Investigating resistance mechanisms to *Phytophthora cactorum* in strawberry and apple

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School of Agriculture and Policy Development

Matteo Luberti

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

Matteo Luberti

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Abstract

Apple (*Malus* x *domestica; Md*) and strawberry (*Fragaria* x *ananassa; Fxa*) are two of the most culturally and economically important cultivated fruit varieties in the world. *Phytophthora cactorum* (*Pc*) isolates cause substantial damages in both plants' growing systems. Its ability to spread infection through waterlogged orchard soils as well as through the irrigation systems used in tabletop strawberry cultivation, coupled with the lack of available chemical management options, reinforce the need for resistant varieties. Despite the extended resistance breeding efforts in both hosts, and the recent mapping of resistance in *Fxa*, much remains to be elucidated of the mechanisms underlying plant resistance to this pathogen.

Resistance in an apple was mapped using a bi-parental population generated from the cross of two popular rootstock varieties ('M.27' and 'M.116'), revealing the presence of a large-effect quantitative trait locus (QTL) on chromosome 6 (*MdRPc1*). Moreover, a preliminary genome-wide association study (GWAS) performed on a panel of 99 apple accessions from the wider germplasm confirmed the presence of the *MdRPc1* locus, as well as two additional loci (*MdRPc2* and *MdRPc3*) on chromosomes 5 and 15. The transcriptional response to infection was studied in both hosts through the whole-transcriptome sequencing of root tissue samples of susceptible and resistant varieties from time course inoculation experiments. This allowed to identify pathways regulated upon *Pc* infection, as well as candidate resistance/susceptibility genes. Finally, the transcriptome analysis of *Pc* during apple infection revealed the regulation of a large effector array and highlighted candidate pathogenicity genes. Further, comparisons with the previously published transcriptome analysis of a *Pc* isolate during infection of strawberry has provided insights into factors determining host specificity. Taken together these findings help elucidate the mechanisms underlying host-*Pc* interactions and provide valuable data to be used in future resistance breeding efforts.

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Chapter 1: General Introduction

While the origins of the domestication of crop plant species can be traced back over 10,000 years ago (Vavilov, 1987), the domestication of fruit crop plants did not follow for several millennia. The first record of fruit crops came from the Chalcolithic Levant, where date-palm and olive trees had been domesticated around 6,000 years ago (Zohary and Hopf, 2000). During the Bronze Age, figs, grapes, and olives emerged as agriculturally important crops in the Levant and Greece. These subsequently spread throughout the Mediterranean basin. Signs of the domestication of apple, cherry, plum, and pear trees appeared much later, possibly due to the fact that propagation of these species relied on the sophisticated technique of grafting (Zohary and Hopf, 2000).

The first conscious and organised efforts to produce fruit bearing plants with improved horticultural traits started during the 19th century, when the mass selection of strawberry and pear was initiated (Janick, 2005). Thomas Andrew Knight was the initiator of the field of fruit-breeding. He employed inter-pollination of clones to improve existing fruit varieties, generating a number of improved cultivars from a variety of plant species (apple, pear, cherry, and strawberry amongst others; Janick, 2005).

1.1 The *Rosaceae* family

The *Rosaceae* family contains a large number of economically important edible fruit and ornamental plant species. Amongst the most important genera within this family are the *Amygdyloideae* (apricot, cherry, almond, and peach), the *Meloideae* (apple, loquat, and pear), and the *Rosoideae* (brambles, roses, and strawberry). A very conservative estimate reported in 2009, produced looking at market values in USD, put the combined economic value (at the farm gate) of the edible species within this family at around 45 billion USD (Hummer and Janick, 2009). Though beyond the scope of this study, a non-comprehensive estimate of the size of the *Rosaceae* family world-market, based on 2020 FAO estimates of production, puts their economic value at over 161 billion USD (FAOSTAT - https://www.fao.org/faostat -

accessed 21/04/2022). Below is Table 1 summarising the most notable species contained in the genera within this family and their uses (Hummer and Janick, 2009).

Table 1.1. List of economically important species of Rosaceae organised by subfamily (Hummer and Janick, 2009).

Subfamily	Genus	Species	Common name	Uses
Amygyloideae	*Prunus*	*armeniaca*	Apricot	Fresh and processed fruit
		avium	Sweet cherry	Fresh and processed fruit
		cerasus	Tart (sour) cherry	Fresh and processed fruit
		domestica	European plum	Fresh and processed fruit
		dulcis	Almond	Fresh and processed nut
		mume	Mume	Ornamental
		persica	Peach, nectarine	Fresh and processed fruit
		serotina	Black cherry	Timber species
Maloideae	*Amelanchier*	*alnifolia*	Saskatoon, serviceberry; shadbush	Landscape ornamental
	Aronia	*melanocarpa*	Black chokeberry	Processed fruit for juice, nutriceutical
	Chaenomales	*japonica*	Japanese quince	Landscape ornamental, processed fruit
	Cotoneaster	*spp.*	Cotoneaster	Landscape ornamental
	Crataegus	*spp.*	Hawthorn, thornapple	Landscape ornamental, craft uses for wood
	Cydonia	*oblonga*	European quince	Fresh and processed fruit, dwarfing rootstock for pear and loquat
	Eriobotrya	*mespilus*	Loquat	Fresh and processed fruit
	Malus	*×domestica (M. pumila)*	Apple	
		spp.	Crabapples	Landscape ornamentals
	Pyrus	*calleryana*	Callery pear	Landscape ornamental
		communis	European pear	Fresh and processed fruit
		serotina	Japanese pear (nashi)	Fresh fruit
		ussurienses	Chinese pear	Fresh fruit
	Mespilus	*germanica*	Medlar	Fresh fruit (bletted)
	Photinia	*spp.*	Photinia	Landscape ornamental
	Pyracantha	*spp.*	Firethorn	Landscape ornamental
	Sorbus	*spp.*	Mountain ah, rowan	Landscape ornamental
Rosoideae	*Fragaria*	*×ananassa*	Strawberry	Fresh and processed fruit
	Geum	*spp.*	Avens	Herbaceous perennial
	Kerria	*japonica*	Kerria	Landscape ornamental
	Potentilla	*spp.*	Cinquefoil	Landscape ornamental
	Rosa	*spp.*	Rose	Cut flowers, landscape ornamental, perfume oil, medicinal
	Rubus	*spp. and hybrids*	Blackberry, raspberry, hybrid berry	Fresh and processed fruit
Spiraeoideae	*Spirea*	*prunifolia*	Bridal wreath	Landscape ornamental
	Exochorda	*racemosa*	Exochorda	Landscape ornamental
	Physocarpus	*opulitolius*	Ninebark	Landscape ornamental

1.1.1 *Fragaria* x *ananassa*

The cultivated strawberry (*Fragaria* x *ananassa; Fxa*) is a perennial, outcrossing, herb-like species that can be asexually propagated using runners. It is reportedly sensitive to inbreeding, therefore breeding programs have been based on the intercrossing of elite parental lines (Hummer and Hancock, 2009). Strawberry species have a vast habitat range that spans from the tropics to the arctic. Despite no physiological limitations, 98% of strawberry cultivation happens in the northern hemisphere (Hummer and Hancock, 2009). Pests and disease are amongst the biggest constraints in strawberry production. Strawberry tarsonemid mite (*Tarsonemus pallidus*), strawberry blossom weevil (*Anthonomus rubi)* and strawberry aphid (*Chaetosiphon fragaefolii*) are amongst the most important pests of strawberry in northern Europe. In the same region the major diseases impacting cultivation are crown rot (*Phytophthora cactorum),* grey mould (*Botrytis cinerea*) and powdery mildew (*Podosphaera aphanis*) (Parikka and Tuovinen, 2014).

