than seen in the earlier conditions, a total of 2249 DEGs were identified in response to PM infection. Several transcription factors were identified, including ERFs and WRKYs. Additionally, genes encoding cytochrome P450 (CYP) were upregulated in old foliage, specifically *CYP94C1* and *CYP716A1*. P450 enzymes are involved with various cellular process including detoxification of xenobiotics, defence against pathogen such as *Phytophthora infestans* potato and activating in response to stimulus from other organisms, such as regulating the production of toxic phytoalexins to resist aphid attack in peach [49], [54], [55]. The *CYP94C1* gene responds to stress and produces a physiological response to wounds through the JA signalling pathway and is known to interact with WRKY transcription factors. While there is no direct evidence that *CYP716A1* is involved with ontogenic resistance, it is known to be involved with plant stress response[49], [56].

Calcium ions play a vital role in the production of intracellular signalling cascades, with many major functions including growth, development and biotic stress responses. In the context of ontogenic resistance, the upregulated gene calmodulin binding transcription activator 3 (*CAMTA3*) is involved with the calcium signalling pathway, which signals the plant defence response [57], [58]. Studies have shown *CAMTA3* mutants have a reduced effectiveness in responding to biotic stress, resulting in increased susceptibility to pathogens such as *P. syringae* and *B. cinerea* [58]. Several WRKY transcription factors were upregulated in ontogenic resistance. The WRKY family are involved with plant growth and stress responses, during biotic stress, WRKYs either establish a response at the site of infection to restrict the pathogens spread or initiate a signalling cascade through the JA, SA and ET which, in-turn, activate genes downstream for immune response [59]. Recent studies have demonstrated an increased immune response involving WRKY transcription factors in grape and apple,

positively contributing to resistance against PM infection [56], [60]. A gene encoding ALD1 is involved in localised pathogen response and with systemic acquired resistance (SAR) which is activated by pathogen associated molecular patterns (PAMPs). ALD1 has only previously been document to be involved with the defence against *P. syringae* in Arabidopsis, here, ALD1 has been shown to play a role in PM infection as well [61].

R genes involved with ontogenic resistance encoding SOBIR1, RPM1 and TAO1, invoking a hypersensitive immune response to the pathogen. SOBIR1 is associated with programmed cell death and R gene signalling whereby acting as receptor complex for receptor like proteins (RLP) and receptor like kinases (RLK) in the presence of pathogens. In tomato, SOBIR1 is involved with immune response against the fungal pathogen *Cladosporium fulvum* [62]. Another R gene, encoding RPM1, recognises pathogen effectors and activates hypersensitive response programmed cell death to stop the infection, for example in *P. syringae* infection of Arabidopsis. Upon activation of cell death, RPM1 is immediately degraded in order to limit the area of cell death [63]. An R gene encoding TAO1 protein with a TIR-NB-LRR domain is involved with defence gene expression and is essential for resistance against *P. syringae* [64]. Also identified was the gene *APRR5* which is activate in high SA accumulation plays a role in plant-pathogen resistance via regulation of cytokines as observed in tomato plants with *P. syringae* infection [65].

It has been previously hypothesized that ontogenic resistance is caused by high levels of cutin acid in young leaves leading to susceptibility [8], [9]. However, the abundance of DEGs identified in ontogenic resistance it can be hypothesised that the prolonged exposure to infections triggers a complex and extensive immune response. This highlights the plant's continuous battle during infection. As several drought related DEGs were identified in this study, its hypothesis that the shared symptom of leaf curling in water stress may also be employed as mechanism used as defence against PM.

Dual pathogen response with PM infection

The analysis of gene expression profiles for both tissue and cultivar responses identified a total of 25 genes that were involved with PM infection. Of these genes, 17 were upregulated and eight down regulated. Among the upregulated genes, three were strongly associated with a stress response: *CAF1-11, DREB1D* and transcription factor *MYC2*. The presence of these genes in 'E10' resistant foliage across both observations suggests they are key genes for resistance to PM in foliage.

Gene expression profiles for shared tissue and ontogenic genes revealed six common upregulated genes, all of which are known to have a role in disease resistance: *WKRY 53,*

DSC1, CAF1-11, SOBIR1 and *ERF105*. The WRKY transcription factors are recognised for playing key roles in pathogen response [66]. *WRKY 53* and *WRKY 70* have been observed to be upregulated in PM infection (*Golovinomyces ambrosiae*) in cannabis, with increased expression during prolonged infection [34]. Comparably, the majority of the WRKY's identified in this study were associated with ontogenic resistance. Disease resistance-like protein (*DSC1*) encodes a disease resistant protein in the TIR-NB-LRR family and has been associated with resistance to *Phytophthora parsiana* in *Pistacia vera*. *DSC1* acts as a hypersensitive response factor, initiating localised cell death and acts as a guard to *CAMTA3*, which was also found among the most significant ontogenic DEGs [67] [68]. The transcription factor *ERF105* is involved with the SA pathway and regulates ET associated genes in pathogen response. Studies knocking out *ERF105* in maize has been shown to decrease the resistance in response to the fungal pathogen *E. turcicum* [45].

Gene expression profiles for cultivar and ontogenic resistance to PM infection identified a total of 35 common genes that were associated with PM infection. Two were associated with pathogen defence were *RBG7* and *DAW1*. The gene *RGB7* identified belongs to the RNA-binding glycine rich superfamily shown to play a role in pathogen defence such as against *P. syringae* and *B. cinerea*. While this role had not directly been demonstrated for RGB7, these genes should be considered in future studies to confirm their potential defence [69]. Also a gene encoding Transducin/WD40 repeat-like superfamily protein (DAW1), which has been shown to repair soybean cells damaged by red leaf blotch caused by *Coniothyrium glycines*. DAW1 may work in a similar fashion in strawberry PM blotching response as observed with the disease progression [70].

Overall, in dual comparisons, two genes *MYC2* and *DREB1D* were identified in 'E10' observations for cultivar and tissue resistance, suggesting they are key genes required for resistance to PM in foliage. Other cross over genes found in the different observations are shown to be important genes in the defence response to PM infection. Similarly, in results from investigating in apple leaves infected with *Podosphaera leucotricha*, there is a strong involvement with transcription factors WRKY, NAC and MYC2, as well as high involvement with ERFs [56].

Susceptibility gene Mildew resistance loci

In view of identifying PM resistance genes, it is noteworthy that six Mildew Locus O (MLO) DEGs belonging to the MLO family were discovered. Mutant MLO genes have demonstrated a broad sense resistance to PM infections, as has been shown in tomato [71], barley [72] and apple [73]. Studies have shown that MLO upregulation occurs during early PM infection,

facilitating the PM to penetrate into plant tissue. This upregulation negatively regulates the actin pathways and it is postulated that PM exploits this actin vesicle transport system for nutrient supply [57], [74], [75], [76]. Actin transport requires calcium for activating, which may be linked to MLO negative regulation, notably, *MLO14* shown to have a functional interaction with gene encoding CML11, a calcium sensor. This suggests that *MLO14* may be a negative regulator involved with or activated by CML11, either possibility suggests *MLO14* as an interesting candidate for PM resistance. *M. domestica MdMLO19* has been shown to be an orthologue of *FvMLO4* [77]. When compared with *FvMLO4* and *FaMLO4* sequences, there was high sequence identity of 97 %. [71]. Gene knockout experiments that targeted the identified susceptibility gene *MdMLO19* in *M. domestica* resulted in the resistance of two cultivars, leading to a reduction in PM susceptibility, with no secondary complications. These findings suggests that this one MLO gene may be sufficient for imparting resistance in apple, as also observed in pea and tomato [71], [73], [78]. This evidence makes *MLO4* a candidate for potential MLO resistance in strawberry and should be a focus in future studies. *FaMLO3* is identified as upregulated in cultivar resistance and downregulated in ontogenic resistance. This gene has approximately 96 % sequence identity with *FvMLO3*, and studies on *FvMLO3* have shown that high levels of expression is associated with PM infection in *F. vesca* [79]. In this study it was demonstrated that MLOs were upregulated in both tissue resistance and cultivar resistance, whereas in ontogenic resistance all four were downregulated. *FaMLO6* was identified in ontogenic resistance; for future studies it may be worth noting that *MLO6* in Arabidopsis has high conservation with Fragaria species, and thus *MLO6* may correspond with *AtMLO6*, which required the addition of *AtMLO2* and *AtMLO12* for knockout, to achieve complete resistance to PM [71]. The downregulation of all four MLOs associated with ontogenic resistance in ‘Hapil’ leads to the hypothesis that this aspect of oncogenic resistance may overcome the negative regulation imposed by MLOs during prolonged exposure.

DEG identified in all resistance expression profiles

Gene expression profiles analysed for all observed resistance expressions showed that the CCR4-associated factor (*CAF1-11*) was present in all observations for resistance. *CAF1* is a subunit of the CCR4-NOT complex, an evolutionary conserved protein complex, that plays a role in the control of transcription and mRNA metabolism. This includes mRNA de-adenylation and RNA-induced gene silencing, which ultimately leads to targeted gene degradation [80]. The initiation of the CCR4-Not complex can result from signaling of the ABA, SA and JA pathways [81]. *CAF1* has been linked with a defence response and resistance to pathogens in several crops [80], [81]. In tomato plants, the *CAF1-11* gene has been demonstrated to be involved with abnormal plant growth and altered pathogen defence response [82]. Tomatoes

modified to over express pepper *CaCAF1-11* were found to have increased levels of resistance to *Phytophthora infestans* [81]. Additionally, overexpression of *CaCAF1* was also found to promote growth of the tomato plant [82]. When *CAF1* mutants (*AtCAF1a and AtCAF1b*) were studied in Arabidopsis they were shown to be associated with a reduction in pathogenesis-related genes (PR) and as a result were more susceptible to *P. syringae* infection [80]. In a hypersensitive mediated resistance response, *CAF1* was demonstrated to be involved with resistance to the bacteria *Xanthomonas citri* in the host *Citrus sinensis* [83]. Our results are consistent with findings in other pathogen studies [80], [82], [83], suggesting that *CAF1-11* plays a key role in multiple pathogen resistance responses. This is the first time *CAF1-11* has been associated with strawberry disease. Moreover, upregulation of this gene has a resistance effect on all tissue types and so may provide protection to strawberries from PM infection.