Strawberry cultivation has a long and interesting history. The *Fragaria vesca* species was already known at the time of the Romans, and *Fragaria silvestris* had been cultivated in Europe since the 14th century as both an ornamental plant and a fruit crop. *F. vesca* has been employed as a model plant for the study of strawberry and for the *Rosaceae* family due to the relative simplicity of its diploid genome, the short reproductive cycle and ease of propagation. Its genome (2n=2x=14, ~240 Mbp) was sequenced in 2011 (Shulaev *et al.*, 2011). The modern cultivated strawberry (*Fragaria* x *ananassa*) is an allo-octoploid (2n=8x=56) with an estimated genome size of 810 Mbp. It probably originated as an accidental hybrid of two wild species, generally dioecious, and native to the American continent: *Fragaria virginiana*, *Fragaria chiloensis* (Edger *et al*., 2019). The latter reached the French city of Brest after it was brought back to Europe by Amédee François Frézier. A French army officer and spy who collected several specimens of the plant in the Chilean town of Concepcíon (Darrow, 1966).
Most of the early breeding efforts were carried on by private breeders. It was not until the beginning of the 20th century that state-funded breeding programmes started to appear. Notable is the Scottish strawberry industry, which started in the 1870s and at its peak in 1908 took up 1,439 acres of land. In 1920, the first strawberry breeding program funded by the US Department of Agriculture was initiated. England would soon follow, with breeding efforts

being carried out at the Cambridge research station. This breeding programme yielded a number of varieties, which by 1962 comprised 80% of the commercial crop in the country. While the commercial production of strawberry in European countries such as France, Italy and Germany did develop at the beginning of the 20th century, these were initially reliant on British and American varieties (Darrow, 1966). It is only more recently that private breeding programs have started (Faedi *et al.*, 2000). Historically, these breeding efforts have focused on a number of traits. These include factors relevant to the consumers such as fruit size, flavour and colour, but also hardiness, disease resistance, ease of propagation and general adaptations to the growing region, which are of great interest to the producers (Faedi *et al.*, 2000).

1.1.2 Malus x domestica

The modern cultivated apple varieties are derived from *Malus* x *domestica* (*Md*), an autotetraploid species with estimated genome size of 740 Mbp and a chromosome number of 17 (Velasco *et al.,* 2010; Li *et al.,* 2016; Daccord *et al.,* 2017; Zhang *et al.,* 2019b). Apples are one of the most widely cultivated fruits in the world, with production extending to virtually all temperate and sub-tropical regions of the world . There are several pests and diseases, as well as environmental factors affecting commercial apple production. Apple fire blight (*Erwinia amylovara*) is one of the most important diseases of apple, with apple scab (*Venturia inaequalis*) and apple canker (*Neonectria ditissima*) all having a substantial impact on production (Harris, 1991; MacHardy, 1996; MacKenzie and Iskra, 2005; Khan *et al*., 2006; Gómez-Cortecero *et al.,* 2015). Root and crown rots caused by *Phytophthora* species are of particular interest to apple rootstock breeding, and a threat to all northern European apple cultivation regions.

The practice of clonally propagating apple scions with desirable traits by grafting them onto unrelated root systems goes back several millennia (Karp and Hu, 2018). The ancient Greeks and Romans systematically employed this technology in their apple orchards. The Greek philosopher Theophrastis reports that Alexander the Great was the first to introduce dwarfing root stock from the “Spring Apple” variety to Europe from Asia Minor. It is possible that some of these early dwarf varieties survived the fall of the Roman empire and became known in

17th century France as the Paradise apple (Fallahi *et al.*, 2002). This is when it was first recognised that rootstocks could be selected for their ability to impart favourable traits to the scion cultivar (Karp and Hu, 2018).

The first modern apple rootstock breeding programme was initiated at the East Malling Research Station in South-Eastern England in 1917. Their efforts mainly focused on dwarfing and early bearing traits. This resulted in the production of the "Malling" series of which 'M.9' is the most famous and most widely adopted member. The joint efforts of the John Innes Institute and the East Malling Research Station later resulted in the production of two woolly apple aphids (WWA) resistant series of apple rootstocks derived from the 'Northern Spy' variety: the Merton Immune and the Malling-Merton series. The apple rootstock breeding programme was revived at East Malling in 1968 with the primary goal of producing crown rot-resistant varieties (Janick, 2005).

In the past century, apple rootstock breeding programmes have been started all over the world. They focused on the selection and development of a number of different traits, based on the specific needs of different growing regions. Historically the principal goals of rootstock breeding programs have been tree size control, canopy architecture, early bearing, root morphology and nutrient absorption, propagation traits, and disease resistance (Jain, 1986).

1.2 The *Phytophthora* genus

The Oomycota are defined as a distinct class of eukaryotic, fungus-like microbes. Despite being morphologically very similar to fungi, they have some unique distinctions (Judelson and Blanco, 2005). The major component of the oomycete cell wall is cellulose, in contrast chitin is the major component of most fungi's cell wall. Oomycete mitochondria can be distinguished from fungal ones by their tubular cristae. Additionally, during their vegetative mycelial stage, oomycetes are diploid, while fungi generally form haploid thalli (Rietman, 2010). The genus *Phytophthora,* from the ancient Greek for 'plant destroyer', is comprised of several pathogenic oomycete species responsible for devastating damage to staple crops worldwide (Erwin and Ribeiro, 1996). The most notable of *Phytophthora* outbreaks is perhaps the Great Irish Famine which lasted from 1845 to 1849 and was caused by a strain of

Phytophthora infestans introduced to Europe from the Mexican peninsula (Rizzo *et al.*, 2005). Sudden oak death (*Phytophthora ramorum*) epidemics have also caused significant economic damage in the past few decades. First detected in California in the 1990s, this plant disease has now spread to Oregon and is estimated to have caused economic losses in the tens of millions of dollars (Rizzo *et al.*, 2005). There are over 150 formally named *Phytophthora* species with different lifestyles depending on the host. *P. ramorum* for example can propagate by aerial spread while *Phytophthora cactorum* (*Pc*) is soilborne. Initial morphology-

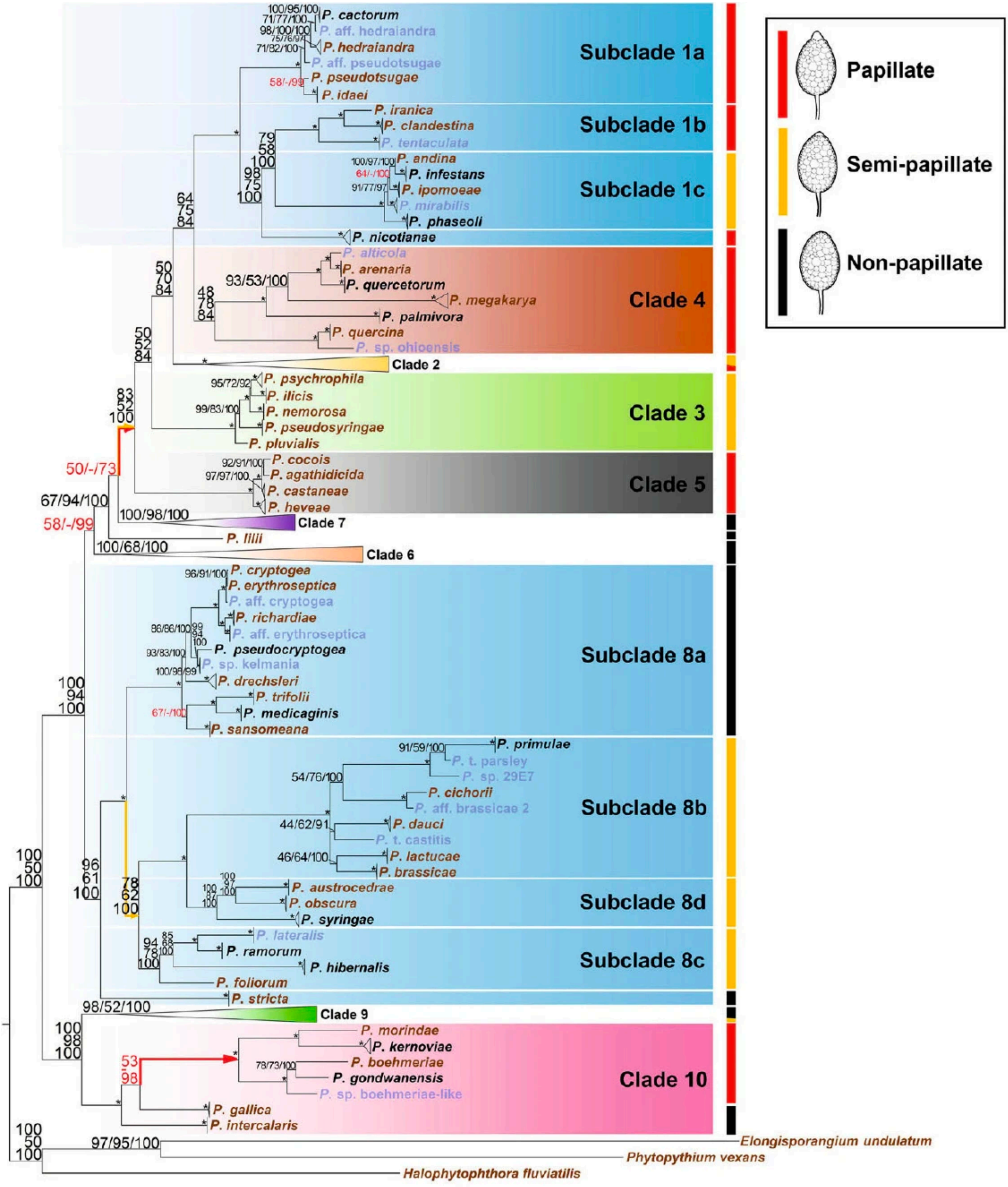


Figure 1.1. Phylogeny of Phytophthora species.

In each clade, papillate, semi- and non-papillate species are indicated in red, yellow and black respectively (Yang *et al.*, 2017).

morphological similarities (Waterhouse, 1963). With the advent of DNA sequence-similarity based phylogenies, the genus has been divided into 10 different clades (Figure 1.1). While some morphological and physiological traits are well conserved within clades (sporangial papillation, growth temperature), others such as sexual organ morphology show no correlation (Yang *et al*., 2017).

1.2.1 Phytophthora cactorum

Pc is a hemi-biotrophic oomycete with isolates able to infect a wide host range. *Pc* is homothallic and it can produce both sexual and asexual spores (Erwin and Ribeiro, 1996). Asexual zoospores are bi-flagellate, motile zoospores released by the sporangia in wet conditions and are able to swim chemotactically towards root exudates from a suitable host to initiate infection (Khew and Zentmyer, 1973). Sexual oospores are resting spores able to persist in the soil for several decades before environmental conditions become favourable to the pathogen again (Sneh and McIntosh, 1974; Maas, 1998). First named *Pernospora cactorum* by Lebert and Cohn (1870) for the cacti plants it was described from (Blackwell, 1943), *Pc* was soon recognised as a generalist pathogen able to infect hundreds of plant hosts (Erwin and Ribeiro, 1996). Isolates of *Pc* has been found to be pathogenic in a number of forest tree species, including both economically and ecologically important ones such as *Pinus sylvestris*, *Picea abies*, *Larix* x *eurolepis*, *Betula pendula*, *Quercus robur*, *Fagus sylvatica*, *Populus trichocarpa* and *Tilia cordata* (Vettraino *et al.,* 2008; Cleary *et al.,* 2017; Nowakowska *et al.,* 2020). As well as horticulturally important plant species such as *Fxa, Md, Panax ginseng, Prunus amygdalus,* species in the *Rhododendron* genus, *Prunus armeniaca* and *Pyrus communis* (Grove *et al.,* 1991; Hantula *et al.,* 2000; Bhat *et al.,* 2006; Rytkönen *et al.,* 2012). Despite their morphological similarities, *Pc* isolates show strong host specificity and are often unable to produce full virulence in other hosts (Darmono *et al.,* 1991; Hantula *et al.,* 2000; Nellist *et al.,* 2021). This, in addition to recent genomic data, suggests that *Pc* may henceforth have to be considered a species complex instead (Nellist *et al.,* 2021).

Pc's ability to persist in infected soils for a prolonged amount of time, as well as being able to spread through irrigation systems make it a continued threat to commercial fruit production. The rise in resistance and the increased restrictions on pesticide use being implemented worldwide mean there are less means than ever available to control *Pc.* While some bio-control agents have shown limited promise, the use of resistant cultivars remains the most cost effective and environmentally conscious way to address the threat posed by *Pc* (Lee *et al.,* 2015; Pánek *et al.,* 2021; Marin *et al.,* 2021; Nyoni *et al.,* 2021; Vettraino *et al.,* 2022).