Conclusion

The results of this study have provided greater insight into resistance mechanisms present in different strawberry resistance types. In this study, an innovative method to in narrow down candidate genes between tissue types offered a novel suite of putative resistance genes in strawberry. With the overall total of 2692 defence DEGs identified, several key upregulated resistance transcription factors were identified, including ERFs, MYB, MHC and WRKYs, and these were found to be highly interconnected. Notably, ontogenic resistance involved significantly larger number of genes compared to the other studies, suggesting a more extensive involvement of the immune response during prolonged infection. Furthermore, only R genes were identified in ontogenic resistance. Several genes identified were found to respond to abiotic stress and drought stress; suggesting that these genes may be triggered by the same mechanism in response to PM infection depleting resources. Additionally, the *FaMLO4*, identified in tissue resistance is a prime candidate for further investigations, as previous knockout studies of its orthologues in apple successfully produced a high level of resistance to PM infections, with no secondary effects. In general, although the immune response is still not clearly understood, the findings reported here support some other studies in response to PM infection and offer some new putative genes for validation in future studies in PM resistance. The most noteworthy of the DEGs is the identification of the *CAF1-11* gene, identified in all examples of resistance, and providing a key candidate for future studies for all tissue resistance in strawberry.

Supplementary

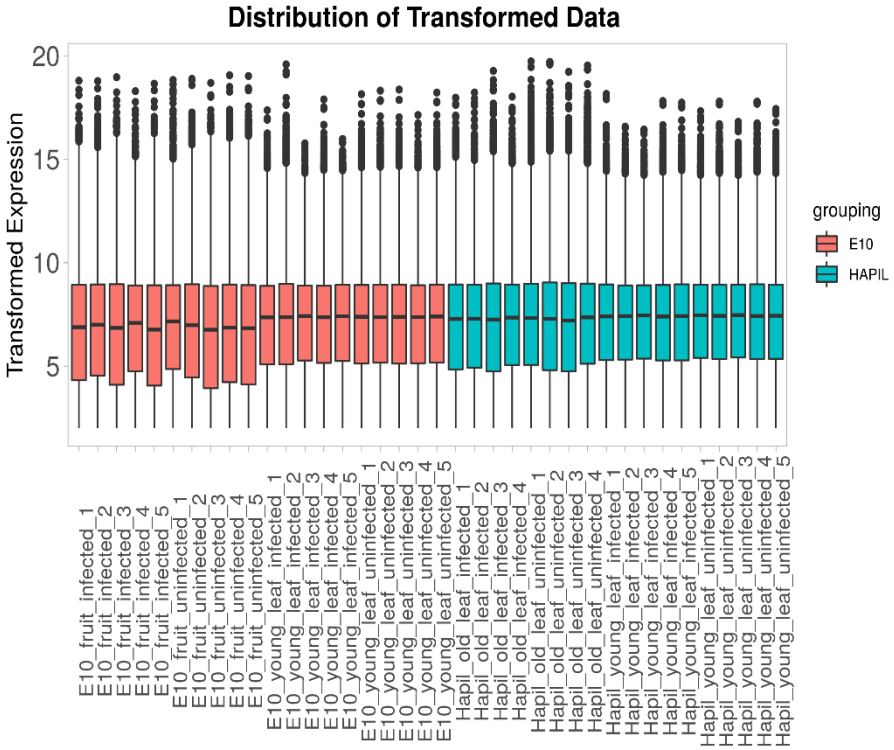
Supplementary Table S1. Experimental design detailing sampling of different tissue types, cultivars and biological replicates numbers.

Tissue type	*Cultivar*	*Powdery Mildew Infected*	*Non infected (control)*
Fruit	Hapil	5	5
	E10	5	5
Young foliage	Hapil	5	5
	E10	5	5
Old foliage	Hapil	4	4

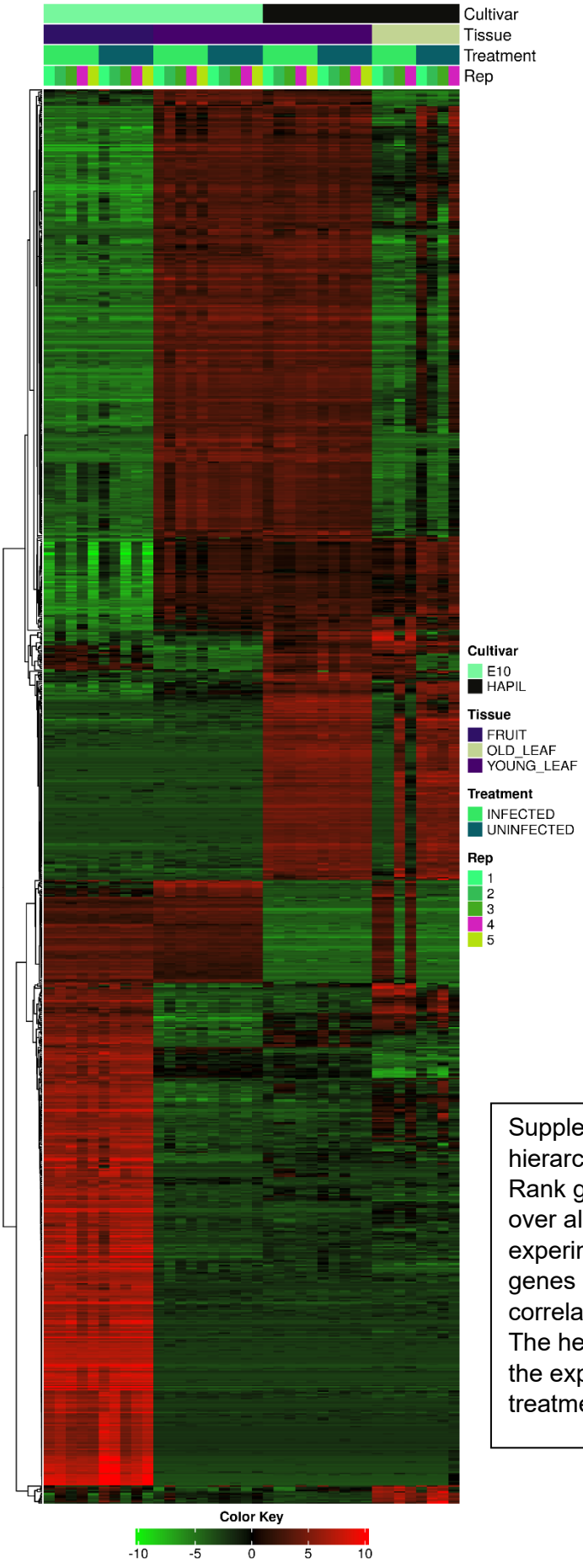
Supplementary Table S2. Total amount of raw reads count, Raw data (Raw reads*sequence length) calculated in G, effective % (Clean reads/Raw reads), Error base rate, GC content and Q20-Q30 percentage based on Phred value/total base count.

	Raw reads	Raw data	Effective(%)	Error(%)	Q20(%)	Q30(%)	GC(%)
Hapil old leaf uninfected 1	1.21E+08	18.2	98.82	0.03	97.73	93.59	47.61
Hapil old leaf uninfected 2	91766802	13.8	99.15	0.03	97.83	93.75	46.34
Hapil old leaf uninfected 3	83537976	12.5	99.01	0.03	97.81	93.73	47.37
Hapil old leaf uninfected 4	92237850	13.8	99.03	0.02	98	94.18	46.36
Hapil old leaf infected 1	1.14E+08	17	98.85	0.03	97.94	94.04	48.45
Hapil old leaf infected 2	96435568	14.5	98.76	0.03	97.74	93.59	47.2
Hapil old leaf infected 3	83331288	12.5	99.02	0.03	97.68	93.47	47.34
Hapil old leaf infected 4	97448934	14.6	98.54	0.03	97.65	93.38	47.58
Hapil infected young leaf 1	92361588	13.9	98.99	0.03	97.88	93.97	46.66
Hapil infected young leaf 2	92236824	13.8	98.83	0.03	96	89.94	47.49
Hapil infected young leaf 3	1E+08	15.1	98.68	0.03	96.19	90.32	47.18
Hapil infected young leaf 4	1.1E+08	16.5	98.26	0.03	96	89.9	46.69
Hapil infected young leaf 5	86731182	13	98.53	0.03	96.11	90.12	47.24
Hapil uninfected young leaf 1	99269056	14.9	98.98	0.03	97.75	93.65	46.92
Hapil uninfected young leaf 2	98200782	14.7	98.53	0.03	96.43	90.73	47.09
Hapil uninfected young leaf 3	80468598	12.1	98.6	0.03	96.15	90.23	46.82
Hapil uninfected young leaf 4	87404476	13.1	98.45	0.03	95.87	89.64	46.84
Hapil uninfected young leaf 5	80614874	12.1	98.47	0.03	96.09	90.09	46.91
E10 infected leaf 1	90469632	13.6	98.95	0.03	97.82	93.76	46.84
E10 infected leaf 2	1.75E+08	26.2	98.42	0.03	96.18	90.24	46.9
E10 infected leaf 3	84366180	12.7	98.66	0.03	95.96	89.81	46.9
E10 infected leaf 4	84982048	12.7	98.7	0.03	96.5	90.8	47
E10 infected leaf 5	96317392	14.4	98.67	0.03	96.59	90.97	46.55
E10 uninfected leaf 1	86612940	13	98.86	0.02	98.15	94.54	46.68
E10 uninfected leaf 2	1.13E+08	16.9	98.61	0.03	96.51	90.83	46.63
E10 uninfected leaf 3	83680246	12.6	98.38	0.03	96.67	91.2	47.28
E10 uninfected leaf 4	84102628	12.6	98.29	0.03	96.35	90.5	46.78
E10 uninfected leaf 5	92428990	13.9	98.4	0.03	96.28	90.34	47.03
E10 strawberry infected 1	84602334	12.7	99.01	0.03	96.13	90.18	47.86
E10 strawberry infected 2	98682224	14.8	98.97	0.03	97.87	93.85	46.44