1.3 Plant resistance mechanisms

Unlike animals, plants do not possess an adaptive immune system. Therefore, they have to rely on an innate suite of defence mechanisms in order to prevent and overcome pathogen infection. This is composed of both physical barriers as well as biochemical mechanisms (Jones and Dangl, 2006). Fungi and oomycetes can enter plant cells by directly employing structures called hyphae. Biotrophic and hemi-biotrophic classes of these filamentous eukaryotes produce haustoria, structures that allow them to interface with the plant cell plasma membrane to exchange nutrients and secrete effector molecules to suppress the host's immune response (Jones and Dangl, 2006). The first layer of plant defences consists of pattern recognition receptors (PRRs), a vast array of transmembrane proteins able to recognise pathogen-associated molecular patterns (PAMPs; Zipfel, 2008). Interactions between PPRs and PAMPs are highly specific. Plants that lack a particular PPR are unable to detect the associated pathogen, while plants that do possess them are able to detect PAMPs at sub-nanomolar concentrations (Boller and He, 2009). The great evolutionary pressure that results from the high interaction-specificity has been a key driver in the large expansion observed in families of PRR genes (Fritz-Laylin *et al.*, 2005; Wang G *et al.*, 2008; Lehti-Shiu *et al.*, 2009; Tör *et al.,* 2009). The best studied plant PRRs include receptor-like kinases (RLKs), which contain a single transmembrane domain, an extracellular domain such as a lysine motif (LysM), leucine rich repeats (LRRs) or lectin, and an intracellular kinase domain; and receptor-like proteins (RLPs), which lack the cytoplasmic kinase domain while retaining the transmembrane and extracellular LRR motifs (Dodds and Rathjen, 2010). Effector recognition in the cytoplasm is mediated by nucleotide-binding (NB) and oligomerization domain (NOD)-like receptor (NLRs) proteins containing two conserved domains, a NB domain and a C-terminal LRR domain (Jones and Dangl, 2006). This protein family can be roughly divided based on the N-terminal domain. CNLs (CC-NB-LRR) contain a coiled-coil (CC) structure, while TNLs (TIR-NB-LRR) contain an N-terminal Toll/interleukin-1 receptor (TIR) domain (Sukarta *et* *al.*, 2016). Mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs) act downstream of these receptors through reversible phosphorylation of proteins, including transcription factors (TFs; Meng and Zhang, 2013). Several TF families are known to modulate the plant immune response, including basic leucine zipper containing domain proteins (bZIP), amino-acid sequence WRKYGQK (WRKY), myelocytomatosis related proteins (MYC), myeloblastosis related proteins (MYB), APETALA2/ ETHYLENE-RESPONSIVE ELEMENT BINDING FACTORS (AP2/EREBP) and no apical meristem (NAM), Arabidopsis transcription activation factor (ATAF), and cup-shaped cotyledon (CUC), together referred to as NAC TFs (Alves *et al.,* 2014). WRKY TFs are among the largest plant TF families and have been associated with regulation of plant responses to both biotic and abiotic stresses (Wani *et al.,* 2021). They serve a complex role and have been extensively associated with both positive and negative regulation of plant immunity (Qiu *et al.,* 2007; Kim *et al.,* 2008; Mao *et al.,* 2011; Chujo *et al.,* 2014), including in plant-*Phytophthora* pathosystems (Naveed *et al.,* 2018; Cui *et al.,* 2019a; Cui *et al.,* 2019b; Cheng *et al.,* 2020). In compatible plant-microbe interactions, these TFs regulate the production of antimicrobial secondary metabolites and hormonal signalling pathways. Salicylic acid (SA), jasmonic acid (JA), ethylene (ET), auxin, abscisic acid (ABA), cytokinins (CKs), and brassinosteroids all play key roles in defence responses and have complex crosstalk relationships (Robert-Seilaniantz *et al.,* 2011).

1.4 Aims and Objectives

The project focused on exploring sources of resistance to *Pc* in two horticulturally important species (*Md* and *Fxa*). The mechanisms underlying resistance in both species were investigated at the transcriptional level in order to identify elements responsible for the variation in resistance observed across different cultivars. Transcriptional changes in the pathogen were also explored, comparing both differences between cultivars and differences between species in an effort to expand our understanding of *Pc* host specificity determinants.

Firstly, the available *Md* germplasm was tested for resistance/susceptibility to *Pc* in order to identify possible sources of resistance (Chapter 2). These were then mapped to three genomic loci using quantitative trait loci (QTL) mapping and a preliminary genome-wide association study (GWAS). A locus putatively associated with resistance, mapped in a segregating bi-parental population originated from a cross of two *Md* cultivars (‘M.27’ and ‘M.116’) particularly relevant to the apple rootstock breeding program was then further explored using KASP marker genotyping in an effort to identify a preliminary panel of markers for eventual deployment in the breeding pipeline (Chapter 3). The early transcriptional response to *Pc* infection in both the ‘M.27’ and ‘M.116’ cultivars was investigated through a time-course inoculation experiment, in an effort to identify elements underlying resistance/susceptibility (Chapter 4). Data from a similar experiment, in which the two *Fxa* cultivars 'Emily' and ‘Fenella’, that had been crossed to produce a segregating population used to map resistance to *Pc* in a previous study, was employed to explore the early transcriptional response to *Pc* in *Fxa* (Chapter 5). Lastly, whole-transcriptome analysis of *Pc* during infection of ‘M.27’ and ‘M.116’ was employed to explore the pathogen’s effector repertoire and to identify host specificity determinants (Chapter 6). Taken together, the results presented in this thesis help elucidate the resistance mechanisms employed by different plant hosts to combat *Pc* infection, as well as the pathogen infection strategies and host specificity factors.

Chapter 2: Response of apple (*Malus* x *domestica*) accessions to UK *Phytophthora cactorum* isolates in cut-shoot tests

Preface

This chapter was originally published in ISHS Acta Horticulturae 1307: XV EUCARPIA Symposium on Fruit Breeding and Genetics, in July 2021 (Luberti *et al.,* 2021). The study presents the results of a resistance screen of the most widely used apple rootstock varieties in UK breeding programs with the aim to assess the state of resistance/susceptibility of the germplasm as well as to identify potential new sources of resistance. This work allowed for the identification of the mapping population discussed in Chapter 3.

2.1 Abstract

Phytophthora cactorum (*Pc*) is a water-borne oomycete pathogen responsible for economically-significant losses in the commercial production of apple and strawberry. In cultivated apple (*Malus domestica*), *Pc* causes bark rots on the scion (collar rot) and rootstock (crown rot), as well as necrosis of the fine root system (root rot) and fruit rots. Reproducibly characterising plant genetic resistance in controlled environments can be difficult; most reports of inheritance in apple have looked at segregations following inoculation of young seedlings whilst cultivar performance is often confirmed in field plantings. This study aimed to test the usefulness of inoculating detached shoots to determine the response of apple accessions to two UK *Pc* isolates. Twenty-nine apple accessions were tested with the intention of determining the feasibility of employing this method to optimise large scale phenotyping of germplasm, breeding lines and mapping populations for UK material. Isolate P295 was markedly less virulent than the recently isolated R36/14. Variation in susceptibility was observed in apple and nine accessions were found to be very resistant to both isolates, with no lesion development recorded. These results highlight useful material for future resistance breeding to UK isolates.

2.2 Introduction

The oomycete genus *Phytophthora* comprises a number of pathogenic species responsible for substantial damage to crops worldwide. The extreme severity of *Phytophthora* outbreaks has generated great interest worldwide in finding sources of resistance. Improving the current understanding of resistance mechanisms to *Phytophthora* species will also be essential in order to generate more durable resistance.

Phytophthora cactorum (Pc) isolates can cause disease in over 160 plant hosts, including economically important horticultural crops such as the cultivated strawberry (*Fragaria* x *ananassa*) and apple (*Malus* x *domestica*; Erwin and Ribeiro, 1996). Management strategies have previously focused on chemical control and soil fumigation. As fungicide resistance increases and fumigation is being restricted by legislation, the identification of sources of resistance has become increasingly important. Resistance to *Pc* in strawberry is known to be polygenic (Denoyes-Rothan *et al.*, 2004; Shaw *et al*., 2006; Shaw *et al*., 2008). Recent work on resistance to *Pc* in strawberry at NIAB EMR has identified three major effect Quantitative Trait Loci (QTL) in a bi-parental cross and additional QTL from a genome-wide association study (Nellist *et al*., 2019). A 2017 study also identified a major resistance locus, *FaRPc2*, in which four single nucleotide polymorphism (SNP) haplotypes were found, suggesting the presence of multiple resistance alleles (Mangandi *et al*., 2017). Unlike in strawberry where resistance is known to be quantitative, little is known about resistance to *Pc* in apple. Experiments by Knight and Alston (1969) suggested the presence of a single major dominant resistance gene, *Pc*, in the 'Northern Spy' cultivar, indicating qualitative resistance could exist in apple. Several systematic investigations into resistance to *Pc* in apple germplasm using cut-shoot assays have been conducted showing separation of cultivars of known resistance (Jeffers *et al.,* 1981; Utkhede, 1986; Browne and Mircetich, 1993; Cassie and Khanizadeh 2006), but nothing has been reported in the last thirteen years. We assessed a range of apple accessions relevant to the rootstock breeding programme at NIAB EMR for resistance/susceptibility to two UK isolates of *Pc*.

2.3 Materials and Methods

2.3.1 Plant material

Dormant first year growth apple shoots were collected from each of the 29 apple accessions investigated in this study, in March 2019 from NIAB EMR's gene bank. The varieties 'Queen Cox' and 'M.116' were used as susceptible and resistant standards. They were cut to a length of 22 cm and surface-sterilised by immersing them in a 10 % bleach solution for 15 minutes and then rinsed three times with sterile distilled water. One centimeter was cut off from each end and then both ends were dipped in molten paraffin wax, to seal them.

2.3.2 Mycelium production

Two *Pc* isolates were used in this study, P295 was isolated in Offham (UK) in April 1984 and R36/14 was isolated at the NIAB EMR site (UK) in June 2014. The *Pc* isolates were revived from long term storage 14 days prior to inoculation. The isolates were grown on V8 agar at 20℃ in the dark, as described in Nellist *et al*. (2019). The isolates were re-subbed after seven days to ensure enough inoculum was produced.

2.3.3 Set-up and inoculation

A cork borer (4 mm diameter) was used to produce a wound in the middle section of each shoot and the outer bark was removed with a scalpel. Agar plugs of the same diameter containing the growing edge of *Pc* mycelium were placed mycelium-side down onto the wound to inoculate the shoots. Four independent replicate inoculations of one shoot of each accession were performed with each isolate. Mock inoculation of one shoot per accession was also performed, using sterile V8 agar plugs. Damp paper towels were placed at the bottom of each box (50cm x 90cm) and the shoots were arranged on raised racks made of rolled aluminium foil (Figure 2.1a). The boxes were sealed in clear plastic bags to maintain humidity and were placed in a controlled environment room, with a constant temperature of 22 ℃ (± 2 ℃) and a 16/8 h, light/dark cycle for four weeks.

2.3.4 Disease assessment

The bark surrounding the wound was carefully removed using a scalpel to reveal the full extent of the lesion (Figure 2.1b). The maximum lesion length was measured using a digital caliper as a measure of resistance/susceptibility. To account for the length of the original wound, 4 mm was subtracted from each measurement.

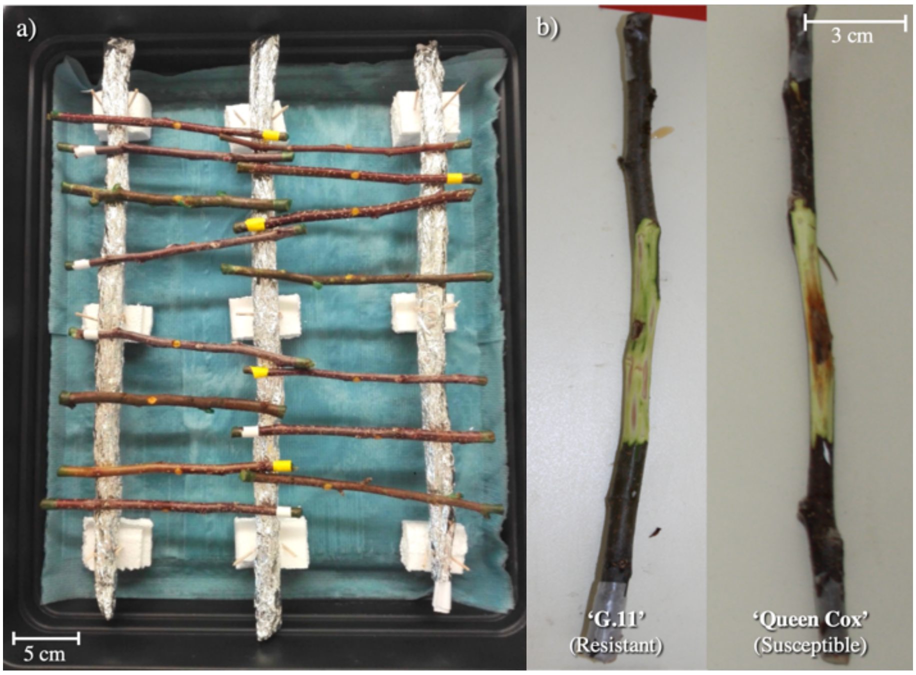


Figure 2.1. Example of experimental set-up and disease assessment (a) Layout of inoculated shoots in box. (b) Disease assessment of shoots inoculated with *Phytophthora cactorum* isolate R36/14.

2.4 Results and Discussion

While anecdotal evidence exists regarding resistance/susceptibility of apple to *Pc* in commercial cultivars, no recent systematic study has been carried out on UK breeding material. Differences in virulence were observed between the two *Pc* isolates tested in this study. Self-fertile 'Queen Cox' was the only apple accession susceptible to P295, with an average lesion length of 16.48 mm (±1.23 mm), which was significantly smaller ($p < 0.001$) than the average lesion caused by R36/14 (26.36 ±1.48 mm). R36/14 was the more pathogenic isolate on the tested germplasm, causing disease on 13 of the tested accessions (Figure 2.2). Of the remaining accessions inoculated with R36/14, nine showed no lesion development, representing useful material for future resistance breeding (Figure 2.2). The remaining seven accessions ('G.202', EMR001, EMR005, EMR006, *Malus hartwigii*, *Malus koreana* and *Malus robusta* 5) were contaminated, and the results were therefore deemed inconclusive. P295 was markedly less virulent than the more recently isolated R36/14. This loss of virulence could be associated with instability in storage as observed in *Phytophthora infestans*, the causal agent of potato late blight disease (Andrivon *et al*. 2010). To date, several isolates of *Pc* have been sequenced, isolated from European Beech, strawberry and ginseng (Grenville-Briggs *et al*., 2017; Armitage *et al*., 2018; Yang *et al*., 2018; Nellist *et al.,* 2021). Large numbers of genes putatively associated with pathogenicity have been identified. Chapter 6 of this thesis explores what genes contribute to virulence in the apple-pathogenic *Pc* isolate R36/14.