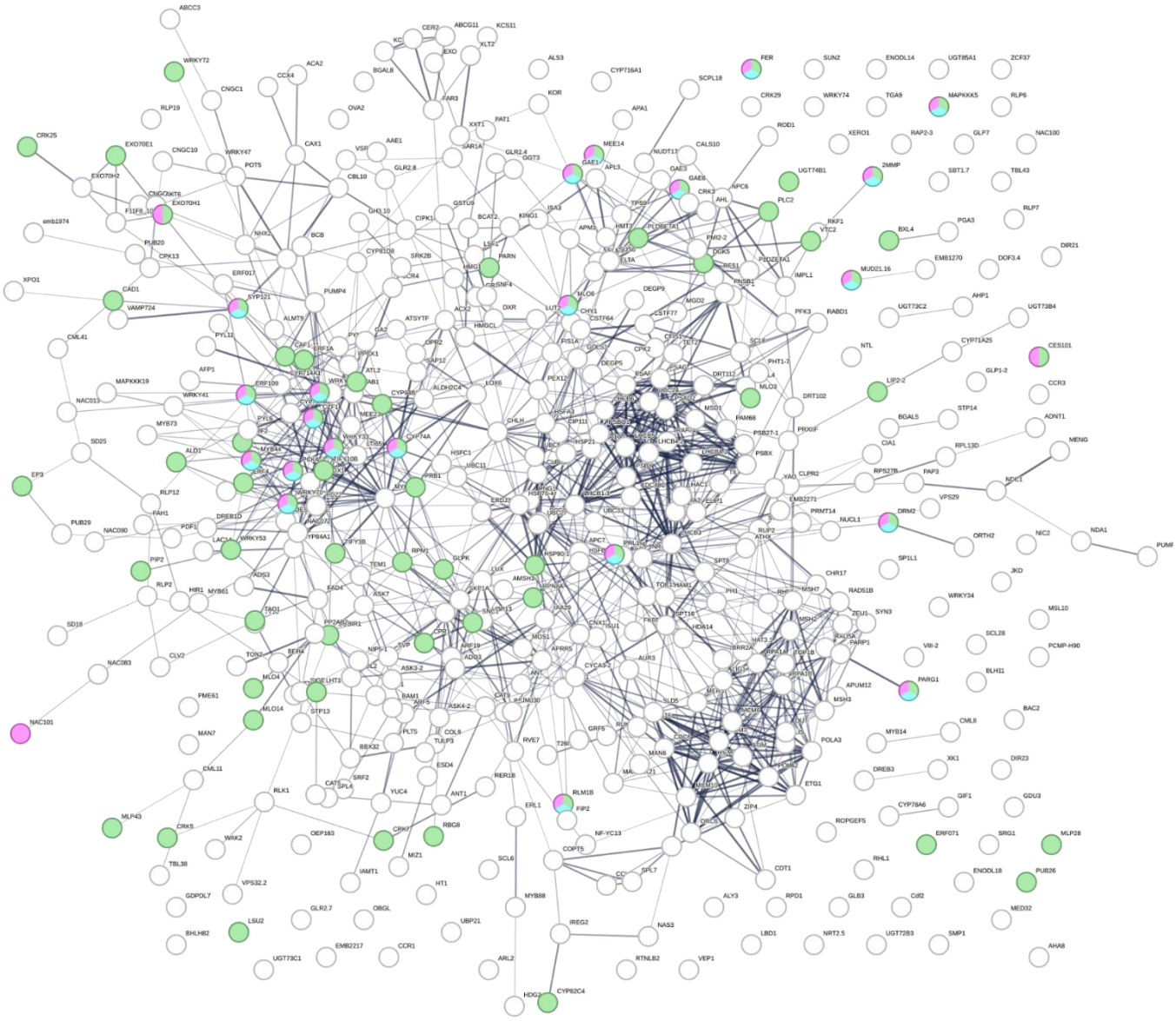
E10 strawberry infected 3	80022734	12	98.84	0.03	96.02	89.92	47.38
E10 strawberry infected 4	85934342	12.9	98.33	0.03	96.85	92.25	47.02
E10 strawberry infected 5	86824282	13	98.76	0.03	95.75	89.42	47.84
E10 strawberry uninfected 1	84330258	12.6	99.13	0.03	97.91	93.96	46.69
E10 strawberry uninfected 2	83677614	12.6	98.76	0.03	96.02	89.97	47.13
E10 strawberry uninfected 3	81656510	12.2	98.76	0.03	96.01	89.92	46.92
E10 strawberry uninfected 4	84063314	12.6	98.73	0.03	96.41	90.75	46.83
E10 strawberry uninfected 5	89048148	13.4	98.7	0.03	95.8	89.53	47.27



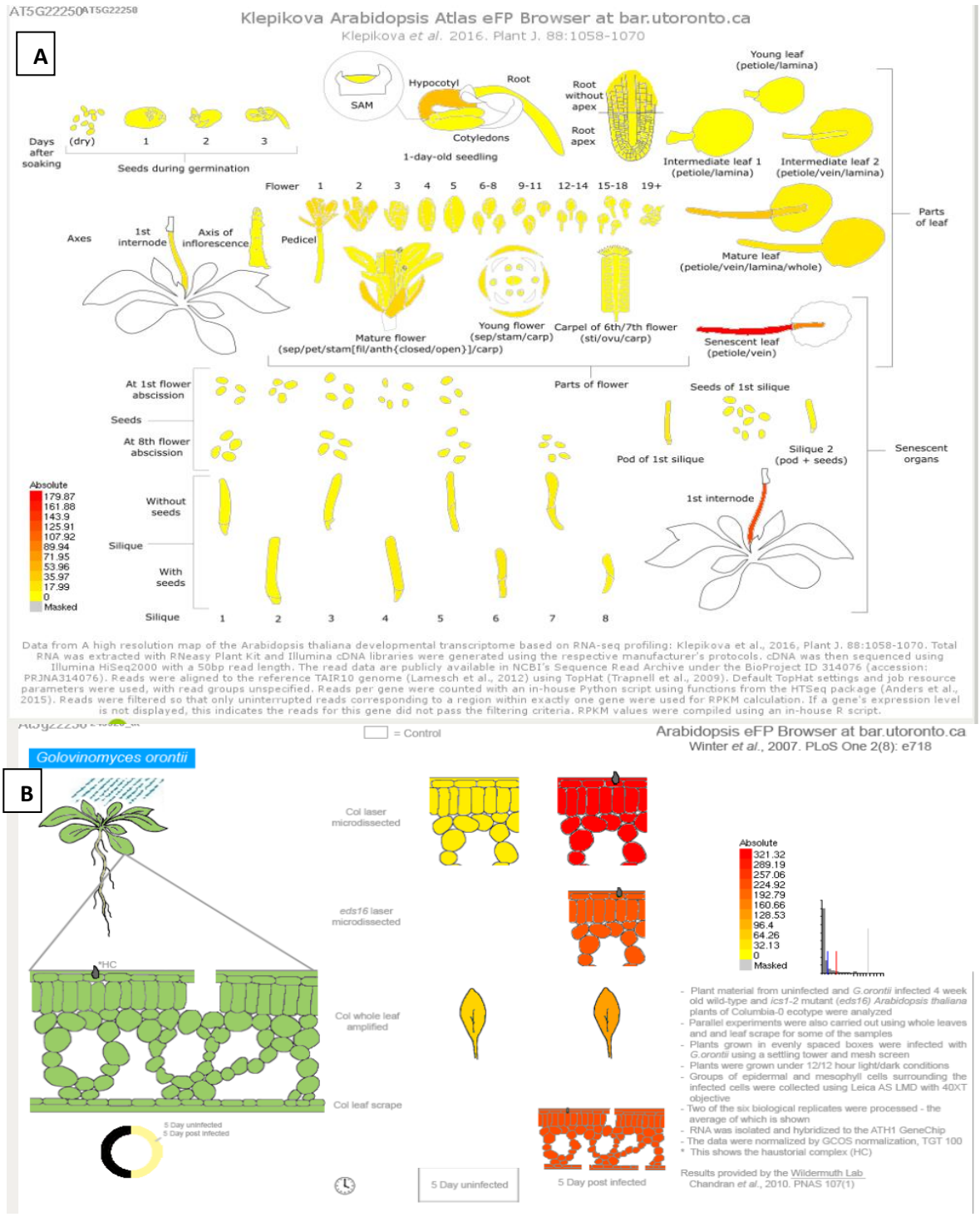
Supplementary Figure S1. Distribution of transformed data. Grouping shows red representing E10 uninfected and infected young foliage/ fruit, Blue representing Hapil uninfected and infected in young/old foliage. Scale 0-20 for transformed expression.



Supplementary Figure S2. The hierarchical clustering with heatmap. Rank genes by standard deviation over all the samples in the experiment, with only the top 1000 genes included. Using Pearson correlation and linking by averages. The heatmap shows all parameters in the experiment incorporating cultivar, treatment, replicates and tissue.



Supplementary Figure S3. Gene interactions network. Functional interactions of all differentially expressed genes identified across the observed assessment using STRING. Nodes represent protein to protein interactions in powdery mildew response uncovered in literature, from transcripts of differentially expressed genes identified. Response node coding: green – defence response (GO:0006952), pink – response to fungus (GO:0009620) and blue – defence response to fungus (GO:0050832)



Supplement Figure S4. *CAF1* expression in tissue. A. Non-infected expression levels in all tissue CAF1 are expressed, B Expression levels of non-infected and infected after 5 days with *Golovinomyces orontii* (powdery mildew). Klepikova Arabidopsis (At5g22250) Atlas with TAIR database [35].

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Chapter 5.

Genetic control of fruit number in octoploid strawberry (*Fragaria × ananassa*)

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Abstract

Enhancing fruit quality and yield production of strawberries has long been a focus of soft-fruit research. Traditional breeding methods have relied on phenotypic selection to identify and select plants with desirable traits for use in breeding programmes. However, recent advancements in genetic technologies have revolutionized the field. In this study a Genomic Wide Association Study (GWAS) was performed to identify genes associated with strawberry flower and fruit numbers for the exploitation in breeding programs to achieve an improvement in yield. Analyses for June bearer and everbearer cultivars were conducted individually and combined across the flowering types. The analysis for combined cultivars identified seven Quantitative Trait Nucleotides (QTNs) associated with fruit number. Individual analysis identified one QTN associated with fruit number in everbearers; however, no QTN were associated with fruit number in June bearers. In addition, a genomic selection analysis was performed to determine the viability of using genetic data to predict fruit number potential. Overall, several putative loci were detected that are known to be involved with flowering habit; however, several novel loci were also identified and associated with fruit number only. These results provide the first steps towards identifying genes that regulate fruit number.

Introduction

Strawberry (*Fragaria* spp) is an economically important crop, with a growing demand for varieties with enhanced quality and yield. [1], [2]. Cultivated strawberry is an octoploid, with a complex evolutionary history; early ancestors can be traced back to America where independent hybridization events occurred to generate *Fragaria virginiana* (North America) and *Fragaria chiloensis* (South America) through the hybridization of four diploid species over 1 million years ago [3], [4], [5]. *F. chiloensis* was originally cultivated by the natives of Mapuche and Picunche [6], [7]. Over 300 years ago *F. chiloensis* was taken to Europe and planted next to cultivated *F. virginiana*, which inadvertently initiated a natural hybridization producing the commercial strawberry species *Fragaria × ananassa* [7], [8], [9].

Strawberry growth is influenced by numerous factors that profoundly affect and enhance growth, such as temperature, light intensity, soil nutrient composition and daylength [10]. Temperature in particular plays an important role in fruit number, with low temperatures

required to exceed the required chill hours to break bud dormancy and initiate floral bud formation [11]. Fragaria species are categorized by flowering habit, either June bearers (short day (SD) flowering plant) flowering once per season and everbearers (long day (LD) and day neutral (DN) flowering plant), flowering recurrently throughout the season. Although everbearing plants have a longer fruiting period, they generally produce fewer fruit at each time point, but with production stretched over a prolonged period of time, they also have more crowns but fewer runners compared to June bearers [12], [13][14]. An investigation comparing cultivars 'Bolero', and 'Everest' (everbearer) to 'Elsanta' (June bearer) found that early flowering emergence timing was similar between these cultivars, despite their different genetic backgrounds, though this may differ between cultivars. Fruiting in June to July showed 'Bolero' and 'Everest' had about 40% lower in yield than 'Elsanta', yet the everbearer had a higher overall yield over the season due to a longer fruiting period [13]. The current production convention typically uses June bearers as the initial crop before extending the cropping season with everbearers, which can produce fruit late into the summer months [10].