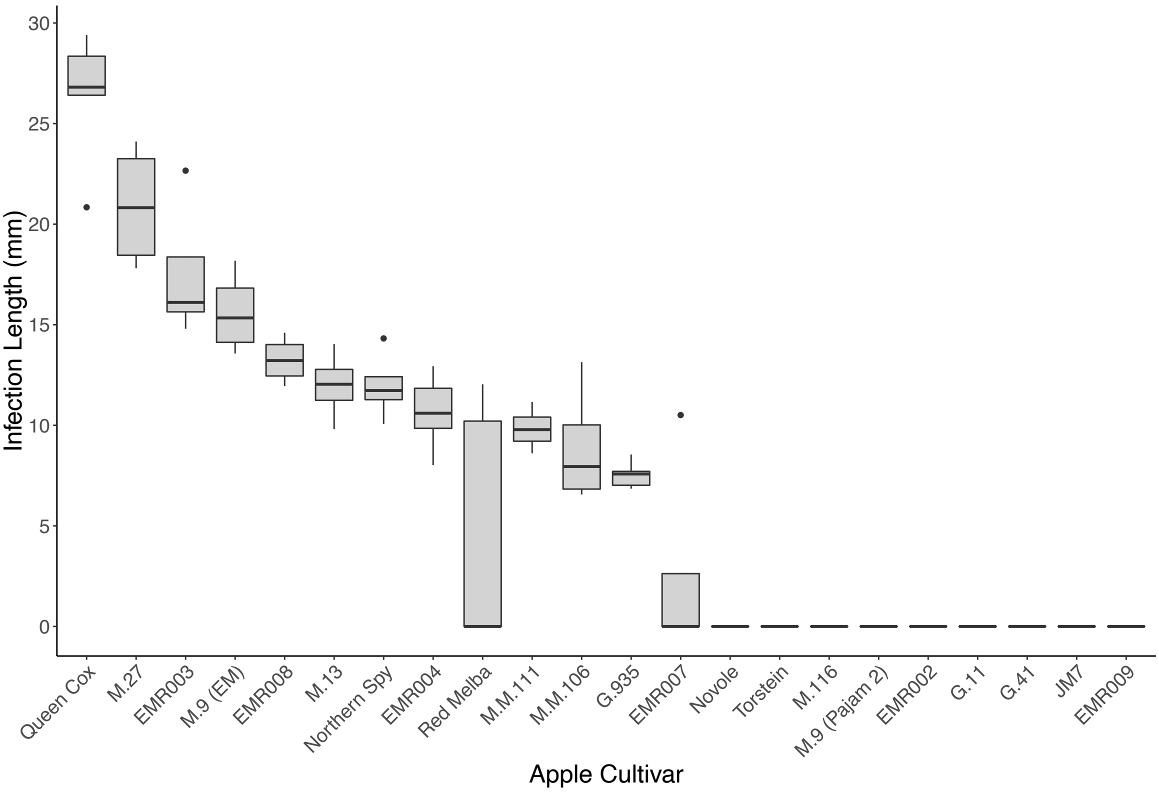


Figure 2.2. Lesion length (mm) on apple detached shoots inoculated with the Phytophthora cactorum isolate R36/14 measured four weeks after inoculation. The error bars indicate the standard error.

Four cultivars, 'Northern Spy', 'Red Melba', 'M.9 (EM)' and 'M.27', that had been previously reported as resistant to *Pc* (Sewell and Wilson, 1959; Alston, 1969; Utkhede, 1986) were found to be susceptible to isolate R36/14 in this study with average lesion sizes of 11.96 mm, 4.50 mm, 15.61 mm and 20.89 mm, respectively. As isolate R36/14 was recently isolated, it may have broken the resistance present in these old rootstocks. A later study reported susceptibility in 'M.9', supporting the idea of resistance breakdown (Bessho and Soejima, 1992). Interestingly, the two clones of 'M.9' behaved differently when inoculated with R36/14, 'Pajam 2' developed no symptoms with either isolate, consistent with resistance reported by Lemoine and Gaudin (1991), while the other clone of 'M.9', 'EM', was susceptible to R36/14. The difference in response observed between the 'M.9' clones, 'EM' (15.61 mm) and 'Pajam 2' (0 mm), might be due to clonal variation or be the result of imperfect inoculation; further testing will be required to clarify this discrepancy. The contamination encountered during the course of this study was fungal in nature. It impacted mostly plant material collected from older trees. The sterilisation time will be increased in future assessments to reduce the levels of contamination.

2.5 Conclusions

We demonstrated how cut-shoot tests can be employed to perform pathogenicity screens on large sets of UK germplasm in a time-efficient and inexpensive manner to identify sources of *Pc* resistance, although the sterilisation procedure still needs improvement to reduce contamination rates. We assessed the current state of resistance to *Pc* in the UK germplasm, with a focus on the material employed for rootstock breeding. Highlighting the breakdown of traditional sources of resistance, and the need to identify new ones in the wider germplasm. The next chapter of this thesis sets-out to map the sources of resistance to *Pc* identified through this germplasm screen.

Chapter 3: Mapping resistance to *Phytophthora cactorum* in the domesticated apple (*Malus* x *domestica*)

3.1 Abstract

Phytophthora cactorum (Pc) is a serious threat to viable commercial apple production systems. The unpredictability of outbreaks, coupled with its long-lasting permanence in the soil and lack of effective chemical control, make this pathogen particularly hard to manage. The use of resistant varieties has been widely hailed as the best option for disease management in many crop plants. Thus, the development of reliable markers for resistance is a critical step toward the breeding of durably resistant apple rootstock varieties. This study reports the first quantitative trait loci (QTL) associated with *Pc* resistance in apple. Using a bi-parental population generated from a 'M.27' X 'M.116' cross for QTL mapping, as well as a panel of apple rootstock and scion accessions for a preliminary genome-wide association study (GWAS), we detected a large effect QTL on chromosome 6 as well as two smaller effect QTL on chromosomes 5 and 15. A panel of SNP markers were also tested on an additional population (MCM007), where ten markers were selected for further validation. This is the first detailed study into understanding resistance to *Pc* in apple.

3.2 Introduction

The domesticated apple (*Malus* x *domestica)* is one of the most widely grown members of the *Rosaceae* family in the world, and it is a both culturally and economically important fruit. In 2020 world production was estimated at 86 million tonnes with a UK market value of 583 million pounds (FAOSTAT - https://www.fao.org/faostat - accessed 21/04/2022). There are several pests and diseases affecting commercial apple production in the northern hemisphere. Root and crown rots caused by the oomycete *Phytophthora cactorum* (*Pc*) can have devastating effects on apple orchards, particularly as the cost of orchard establishment is a key factor in economic viability of apple production (Harris, 1991; MacHardy, 1996; MacKenzie and Iskra, 2005). Thus, resistance to *Pc* is an important target for apple rootstock breeding programs.

Malus x *domestica* (*Md*) is an allotetraploid with a genome size of approximately 740Mb and a chromosome number of 17 (Velasco *et al.*, 2010, Daccord *et al.,* 2017; Zhang *et al.,* 2019b). Phylogenetic analyses have identified *Malus sieversii*, a wild-apple species found in Central Asia, as the primary progenitor of the domesticated apple (Cornille *et al*., 2014; Sun *et al.,* 2021). Substantial contributions from other *Malus* species were also found, and in particular from *Malus sylvestris* (Cornille *et al*., 2012; Sun *et al.,* 2021). Despite the existence of 25 *Malus* species and over 7,000 domesticated apple varieties, modern breeding programs utilise very few of them as founding clones, resulting in limited genetic diversity (Liang *et al.,* 2015; Lassois *et al.,* 2016; Urrestarazu *et al.,* 2016). While interspecific crosses between *Md* and other *Malus* species have reportedly been utilised to introduce traits such as red flesh and disease resistance in commercial cultivars, the limited genetic diversity in the elite germplasm makes it hard to introgress novel traits in breeding lines (Cornille *et al.,* 2012). Apple trait mapping has mainly focused on plant architecture both above and below ground as well as some physiological traits (Kenis and Keulemans*,* 2010; Van Dyk *et al.,* 2010; Fazio *et al.,* 2013; Liu *et al.,* 2020). Perhaps the most notable example of a trait that has revolutionised apple production is the introduction of dwarfing rootstocks in the early 20th century. Recently mapped to two loci (*Dw1* and *Dw2*) located on chromosome 5 and 11, dwarfing has allowed for the development of modern commercial orchard production (Foster *et al.,* 2015). Pathogen resistance is a major target in apple scion breeding programs. Several quantitative trait loci (QTL) for resistance to fire blight (*Erwinia amylovora*), apple scab (*Venturia inequalis*) and powdery mildew (*Podosphaera leucotricha*) have been identified in recent years (Calenge and Durel, 2006; Caffier *et al.,* 2016; Khajuria *et al.,* 2018; Kostick *et al.,* 2021). Efforts have also been put towards breeding for pathogen resistant rootstocks, with a QTL for resistance to crown gall disease having been reported recently (Moriya *et al.,* 2021).

Apple rootstock breeding can be a very lengthy and resource intensive process. This is due to of a juvenility period, which can last between 2-7 years and results in long generation times, as well as the evaluation process required to assess the new selections' performance across a number of traits and locations (Fazio *et al.,* 2021). Marker assisted selection has revolutionised the approach to plant breeding, by allowing to select for traits without the need for trial assessments in the early selection stages. Although this method requires the prior determination of the reliability of said markers as well as later field trails to assess the effect of environmental factor on the traits of interest (Hasan *et al.,* 2021). There are a number of available genetic markers that can be used to aid breeding, but microsatellite (or SSRs) and single nucleotide polymorphism (SNP) markers are by far the most commonly used. SSRs are PCR based markers which require the amplified DNA fragments to be resolved on a gel for genotyping. This makes them relatively cheap to implement, though also relatively low throughput (Nadeem *et al.,* 2017; Hasan *et al.,* 2021). SNP marker-based genotyping on the other hand can achieve much higher throughput levels. This sequencing-based genotyping technology is more sophisticated and, in many cases, more expensive than PCR based techniques. Despite this, the development on increasingly dense SNP genotyping arrays has great facilitated QTL mapping efforts (Rasheed *et al.,* 2017; Nadeem *et al.,* 2017; Hasan *et al.,* 2021; Fazio *et al.,* 2021). Currently there are three main SNP genotyping arrays for apple. The International RosBREED SNP Consortium (IRSC) 8K SNP array (Chagné *et al*., 2012), the Illumina Infinium® 20 K SNP array (built on the previous 8k SNP array), and Affymetrix Axiom® 480 K SNP array (Bianco *et al.,* 2014; Bianco *et al.,* 2016).

The hemi-biotrophic oomycete *Phytophthora cactorum* (*Pc*) can be particularly damaging to commercial apple production. It can cause crown, collar, and root rot (Erwin and Ribeiro, 1996). The first recorded case of apple collar rot attributed to *Pc* was reported in 1912, and it was soon recognised as an issue in apple growing regions all over the world (Harris, 1991).

The disease can enter the tree through wounds, and it reportedly often initiates infection at the graft union site, producing a moist rot. Necrotic bark tissues above ground eventually dry out and split away from the wood, while below ground the bark tends to turn black and is decomposed by soil microorganisms. Orange or brown stripes are found on the wood underneath necrotic bark, which can extend beyond the edge of the lesion (Harris, 1991). Diseases caused by *Pc* have long been recognised as a problem of apple production, particularly due to high cost of orchard establishment. Worldwide breeding efforts have yielded several varieties resistant to *Pc* (Carisse *et al.,* 2006; Luberti *et al.,* 2021; Verma *et al.,* 2021; Choi *et al.,* 2021)

Despite the economic impact *Pc* can have in commercial apple production systems, and the prolonged breeding efforts to introduce durable resistance to the pathogen, the genetic elements underlying resistance to *Pc* remain unknown. Only one report from 1969 indicates the potential for a major resistance gene in the cultivar 'Northern Spy' (Knight and Alston, 1969). This study sets out to elucidate the nature of resistance to *Pc* in apple. A bi-parental cross between two widely used rootstock varieties, moderately susceptible 'M.27' and resistant 'M.116' (M432), was employed in an effort to map resistance to the pathogen. Moreover, a preliminary genome-wide association study (GWAS) was performed on 99 apple rootstock and scion varieties to assess the levels of susceptibility to *Pc* of in the wider germplasm and identify novel sources of resistance. Here we report the first detection of three putative QTL associated with resistance to *Pc.* An additional cross between 'M.27' and the moderately resistant 'MM.106' was employed to identify a panel of markers for further validation, with the aim of eventual deployment in apple rootstock breeding programs.

3.3 Materials and methods

3.3.1 Plant material

First year growth *Md* shoots were collected, up to five shoots per genotype, from each phenotyped individual. A total of 61 individuals from the 'M.27' X 'M.116' cross (M432 population) in 2019, 86 individuals from the M432 population in 2020 (including all individuals from 2019), 126 individuals from the MCM007 population in 2021, along with 29 individuals from the M432 population and nine genotypes in the pedigree of both populations, were phenotyped from the NIAB's East Malling site to be employed for the QTL mapping and marker validation. An additional 99 accessions from both NIAB's apple genebank at East Malling and the National Fruit Collection at Brogdale (Supplementary Table 1), were phenotyped in 2020 for the preliminary genome-wide association study (GWAS). All shoots were cut to a length of 22 cm and surface-sterilised by immersion in a 10% bleach solution for 15 minutes. The shoots were then rinsed three times with sterile dH_2O. One centimeter was cut off from each end and both ends were sealed by dipping in molten paraffin wax to avoid drying out during the incubation period (Luberti *et al.,* 2021).

3.3.2 Inoculum production

Pc isolate R36/14, isolated at the NIAB East Malling site (UK) in June 2014, was employed in the assessment (Nellist *et al.,* 2021). The *Pc* isolate was revived from long term storage 14 days prior to inoculation. The isolate was grown on V8 agar at 20°C $\pm$1°C in the dark, as described by Nellist *et al*. (2019). The isolate was re-subbed after seven days on fresh V8 agar plates to ensure enough inoculum was produced.

3.3.3 Set-up and inoculation

Inoculation of the apple shoots was performed following the methods described in Luberti *et al*. (2021). In brief, a cork borer (4 mm diameter) was used to produce a wound in the middle section of each shoot and the outer bark was removed with a scalpel. Agar plugs of the same diameter containing the growing edge of *Pc* R36/14 mycelium were placed mycelium-side down onto the wound to inoculate the shoots. The apple accession 'Queen Cox' was used as a susceptible control and mock for the assessment (Luberti *et al.,* 2021). The control samples were inoculated using sterile V8 agar plugs; this was due to the restricted availability of plant material. Damp paper towels were placed at the bottom of each box and the shoots were arranged on raised racks made of rolled aluminium foil (see Chapter 1). The boxes were sealed in clear plastic bags to maintain humidity and were placed in a controlled environment room, with a constant temperature of 21 °C (± 2 °C) and a 16/8 h, light/dark cycle. Shoots were assessed 28 days post inoculation.

3.4 Disease assessment

Disease assessment of the five independent replicates was performed following the methods outlined in Luberti *et al.* (2021). Briefly, the bark surrounding the wound was carefully removed using a scalpel to reveal the full extent of the lesion (Figure 3.1). The maximum lesion length was measured using a digital caliper as a measure for resistance/susceptibility. The length of the original wound (4 mm) was subtracted to generate the final measurement.

Figure 3.1. Example of *Phytophthora cactorum* disease assessment in excised apple shoots.

Disease assessment of shoots inoculated with *Phytophthora cactorum* isolate R36/14 (b). The shoots pictured had the outer layer of bark removed to expose the infection area for measurement (Luberti *et al*., 2021).