The discovery of genetic and environmental factors that influence crop traits is important for breeders to enhance yields, increase resistance to disease and ensure resilience under the varying conditions caused by climate change [11]. Strawberry plants are known to be sensitive to environmental stresses, in particular during flowering/fruiting season, especially in the event of heavy rain, prolonged exposure to heat and strong winds [15]. The domestication of strawberry cultivars has involved investigating the genetic diversity of adapted cultivars to specific environments that can be utilized for cultivation. Examples of those are 'Elsanta' and 'Camarosa', known for their tolerance to high temperatures and disease resistance [16], [17], [18]. The rapid changes to global climate conditions and shifts in weather patterns can have a negative impact on the horticultural industry. Indeed, factors such as warmer winters affecting dormancy and extreme heat accelerates the plant development by negatively impacting fruit size and quality. Moreover, extreme weather conditions such as droughts or stormy conditions with heavy downfalls leading to flooding, as well as destructive wind and hail, can all have a detrimental impact on fruit yield. These conditions not only put the plant under stress but can also accelerate potential disease niches [18], [19]. It is important to identify the genes involved in climate resilient stress responses, whilst also retaining traits such as high seasonal yields (including fruit number) and quality such as fruit firmness, taste and shelf life [16].

Plants have a complex regulatory system controlling flowering time, which includes perception of light and temperature throughout the season, referred to as photoperiodism [20]. It is this photoperiodism response that controls flowering habit, classified as short-day flowering, long-day flowering or day neutral flowering plants that flower irrespective of day length [20]. As the

plant develops, juvenile plants are in a vegetive state of growth. After the plant matures and reaches the reproductive stage, there is a developmental transition from vegetative growth to reproductive growth [20]. This developmental transition then shifts from leaf and stem development to floral meristem production to initiate flower formation [21]. This developmental transition is triggered by photoperiodic environmental cues that activate flowering genes [21]. Apical meristems form and cells differentiate either to produce flowers or stolons [18]. It is this process that is mediated by environmental cues, which initiates and regulates flower production through the Shoot Apical Meristem (SAM) [22], [23]. The mechanisms involved in mediating cultivated strawberry flowering are still largely unclear and the majority of molecular research in *Fragaria* has been performed in *F. vesca*, with limited research conducted in *Fragaria* × *ananassa* [18], [24]. Investment in research aimed at identifying key components in flowering initiation and development in *Fragaria* × *ananassa* would allow breeders to accurately select flowering habits.

Traditionally, breeders focused on phenotypic selection to identify individuals with favorable traits for further breeding [25]. In recent decades, research efforts have predominantly employed linkage mapping, through utilizing bi parental populations to map loci of interest within a narrow genetic range [26]. Linkage mapping is achieved through looking for associations between phenotypic and genotypic data to identify regions in a genome that influence the desire trait. The method employs a population generated by crossing two individuals that have contrasting phenotypes, enabling the determination of linkage between the trait of interest and markers in the population. This technique facilitates the identification of genetic regions in the genome that control desirable traits, even when only a limited number of genetic markers are screened across the population [27]. Subsequently, these markers can then be utilized to identify cultivars with the desired trait without the necessity for extensive phenotyping. This results in a significant improvement in the reduction of time and resources compared to the traditional approach of phenotyping selection [28].

Previous fruit quality studies employing linkage mapping have been conducted to elucidate genetic components controlling flower number. In a study employing linkage mapping conducted by Zorrilla-Fontanesi *et al.* (2011), two strawberry lines were crossed and progeny were assessed over three years for firmness and flowering traits. The study revealed the presence of two stable QTL associated with fruit number, localized within distinct genomic regions [31]. However, this study was limited to a bi-parental cross that does not necessarily translate to a wider population [32]. The majority of the research has employed the model plant Arabidopsis as a proxy for most developmental studies. Studying Arabidopsis has provided six major pathways for flowering, involving genes such as Flowering Locus T (*FT*) and Terminal Flower Locus 1 (*TFL1*) [33]. Evidence accumulated has determined that these

genes acts as floral promotors and inhibitors, which respond to environmental cues [34], [35]. *TFL1* has been demonstrated to act as a suppressor of flowering in Arabidopsis with little effect on flowering time [34]. However, in SD *F. vesca*, the *TFL1* orthologue regulates flowering time, controlling the switch from the vegetative state to the flowering phase at the shoot apical meristem and as such *TFL1* is considered essential for flower induction [18], [33]. Conversely, LD *F. vesca* species consists of a 2 bp deletion in the *TFL1* gene and is continually upregulated throughout seasonal flowering, exhibiting the everbearing trait [33]. This highlights the subtle differences between Arabidopsis and diploid *F. vesca* flowering mechanisms. Furthermore, in *F. ananassa, FaTFL1* also acts as a suppressor in connection with Suppressor of Overexpression of Constans (*SOC1*). *FaSOC1* is associated with runner formation, however the precise mechanism in *F. ananassa* remains to be elucidated [34]. Additionally, in *Fragaria x ananassa*, *FaTFL1* expression was identified as cultivar dependent and there was a suggestion that expression was age dependent, with young cultivars expressing higher levels [34]. Although the *FaTFL1-FaSOC1* pathway is associated with commercial Fragaria flowering, other regulators are probably involved and therefore much more research is still needed to determine the complexity of flowering in octoploid strawberry [18], [34]. Notably, there have been very few studies directly working in octoploid species.

The method of Linkage mapping has limitations in terms of low resolution power and restricting the translational ability across a wider population [32]. Unlike Linkage mapping, a Genome Wide Association Study (GWAS) involves employing a population of numerous unrelated individuals to identify genetic markers that are strongly linked to the genetic components controlling phenotypic traits [36]. The application of a GWAS can significantly enhance the statistical power to identify complex traits to identify molecular markers of the trait of interest [37]. A GWAS facilitates the identification of correlations between genetic markers and traits across the entire genome. As fruit quality and yield are complex traits, it is necessary to perform a robust phenotypic assessment, as was demonstrated with the associated perpetual flowering and runner gene (*FPRU*). *FaPFRU* is known to be involved as a flowering activator in LD/DN octoploid cultivars and has been the subject of studies aiming to uncover the major gene for controlling the perpetually flowering habit. Recently it was postulated that *PFRU* was involved with epistatic interactions [38]. This hypothesis was confirmed in a GWAS, which revealed two epistatic modifiers most likely inhibiting the *FaTFL1* in octoploid species, thereby promoting perpetual flowering [14]. A GWAS can overcome the limitation of linkage mapping as a powerful method to dissect complex fruit traits and identify new associated loci [32], [39]. Although important control and regulators have been established, many components involved remain elusive and the gene or genes involved in controlling for fruit number have not yet been

discerned. Unraveling this would require a more robust technique such as GWAS to undercover core genes involved in fruit production [18], [40].

An alternative breeding approach that can be used to improve a trait in breeding is Genomic Selection (GS) [44]. GS uses information from all quantified genetic markers to predict the phenotype of an individual that has not been phenotyped. Many different GS models such as (G-BLUP, Bayes B and LASSO regression), have been employed to assess the impact of using GS to improve fruit size and average fruit yield [44]. A GS model test uses a training set of known genotyped and phenotyped individuals in order to predict the phenotypes of individuals that have not been phenotyped based on combining information across marker effects. The results can be combined across estimates for multiple traits to produce a genomic estimated breeding value for a given individual (GEBV). Here the model will be used to calculate the correlation between the predicted and actual phenotypes, to calculate the predictive accuracy for GS for improvement of the desired trait [25].

The global demand for increased crop yields has been the key focus for many crops improvement in industry. The importance of identifying the genetic components underlying the complex trait of flowering and fruit number is an important step to attain this objective [26]. In this study a GWAS was performed to identify genes associated with fruit number in an effort to narrow down important genes with a role in enhancement of yield.

Method

Experiment design

A genome wide association study (GWAS) was performed using 328 strawberry genotypes containing 244 June bearers (short day) and 84 everbearers. The accessions used for this study included NIAB breeding lines and commercially important varieties. For each cultivar, five runners were transferred from the polytunnel to the glasshouse and propagated as misted tips (in 9 cm pots), in a heated glasshouse compartment at 25 ºC, 16/8 hr day cycle, 100 % humidity for two weeks then reduced biweekly to 80 % and 60 % respectively. Plant material was then relocated to an open field at NIAB, East Malling, Kent (51˚17'20.1"N 0˚27'11.0"E) in 2020. Plants were planted into polythene raised beds (previously fumigated) with a row length of 100m and 1 meter spacing between rows. Each genotype was planted out in replicates of 5 across five randomized blocks and provided with irrigation. The plot had a hedge running along the east side of the rows, leading to 20% of the plot being shaded (block 1) until mid-morning (10.30 am).

Phenotyping

The fruit number count was carried out on 328 cultivars (244 June bearers and 84 everbearers) in the field plot, measured through visual counting and recording of all flowers (including buds) and emerging fruit on for all five blocks at one time point in the second year of planting (2022). In total, 1640 individuals were counted. The assessment involved a team of five, over the duration of five days, to collect the complete data set.

Genotyping

DNA extraction was performed using the Qiagen DNAeasy plant mini extraction kit (Qiagen Ltd, UK) on newly emerging leaves. The Affymetrix Istarw90 Axiom array (90,000 genetic markers) was used to genotype all cultivars (i90k) [45]. The consensus linkage map (created across five biparental mapping populations [Lynn *et al*. (2023) unpublished] was used to define the location of Single Nucleotide Polymorphisms (SNPs) and 'pseudo-octoploid' chromosomes were assigned to enable surrogate physical mapping onto *F.× ananassa* [46]. The consensus linkage map is composed of 28 linkage groups of chromosomes 1-7, with sub genome group assigned A-D [47]. Genes underlying the identified QTN were characterised for molecular and biological functions using Genome Database for Rosaceae (GDR), NCBI and Uniprot 'BLASTx' [48], [49], [50].

Statistical Analysis

Analysis was performed independently with June bearers and everbearers as well as on combined flowering types across all genotypes. Spatial modelling corrected for heterogeneity across the field, using two-dimensional P-spline modelling (SpATS package) [51]. Broad sense heritability (H^2) for genetic associations was calculated using the SpATS package [52]. Best Linear Unbiased Estimates (BLUE) were generated in R package 'lme4' using a mixed linear effect model where genotype was specified as a fixed effect and block a random effect [53]. The BLUEs analysis was used to produce an overall fruit quantity score value for each genotype; these scores were used in the GWAS analysis.

Genetic Analysis

A genome wide association study (GWAS) analysis was performed using BLUE strawberry fruit number, using PLINK as detailed on GitHub [54], [55]. SNPs were filtered in order to remove minor alleles present in less than 5% of the population, as well as any SNP missing from more than 50% of the population. *p*-values were corrected using the Bonferroni method to correct for multiple testing and plotted as a Manhattan plot using 'CMplot' package in R [56]. Quantitative Trait Nucleotides (QTN) above the significant threshold were represented in a Manhattan plot across all octoploid chromosomes.

Genomic prediction

Genomic prediction for potential genetic informed breeding was calculated using ridge regression best linear unbiased prediction "rrBLUP" using the R package [57].

$$y = \mathcal{Z}g + \mathcal{S}\tau + \beta$$

Where β is the population structure shown by the $\mathcal{Z}$ and $\mathcal{S}$ which represent the 0, 1 matrices represented by a fixed effect. τ represents the additive SNPs and g the genetic background. A training sample of 60 % and test sample of 40 % was used to calculate predictive accuracy. Values for the average predictive accuracy were obtained through 100 permutations of the model, for each permutation a random selection of genotypes were selected for the training data to predict the score of the test data [44]. Predictive ability was calculated through multiplying predictive accuracy and broad sense heritability.

For full methods refer to Chapter 2: 2.1, 2.3, 2.5 – 2.7.

Results

At a single timepoint in mid-May, 244 June bearers and 84 everbearers were assessed for fruit number. The spatial analysis shows 75% of the plot is uniform for both June bearer and everbearers, the remaining 25 % shows some variation for both flowering types (Figure 1). Broad sense heritability values for fruit number across all cultivars was 0.66, with broad sense heritability for June bearers and everbearers were calculated to be 0.59 and 0.63 respectively. The BLUES histogram illustrates that the June bearers had higher fruit numbers compared to the everbearers (Figure 2).

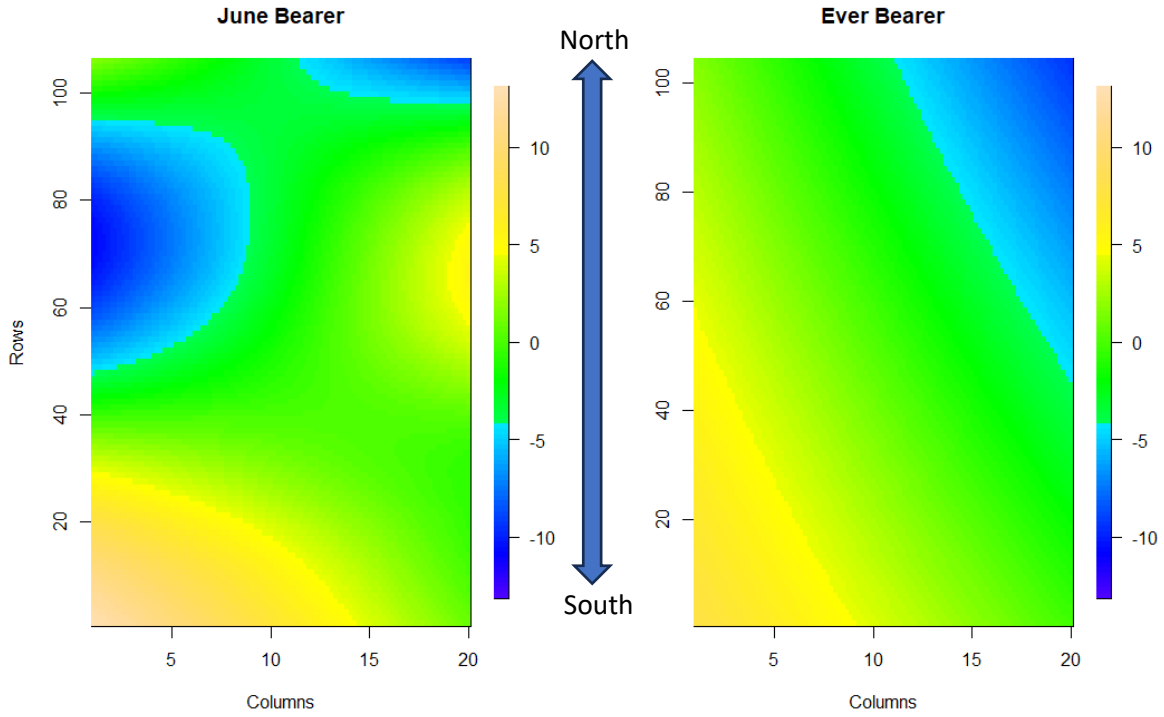

Figure 1. Spatial model analysis of fruit number across the experimental field plot of strawberry plants with a contour plot of the estimated smooth spatial trend. 1A Spatial trend for June bearers. 1B, Spatial trend for everbearers. All fruit numbers were recorded in 2022. Columns denote raised beds; rows denote the position of each plant along the beds. The scale bar represents strawberry fruit number – yellow 10, blue -10. Arrow represents orientation of the plot.

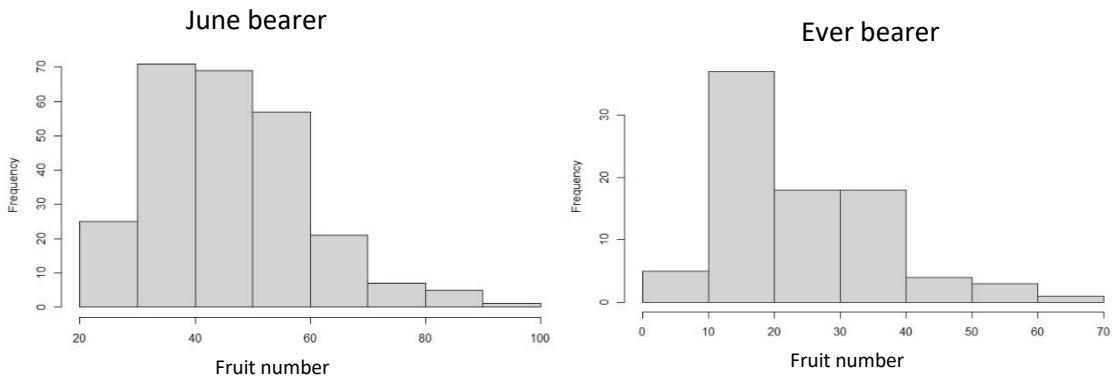

Figure 2. Frequency distribution of Best Linear Unbiased Estimates (BLUES) for Strawberry fruit number, A, June bearer and B, everbearer. x axis represents the fruit number, y axis represents the frequency of plant numbers with each given fruit number.

Table 1. Significant Single Nucleotide Polymorphism (SNP) associated with fruit number identified through a GWAS analysis. Analysis comprised of June bearers and everbearers. Quantitative Trait Nucleotide (QTN) name, linkage group, position and closest SNP and Gene description.

QTN	Linkage group	Position (Mb)	Closest SNP	Gene description
Fafl1A	1A	9.3	AX-89841395	Transcription factor SPATULA
Fafl3B	3B	34.2	AX-89882039	F-Box protein/kelch-repeat protein
Fafl3C	3C	21.8	AX-89879350	Heterogeneous nuclear ribonucleoprotein H (hnRNP)
Fafl5B	5B	20.3	AX-89792975	TOC75-3, Chloroplastic
Fafl5D	5D	17.9	AX-89849036	Squamosa promoter-binding-like protein 14
Fafl7A	7A	22.0	AX-89801912	Scarecrow-like protein 8
Fafl7B	7B	15.0	AX-89846535	Dicer homolog 1

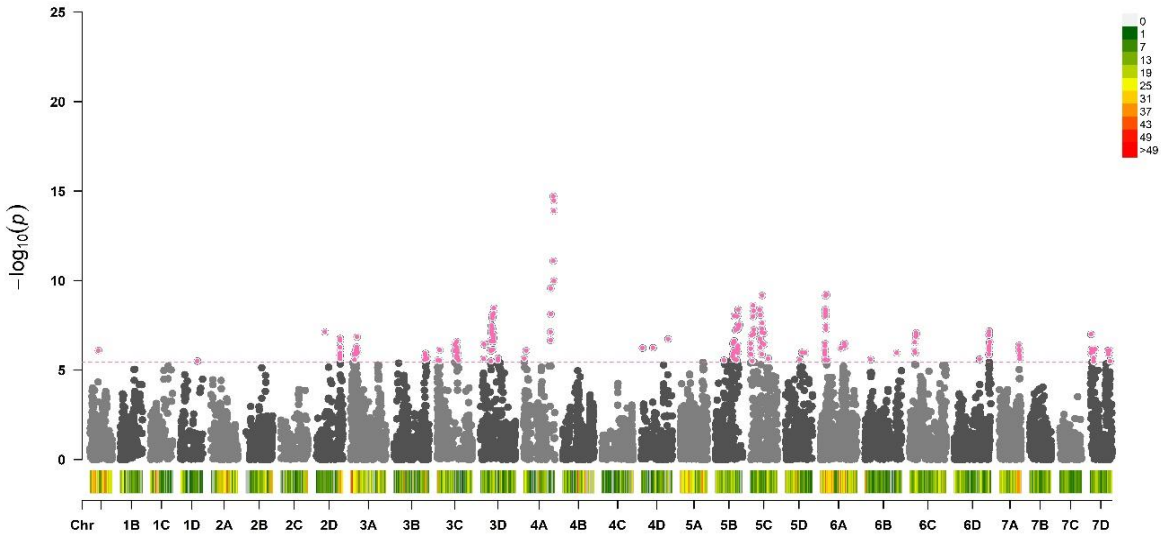

Figure 3. Manhattan plot of single nucleotide polymorphisms (SNPs) association with fruit number in octoploid strawberry after GWAS analysis across 328 accessions. The consensus octoploid map was used to scale marker positions along the 28 chromosomes. Chromosomes are denoted 1-7, with A-D representing octoploid sub genomes (van Dijk 2014). Grey points represent non-significant SNPs, pink points above the threshold line represent significant SNPs.

A GWAS analysis was performed to determine key flowering/fruit number qualitive trait nucleotides (QTN), across all accessions. Several significant QTN were identified for fruit number (Figure 3). Focal SNPs identified 18 genes that were associated with fruit number; the six most significant QTNs were positioned inside candidate fruit number genes (FaFl: 1A, 3B, 3C, 5B, 5D and 7A), with one neighboring (7B). The SNP on chromosome 4A identified all three major loci (PFRU) know to be associated with the everbearing flowering trait [14].

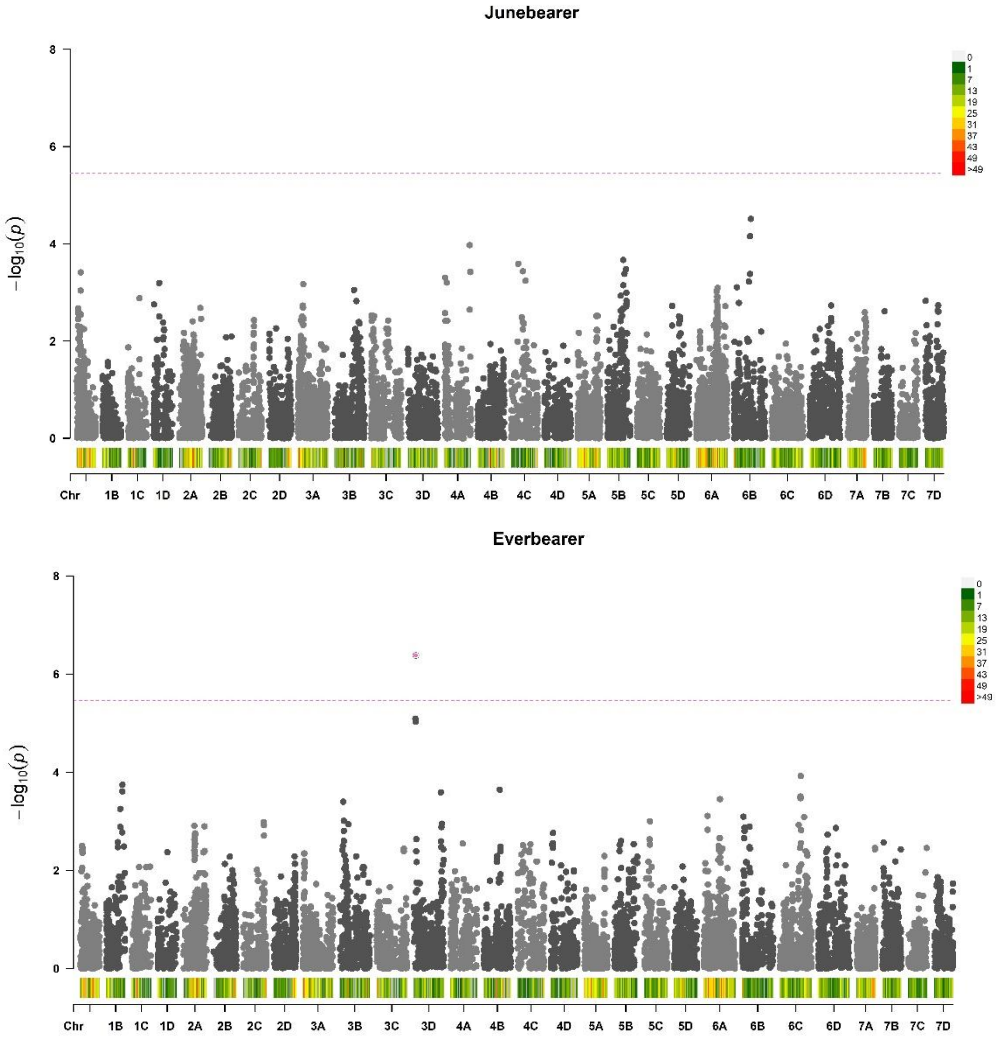

Figure 4. Manhattan plot of single nucleotide polymorphisms (SNPs) associated with fruit number in June bearer and everbearer octoploid strawberry. 4A Manhattan plot for June bearers, 4B Manhattan plot for everbearers. The consensus map was used to scale marker positions along the 28 chromosomes of octoploid strawberry. Chromosomes are denoted 1-7, with A-D representing octoploid sub genomes (van Dijk 2014). Grey points represent non-significant SNPs, pink points above the threshold line represent significant SNPs.

The different flowering habits present in the population of strawberry were seen to confound the results obtained. Everbearers produce fewer strawberries in a given period and thus a combined analysis led to the identification of QTN associated with flowering habit. In order to identify markers that were associated with fruit number only, a separate analysis was performed for June bearers and everbearers. Analysis showed no significant SNPs were associated with fruit number for the June bearers (Figure 4). However, several markers just below the threshold in the June bearer plot were identified as significant in the combined dataset (e.g., 5B, 7A). In contrast, the everbearers did yield a significant SNP inside a gene on chromosome 3D (Figure 4) (Table. 2). This significant gene was listed as an

uncharacterised gene in the *F. vesca* gene models. A GDR search was used to identify the encoded protein as putative nucleoporin protein Ndc1-Nup with a 93.6 % identity to a homologue in *Rosa Chinensis* and 67.4 % in *Prunus armeniaca*. Ndc1 is nucleoporin protein involved with the Nuclear Pore Complex, a highly conserved family.

Table 2. Significant Single Nucleotide Polymorphism (SNP) associated with fruit number identified through a GWAS analysis with everbearers only. Quantitative Trait Nucleotide (QTN) name, linkage group, position and closest SNP. Gene description of gene located on the SNP.

QTN	Linkage group	Position	Closest SNP	Gene description
Fafl3D	3D	28.5	AX-89882377	Putative nucleoporin protein Ndc1-Nup

Genomic Selection

Genomic selection analysis for fruit number indicated a predictive accuracy of 0.23 and predictive ability of 0.15 for all cultivars. Individual genomic selection analyses led to a predictive accuracy of 0.032 and predictive ability of 0.019 for June bearers and predictive accuracy 0.14 and predictive ability 0.08 for everbearers. These values suggest there is limited potential of increasing fruit number through genomic selection in the study population.

Discussion

The aim of this study was to identify genetic components associated with high fruit number. GWAS analyses were conducted on June bearers and everbearers separately as well as on all genotypes combined. In total, 328 strawberry cultivars were analysed in a GWAS, with 244 June bearers and 84 ever bearers. The QTN present on chromosome 4A, 5C and 6A identified in the combined analysis were previously characterized as being associated with the everbearing trait and thus were discounted as true fruit number QTN [14]. For the remaining chromosomes, four significant QTN were identified that exceeded the significance threshold and did not correspond to everbearing QTN (Table 1). The most significant QTN was located on chromosome 1A, inside the *Fafl1A* QTN, identified as transcription factor *SPATULA* (*SPT*) involved with floral organogenesis. *SPT* is associated with cell proliferation, germination, flower tissue growth and fruit development [58], [59]. The *SPT* gene is expressed in all flower organs and leaves, and expression varies at different development stages during fruit development and maturation [58]. During the development of fruit, *SPT* has also been associated with promoting growth and seed dormancy [60]. One of the main roles of *SPT* is involvement in the differentiation of specialized tissues involved in flower formation. Such specialized tissue formation occurs within the carpel development alongside the development of marginal tissue such as the stigma, ovules, septum and apical region. For development of the gynoecia (carpels), *SPT* is involved in the auxin signaling pathway [60]. To elucidate *SPT* involvement in gynoecium development, knockout studies have shown that *SPT*, in tandem with *AGAMOUS*, is essential for achieving full maturation of the gynoecium [59]. Another study involved with knockout mutation of *SPT* and *ALCATRAZ* resulted in a reduction in the number of flowers/petal size and lignification localization in the pericarp [58].

The QTN *Fafl3C* was situated on chromosome 3C inside the *Syncrip* gene, a heterogenous nuclear ribonucleoprotein H (HNP) which is part of the RNA Binding Protein (RBP) family. HNP is involved in plant growth and the regulation of flower gene expression [61]. HNP also plays an important role for successful floral transition, switching from a vegetative state to reproductive growth [62]. On chromosome 5B, *Fafl5B* was associated with the Translocon outer membrane complex (*TOC75-3)* gene, which is involved in embryonic morphogenesis and is essential for successful embryonic growth reaching maturity [63]. Genes in the *TOC* family are involved in the timing of expression and regulation of the circadian clock [24]. *Fafl7A* was associated with the gene *SCL8* a transcription factor Scarecrow protein, which is involved with plant development, volatile terpenes biosynthesis, floral scent and is implicated in seed growth [64]. Furthermore, QTNs *Fafl3B* F box/Kelch repeat and *Fafl5D* SQUAMOSA PROMOTOR BINDING-LIKE are recognized as floral regulators although additional research is warranted to comprehensively understand their roles [65], [66]. Additionally, Fafl5B,

identified as Dicer homologue 1 operates with Dicer-like3 to promote flowering by repressing the FLOWERING LOCUS C (FLC) [67]. The putative floral regulators F-box/Kelch, SQUAMOSA have been cloned though further analysis is required to achieve their full function. For instance SQUAMOSA has been found to have diverse pattern in tissue, whereby an homologue was found to delay flowering time in Alfalfa but involved with *F. vesca* fruit development [66], [68]. Overall, of the QTN associated genes identified, *SPT* is a novel candidate worthy of future investigation.

In the everbearer, the SNP identified in the analysis was on chromosome 3D, present in a *Nuclear porin* gene (*NUP*), which makes up part of the Nuclear Pore Complex (NPC). Across different NPC subcomplexes, there are over 30 known kinds of *NUPs*, all with distinct cellular roles [69]. Known roles include transport of biomolecules between the nucleus and cytoplasm, as well as possible roles controlling gene activation. Some NUPs have been found to be involved with regulating flowering time (*NUP96, NUP160, HOS1*), mediating flower production both in June bearers and everbearers (*NUA*) and pollen and ovule development (*NUP1, NUP88*) [69]. Since this protein has yet to be fully characterized in *Fragaria*, understanding its underlying function may lead to the discovery of a gene associated with flower/fruit number. This in turn would contribute to potential yield improvements such as fruit size or stress resistance.

As fruit number is a highly valued trait, using genomic selection to improve this trait would provide a substantial advantage for crop yield production. To further assess the feasibility of using genomic selection for the trait of fruit number, predictive accuracy was calculated to identify the potential power of effectiveness. The combined dataset of June bearers and everbearers showed a broad sense heritability score for fruit number of 66%, suggesting a strong genetic component controlling the observed variation. However, genomic prediction was associated with a predictive ability for fruit number at 15 %. Individual analysis revealed a low predictive ability for June bearers and everbearers at 1.9% and 0.8% respectively. These low values suggest that fruit numbers may not be a suitable candidate for genomic selection.

In this study, cultivars ‘Alice’, ‘Fenella’ and ‘Perfection’ were among the June bearers that exhibited the highest fruit number, while ‘White Carolina’, ‘Emily’ and ‘Vibrant’ were among the least. Among the everbearers, ‘Bolero’, ‘Calypso’ and ‘Selva’ displayed high fruit numbers, whereas ‘Albion’ and ‘Buddy’ were amongst the lowest. The high fruit number in ‘Bolero’ and ‘Selva’ was expected as these cultivars are known for their high flowering/fruit production [13].

Conclusion

The integrated analysis across all genotypes identified seven QTN, which could be linked to putative genes likely to have a function related to flower and fruit number. This investigation not only identified novel candidates but also substantiated the involvement of previously recognized genes considered putative floral regulators. Notably, the identification of *Fafl1A* identified within the SPT gene, which is associated with flower and fruit development, provides a potential candidate for flower/fruit number and should be investigated further in future studies. Interestingly, when segregating June bearers and everbearers for analysis, only one significant QTN emerged within the everbearers. The success of the GWAS in identifying multiple QTN related with flowering demonstrates its potential efficiency, especially in identifying low-effect traits influenced by environmental conditions [70]. It would also be worth performing these studies for consecutive years to account for potential epistatic effects influencing flower/fruit number and provide a more robust insight into the genetic control of this trait. The investigation also employed genomic selection values exhibited a diminished predictive capacity associated with fruit number. This may be attributed to the nature of the genetic components controlling the trait, or the limited predictive power associated with the assessment of a relatively small population.

In this study the QTN associated with genes identified - *SPT*, *HPN* and *NUP* emerge as promising candidates for further genomic validation by overexpression analysis to elucidate their roles in flower and fruit number. These findings provide a foundation for future GWAS investigations to ascertain complex molecular mechanisms underlying the regulation of flower/fruit number in Fragaria species.

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Chapter 6 Discussion

6.1 Investigation into Strawberry powdery mildew and fruit number

This thesis presents a robust investigation into strawberry powdery mildew resistance and fruit number for application in the advancement of strawberry breeding programmes. Previous studies have employed linkage mapping to elucidate molecular markers associated with key traits. However, powdery mildew resistance and fruit number traits still have not discerned definitive causal genes. In order to achieve this goal a GWAS and RNA sequencing experiment were undertaken to elucidate genetic markers associated with these traits. A GWAS is a powerful approach to identifying allelic variants associated with a trait, which retain relationship across a large population of related germplasm. In addition, a genetic analysis performed over multiple phenotyping events can account for any genetic and environmental interactions (GXE) to identify stable and potentially more durable candidate genes. The application of RNA sequencing experiment provided the platform to analyze tissue specific immunogenetic resistance responses for a novel tissue comparative analysis.

6.2 GWAS powdery mildew resistance genes

The development of advanced methods for investigating disease resistance traits has enabled the incorporation of molecular markers into breeding programmes. Pre-breeding research has underpinned the development of molecular markers. For example, quantitative trait loci (QTL) mapping has allowed the detection of genetic markers associated with disease resistance. Specifically in strawberry, QTL mapping has led to the successful identification of genetic loci associated with certain diseases [1], [2], [3]. However, the application of QTL mapping is restricted, as the transferability of alleles are typically limited to a small number of related lines [4]. Furthermore, although QTL mapping has proven highly effective for capturing variability controlled by a single gene resistance, exploitation of results when dealing with polygenic traits has shown limited promise as seen in efforts to capture resistance to PM in strawberry [5], [6], [7]. This is because QTL were associated with a small effect size and the majority of markers did not retain an association with alleles of interest across the wider germplasm.

To account for the complexity of the PM resistance trait a more in-depth analysis would be required to scan a diverse panel of individuals to identify genetic loci that retain linkage with causative alleles across breeding populations. In this thesis a genome wide association study (GWAS) was designed to identify genetic variations associated with complex traits, with hundreds of accessions used to determine allele variants associated with desired traits. The only published GWAS, conducted by Cockerton *et al.* (2018), found a single QTN associated with disease resistance to strawberry PM. However, in this study only 74 accessions were

used and so there was limited power to detect genetic loci present within the population [5]. In order to achieve high enough statistical power for a GWAS analysis, it is recommend to use over 300 individuals that are to detect alleles that are associated with the trait of interest but present at a low frequency within the population [5]. In order to obtain greater statistical power through collecting a comprehensive dataset, it was deemed pragmatic to conduct a large scale GWAS.

The results in chapter 3 presented, a GWAS was conducted, using 331 different cultivars with five replicates, totaling 1655 experimental individuals. The results of the GWAS yielded multiple genetic loci associated with PM resistance in foliage, with several QTN displaying a high effect size of over 50%. Furthermore, six stable QTN were identified across both years of assessment, in particular *FaRPa7Dab* was associated with a 61 % effect size. These QTN can be incorporated into a breeding program as molecular markers to assist breeders to produce durable resistance against PM. The capture of polygenic resistance may provide a more durable source of disease resistance [8]. Indeed, monogenic resistance has frequently been observed to break down in the field [9], [10]. An example of resistance breakdown in strawberry was observed in the cultivar 'Korona'. 'Korona' was introduced as a PM variety, however, a few years after introduction breakdown of this resistance was observed and 'Korona' is now known to be highly susceptible to PM, which was also noted in this study [11]. The hypothesis was generated that this breakdown was caused by mutations in the PM overcoming the host resistance [11]. A more durable cultivar could be obtained by incorporating several alleles into a single cultivar to achieve polygenic resistance. Polygenic resistance would require PM to accumulate multiple resistance breaking mutations to override the resistance [8].

A transient QTN identified in 2022 *FaRPa6Db*, corresponded with a marker previously found in a QTL mapping study by Cockerton *et al.* (2018) [5]. Although this marker was not found in the preliminary GWAS presented in the same study, it was found in the GWAS detailed here, these results illustrate the importance of using a large pool of accession in a GWAS study to identify all genetic resistance present within a population. In this study twenty-six novel transient QTN were identified; the high number of QTN identified and the transient nature of the QTN emphasizes the complex mechanism involved in mediating resistance to PM. Previous linkage mapping also observed transient loci, over different phenotyping events and through different locations [5], [6], [7], [12]. Further studies into the transient QTN would be beneficial to understand the impact that GxE interactions and/or pathogen race type have on disease response. One hypothesis that may explain the observation of transient QTN is the potential that the plants immunity shifts in response to temperature. These previous studies have shown that different temperatures can lead to a switch between either pattern triggered

immunity (PTI) or effector-triggered immunity (ETI) response. As such, where seasonal temperatures differ annually this may trigger a different immune response to PM [13]. This would account for the transient behaviour in this study, as weather patterns were extremely high in 2022, something that was reflected in the immunity shift in response. For future breeding this is something to take into consideration with respect to global weather changes.

In addition, tissue specific resistance responses to PM were investigated, through identifying QTN in fruit and foliage and subsequent comparison of resistance responses. In the GWAS analysis conducted on fruit affected by PM, there were no significant QTN identified; we hypothesis that the discrepancy between the number of fruit and foliage QTN may be due to a tissue specific disease response. As such, we hypothesise that resistance in fruit is facilitated by a different mechanism of defence. Alternatively, it is possible that the low level of susceptibility seen in the strawberry fruit (Figure 1) meant that there was insufficient phenotypic variation present in the population to allow resistance QTN to be detected. It is therefore clear that fruit resistance requires further investigations in a population that contains a greater distribution of disease scores before the underlying genetic mechanism of resistance can be elucidated.

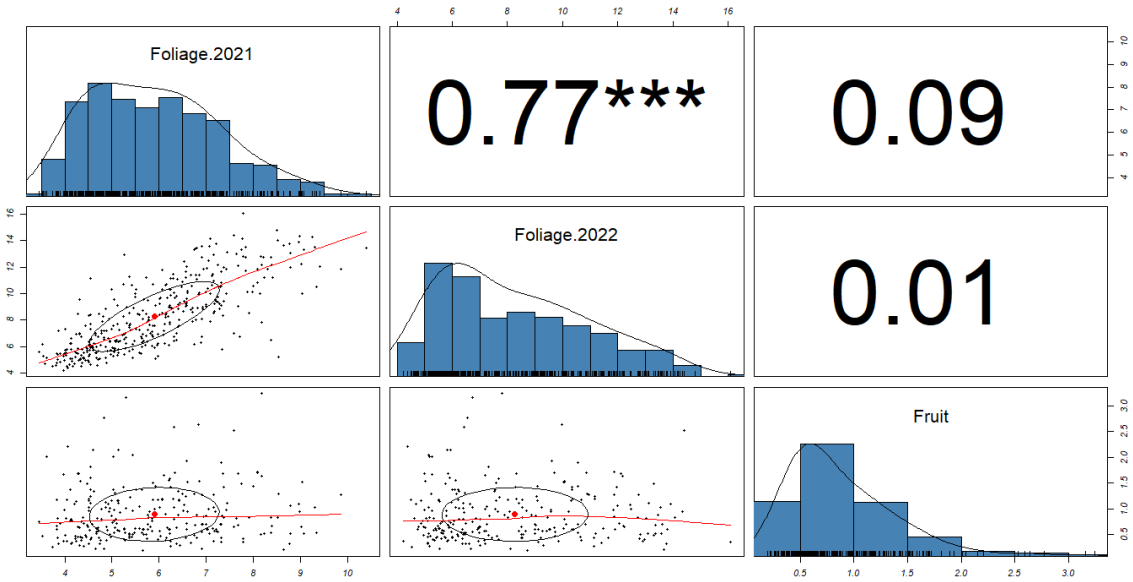

Figure 1 Spearman correlation matrix for powdery mildew phenotype data for foliage phenotypes for 2021 and 2022 and fruit scores 2022. Genotype correlation, significance (*p*) values are denoted by red stars: * < 0.05, ** <0.01, ***<0.001, numbers are Spearman correlation coefficients (r values).

6.3 Differentially expressed resistance genes

RNA sequencing is a valuable tool that allows a deeper insight into the genetic information controlling the functional characteristics of desired traits. Transcriptome analysis can provide a list of differentially expressed genes (DEGs) associated with a trait. The application of this method has allowed the identification of DEGs that are expressed in tissue exposed to the disease. In-house observations have been made that differential resistance levels exist in tissues within the same cultivar; showing that fruit can be susceptible in contrast to resistant foliage or vice versa [unpublished]. Tissue specific responses to pathogens have also been reported in different cultivars for example the fruit of *F. vesca* elicits a defence response to *Botrytis cinerea* and 'Apollo' petiole specific resistance to *Colletotrichum fragariae* via thickening of the cell wall [14], [15], [16]. Conversely, the cultivar 'Alba' is more susceptible to *Colletotrichum acutatum* when the fruit matures [17]. These specific tissue responses reveal the need to understand the PM response on different tissue and a potential for identifying significant candidate genes. However, to date no studies have been conducted investigating different tissue types to elucidate a potential universal response. Chapter 4 employed a novel approach by analyzing different tissue types with the aim of identifying genes related to all tissue types in response to disease resistance. The approach undertaken in this study also allowed the identification of essential regulatory pathways and mechanisms involved in PM resistance. The focus of the study was across three types of resistant 1. Cultivar resistance, 2. Tissue resistance and 3. Ontogenic resistance (Chapter 4). Of all the differentially expressed genes identified in response to resistance, only one universal gene was detected in all resistance types – CCR4 associated factor 1 (*CAF1-11*). This gene is normally expressed at low levels inside uninfected foliage and flower tissue. The results in chapter 4 show that CAF1-11 is highly expressed in all three different resistance responses to PM. This evidence is supported by other crop studies, whereby *CAF1-11* has also been shown to play a role in *Citrus sinensis* resistance to citrus canker and *Capsicum annuum* resistance to *Phytophthora infestans* [18], [19]. These findings, when taken with the association across three resistance types indicate that overexpressing *CAF1-11* in strawberry may be a mechanism for achieving comprehensive resistance to PM.

The results generated from the different resistances for the three resistance types studied found that many of the DEGs were unique to each PM resistance response such as transcription factors DREB and LUX. However, there were some correlations with other PM studies from Jambagi and Dunwell (2015) in *F. vesca*, Tian *et al.* (2019) in *Malus domestica* and Adhikary *et al.* (2022) in *Cannabis sativa L* [20], [21], [22]. All three studies identified transcription factors (TF) such as Myelocytomatosis (MYC2), WRKY and Ethylene response factors (ERFs) that were associated with PM infection. Interestingly. Tian ***et al.'s*** (2019) study

in apple, also identified the importance of cytochrome P450, which was also identified in this study, suggesting the overall importance cytochrome's in relation to pathogen defense [21]. However, the results reported here suggest that the P450 response was ontogenic specific and activation occurs at a later developmental stage and/or after prolonged exposure to the PM infection. Another important family of transcription factor genes that has been reported in recent studies, encoded ERFs. In this study several such genes were identified, suggesting that they play a strong role in PM resistance. The comparison of tissue specificity revealed the differences in the resistance responses (cultivar, tissue and ontogenic), and it was clear that that there were many unique elements associated with each of the immunogenetic resistance responses. For a full comparison set, future studies should include fruit with documented resistance; although the samples were obtained for this experiment, levels of 'Hapil' resistance were not sufficiently documented to be included in the analysis. Additionally, comparison of the infection at different fruit development stages would allow the study of fruit ontogenic resistance. This data would provide a more complete outline of the complex components underlying the resistance response to PM.

Another interesting observation was that ontogenic resistance comprised of 10 times more DEGs. Furthermore, ontogenic resistance was the only parameter to identify disease resistance genes (R genes) such as *SOBIR1* and *DSC1*. This would provide an explanation to the ontogenic resistance observed in previous studies [23], [24], [25]. A similar change in ontogenic defence has also been noted in anti-herbivore defence, whereby the ontogenic resistance may involve the host switching defence strategies or changes in metabolism to balance the fitness over benefit cost [26]. However, this switching of defence strategies in herbivore attacks is associated with the plant switching from resistant to tolerance mechanisms of defence, whereas in this study the trend appears to be reversed, with tolerance defense switching to resistance [27]. Conversely, strawberry fruit resistance has observed that immature fruit blocks growth with upregulation of pathogen resistance (PR) proteins in contrast to mature fruit, against pathogen *B. cinerea* [28]. This shows the importance of investigating different tissue responses at different time points in plant development, to discover and understand the different defence mechanisms exerted by the host. After the comparison of the transcriptome analysis and the GWAS, there was one gene that was identified in both experiments, this was the transcription factor *R2R3 MYB*. The *R2R3 MYB* gene is known to be involved with SA, ABA and JA ET pathways regulating plant defence [29], [30].

6.4 Flower/Fruit number

A major factor in crop breeding is yield improvement, an important component of strawberry yield is flower/fruit number. The identification of genes associated with high flower/fruit number would be a valuable resource for the industry as consumer demands continue to rise. In this thesis a GWAS study was conducted on 328 octoploid cultivars to identify genetic regions associated with flowering/fruit number (Chapter 5). The results identified six QTN, all of which were found to be associated with flowering, for instance, *SPATULAR (SPT*) which has been shown to be involved with flower tissue growth, development, and has a possible influence on flower number [31].

As the everbearer and June bearer have different developmental patterns, flowering habit obscured the ability to discern flower/fruit number QTN in certain chromosomes. To address this the data was separated by flowering habit to enable individual assessments. The June bearer analysis did not reveal any significant QTN, whereas the everbearer analysis did identify one putative QTN that was inside a potential *Nuclear porin* gene (*NUP*), though characterization is required to determine a definitive function. However, other NUPs have been reported to be involved with mediating flower production and ovule development [32], [33]. It was believed the analysis of the June bearer and everbearer results separately would uncover different flowering mechanisms. However, separation of individuals by flowering habit led to a reduced number of individuals used in each analysis, thereby reducing the power to detect genetic regions associated with the trait of interest. Future work should comprise of additional cultivars for June and everbearers, as well as be performed over more phenotyping events to generate a more comprehensive dataset. However, the putative fruit number genes identified do represent potential candidates for use in breeding. Further studies should validate these marker associations and gene function. Further work through gene editing could determine whether the genes reported here in this study are involved in contributing to increased flower/fruit number and thus improve yield [34], [35].

6.5 Comparison of RNA sequencing and GWAS

In this study two different methods (GWAS and RNA sequencing) were utilized to identify genetic regions associated with PM resistance. GWAS and RNA sequencing are two discrete approaches that can be used to examine the entire genome for genetic information associated with PM infection. A GWAS is designed to identify regions of the genome that are associated with the trait of interest [36], whereas an RNA sequencing experiment can be used to identify up and down regulated genes and can be used to quantify differential aspects involved in the molecular resistance mechanisms, including related biological pathways involved in disease response [37]. The time required to conduct the GWAS was three years, to propagate plants

and then accumulate a robust dataset for precise identification of QTN. In contrast, the RNA could be collected in one year and was designed to answer more complex questions – i.e. what are the factors involved in cultivar resistance, tissue resistance and ontogenic resistance? However, the RNA seq could only be applied to two cultivars, whereas the GWAS could assess a full array of accessions. The application of both the RNA seq and GWAS provided novel putative genes for potential markers, as well as a deeper understanding of resistance types that can provide valuable knowledge about the plant-pathogen interaction.

Overall, findings in this study showed that RNA seq and GWAS are complementary approaches that can be used in conjunction to gain a comprehensive understanding of the genetic basis of disease resistance. RNA seq provides functional insights into gene expression while a GWAS identifies genetic allelic variants associated with traits.

6.6 Genomic selection

Genomic selection (GS) is a breeding strategy that utilizes information from all genetic markers from across the entire genome to predict the genetic merit of an individual for a specific trait [38]. GS is a breeding approach that leverages genetic information to assist accurate and efficient selection decisions for breeders, leading to faster and more accurate genetic gain in the targeted traits. GS provides the ability to select desirable plants at an early timepoint, predicting the individual's genetic potential at an early stage before the trait is expressed, this reduces the need for multiple generations of phenotypic selections [39], [40]. GS is particularly effective for improving traits controlled by polygenic traits as well as for capturing dominant and epistasis effects. In this study the GS was conducted to determine whether PM (Chapter 3) and flower/fruit number (Chapter 5) were good candidate traits for GS. The predictive accuracy scores associated with PM foliage resistance indicated a high potential for successful implementation of genomic selection in the study population. In contrast, the predictive accuracy was very low for fruit PM resistance and for fruit number (including separate analysis with June and everbearers), indicating that these traits are not suitable for genomic selection breeding within this population. Therefore, hypothesizing that fruit traits have very complex genetic mechanisms and may involve complex epistatic interactions, especially in regard to fruit number.

6.7 Mildew Loci O (MLO)

Over recent years, the MLO susceptibility genes have gained prominent interest as a potential target for generating resistance to PM. The first MLO gene identified was discovered as a source of resistance against PM, where a natural mutation of an gene was described and found to enhance resistance considerably [41]. Since then, many MLO genes have been

identified in a variety of crops, with 68 found in *Fragaria* × *ananassa* [42]. In barley, the mutation of only one MLO gene was found to be sufficient to provide durable resistance. However investigations into other plants have found the level of resistance endowed by the MLO genes to vary, for example in Arabidopsis, whereby three different MLO genes need to be knocked out in order to obtain full resistance [43]. In chapter 3, the GWAS identified the *FaMLO16* gene as underlying a transient QTN, which also corresponded to the MLO identified by both Jambagi and Dunwell (2017) and Pessina *et al.* (2016) [44], [45]. However, the RNA sequencing analysis in chapter 4, found four different MLO associated with PM across the different resistance types investigated. The RNA sequencing analysis identified DEGs for *FaMLO4*, *FaMLO3*, *FaMLO6* and *FaMLO14*. Interestingly when investigating the gene interactions, functional connections between the MLO genes and other genes that are involved were identified such as tetraspanin-2 (*TET2*), that have not been previously established. The most noteworthy MLO identified was *FaMLO4*, that has a 98% identity to a previously reported *MdMLO19* which has been successfully knocked out in apple by Pessina *et al.* (2016). Knocking out *MdMLO19* led to strong PM resistance without any pleiotropic fitness cost associated with the knockout. This evidence suggests that *FaMLO4* is a prime candidate for future validation investigations with gene editing in strawberry.

6.8 Summary

In this thesis, the aim was to determine genetic markers associated with PM resistance and fruit number in strawberry. These results could provide significant advancement for the strawberry breeders' power to provide elite cultivars benefiting the industry. Here, multiple candidate genes and molecular markers were identified through conducting a genome wide association study (GWAS) and RNA sequencing.

The genetic markers to determining resistance associated with PM was achieved by conducting a GWAS over the course of two years (Chapter 3). The major outcome of the GWAS was the identification of six stable QTN identified in both years. The identification of these QTN provides stability with genetic interactions and several potential candidates for future stacking to produce a robust resistant cultivar. These QTN, associated with resistance genes, represent promising genetic markers for PM to support future elite lines, in particular, *FaRPa7Dab,* identified with an effect size of 61%. However, resistance in fruit identified no significant QTN, leading to the hypothesis that fruit resistance operates through a different mechanism and warrants further investigation. The GS analysis conducted for PM resistance produced positive results, indicating that PM resistance in foliage is a good candidate for GS and therefore could be utilized by the breeders to select for more resistance cultivars.

The RNA sequencing approach in this thesis involved a novel method to identify tissue specificity (Chapter 4). This was achieved by analysing different tissue comparisons and comparing the overlapping genes involved with resistance. The unique method used in this study, of comparing the resistance types to narrow down the immunogenic response, facilitated the identification of a reoccurring universal resistance gene (*CAF1-11*) associated with PM. Evidence of the *CAF1-11* involvement with resistance to other pathogens presents a highly promising candidate for a future genetic marker.

The GWAS preliminary study was performed to provide validation for using this robust method to identify key components in flower/fruit number genes (Chapter 5). The results from the flower and fruit number GWAS, resulted in identifying seven putative genes for potential genetic markers. This highlights the potential of the GWAS for identifying novel key components for this trait and should be repeated over two or more years to gain the full benefit of the data.

The candidate genes detailed in this thesis provides a foundation for future development of genetic markers. Future exploitation of the genes identified in this thesis would require validation of the gene functions. To explore their potential as markers these genes can be validated for function through expression studies or applications of techniques like CRISPR Cas to knock out genes. The future focus from this project will be to find a pathway, whereby these validation methods can be applied to the candidate genes reported here, for implementation into strawberry breeding programmes to enable the practical application in the near future.

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Data availability

The data in this thesis is available upon request to the author Samantha C Lynn via ResearchGate