3.4.1 Interval mapping

All individuals in the M432 population as well as the parental genotypes were genotyped employing the International RosBREED SNP Consortium (IRSC) 8K SNP array (Chagné *et al*., 2012). A linkage map was produced using JoinMap4® following user manual specification (Van Ooijen, 2004; (Supplementary Table 2). The interval mapping was performed employing MapQTL5® software with the recommended settings (Van Ooijen, 2005). The best linear unbiased estimator (BLUE) value (Goldberger, 1962) of infection length for each genotype was used in the calculation. The same software was employed to perform a 1000 permutation test to determine a threshold of significance across the genome, specific to each round of phenotyping.

A preliminary panel of MCM007 individuals (Supplementary Table 1) was genotyped using a 50 KASP markers panel covering the *MdRPc1* region (Supplementary Table 3). Interval mapping was then performed using JoinMap4® following user manual specification (Van Ooijen, 2004).

3.4.2 Preliminary genome-wide association study

All the individual apple accessions screened had been previously genotyped using the apple 20k SNP array (Supplementary Table 1; Bianco *et al*., 2014). The linkage map employed in this study was developed by Di Pierro *et al*. (2016). The GWAS analysis was performed using the R package GWASpoly (version 2.11), the 'general' model (no assumptions on dominance) was selected and population structure accounted for using a random polygenic effect to control for population structure. The BLUE value of infection length for each accession was used in the calculation, calculated using lme4.

3.4.3 Genome functional annotation

The apple (*Malus* x *domestica*) genome (*Malus* x *domestica* HFTH1 Whole Genome v1.0 - https://www.rosaceae.org/species/malus_x_domestica_HFTH1/genome_v1.0; Zhang *et al.,* 2019b) was employed to explore the genes within the putative QTL identified in this study. General functional annotation of the whole genome was performed using eggNOG (Huerta-Cepas *et al,* 2019), and complemented by the annotations provided by Zhang *et al.* (2019). The 'plant resistance gene database' DRAGO2 (Osuna-cruz *et al.,* 2017) annotation tool was used to identify genes containing resistance-associated motifs (RAMs). Transcription factor prediction was performed using the annotation tool provided by the Plant Transcription Factor database (Tian *et al.,* 2020).

3.5 Results

3.5.1 Whole-genome linkage mapping

A whole-genome linkage map was assembled using JoinMap4® using a total of 7,867 SNP markers from the IRSC 8k SNP array v1 and an array of selected simple sequence repeat (SSR) markers. It comprises 1,431 informative markers, assembled into 17 linkage groups representing the 17 chromosomes present in the *Malus* x *domestica* genome (Supplementary Table 2).

3.5.2 Two rounds of interval mapping suggest the presence of a QTL on chromosome six

All available individuals from the 'M432' population, a cross of moderately susceptible 'M.27' and resistant 'M.116' were phenotyped for two consecutive years (2019 and 2020). The distribution of the phenotypic data shows a binomial distribution of resistant/susceptible phenotypes, indicating a major effect locus (Figure 3.2).

Interval mapping was performed on the phenotypic data gathered in 2019 and 2020 using MapQTL5® and revealed a large effect locus highly associated with resistance on chromosome six , named *MdRPc1*. A permutation test was used to determine a genome-wide significance threshold of LOD = 4.5 for 2019 and LOD = 11.6 for 2020. The putative resistance QTL was found to explain an estimated 54.6% and 58.2% of observed variation in 2019 and 2020, respectively (Figure 3.3). Eight markers, spanning approximately 3 Mbp, remained significantly associated to resistance in both years.

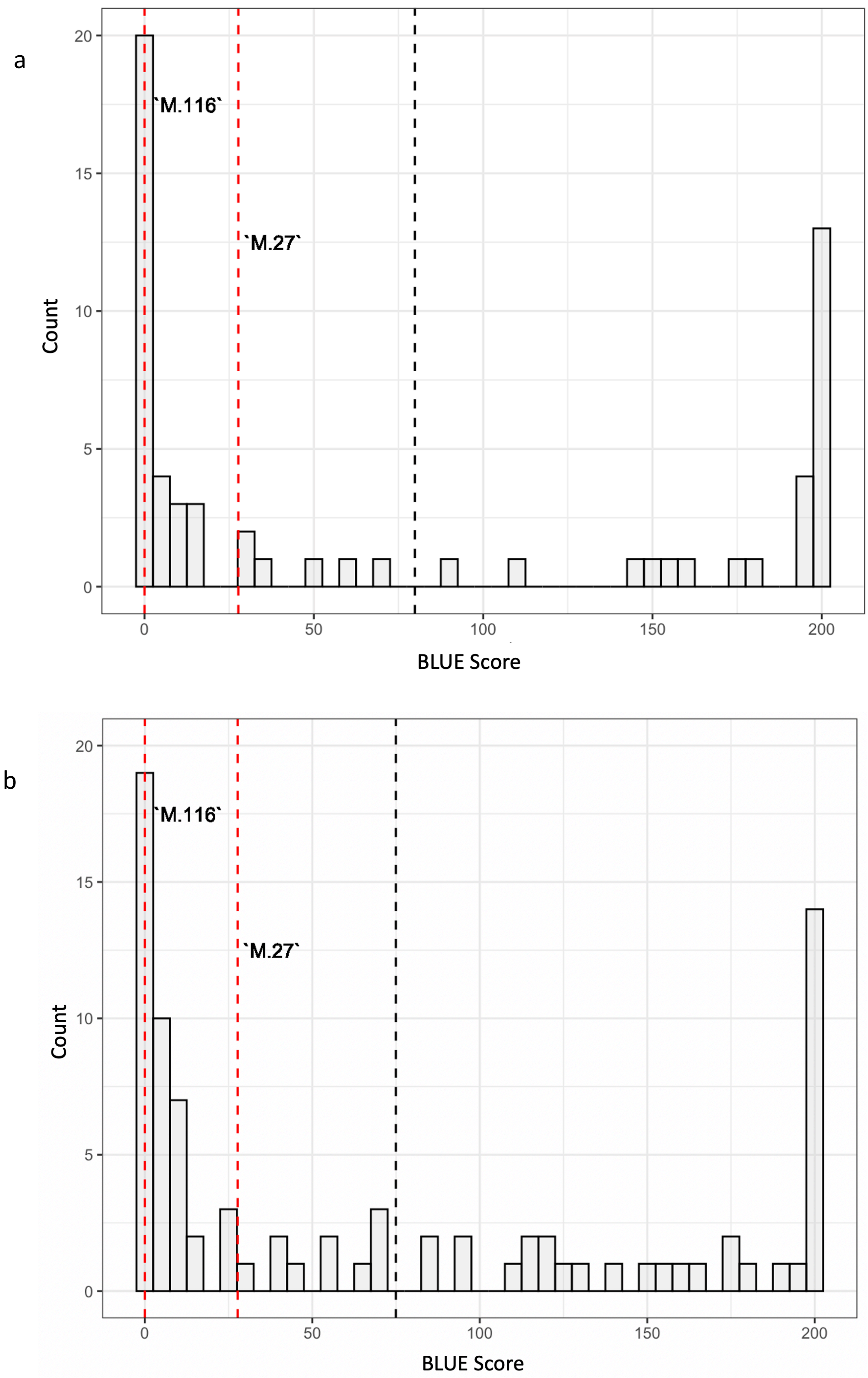


Figure 3.2. Distribution of the values for the best linear unbiased estimator (BLUE) of length of infection scores of each individual assessed in the phenotyping experiments performed in 2019 (a) and 2020 (b). The black dotted line indicates the median. The dotted red lines indicate the BLUE score of the parents.

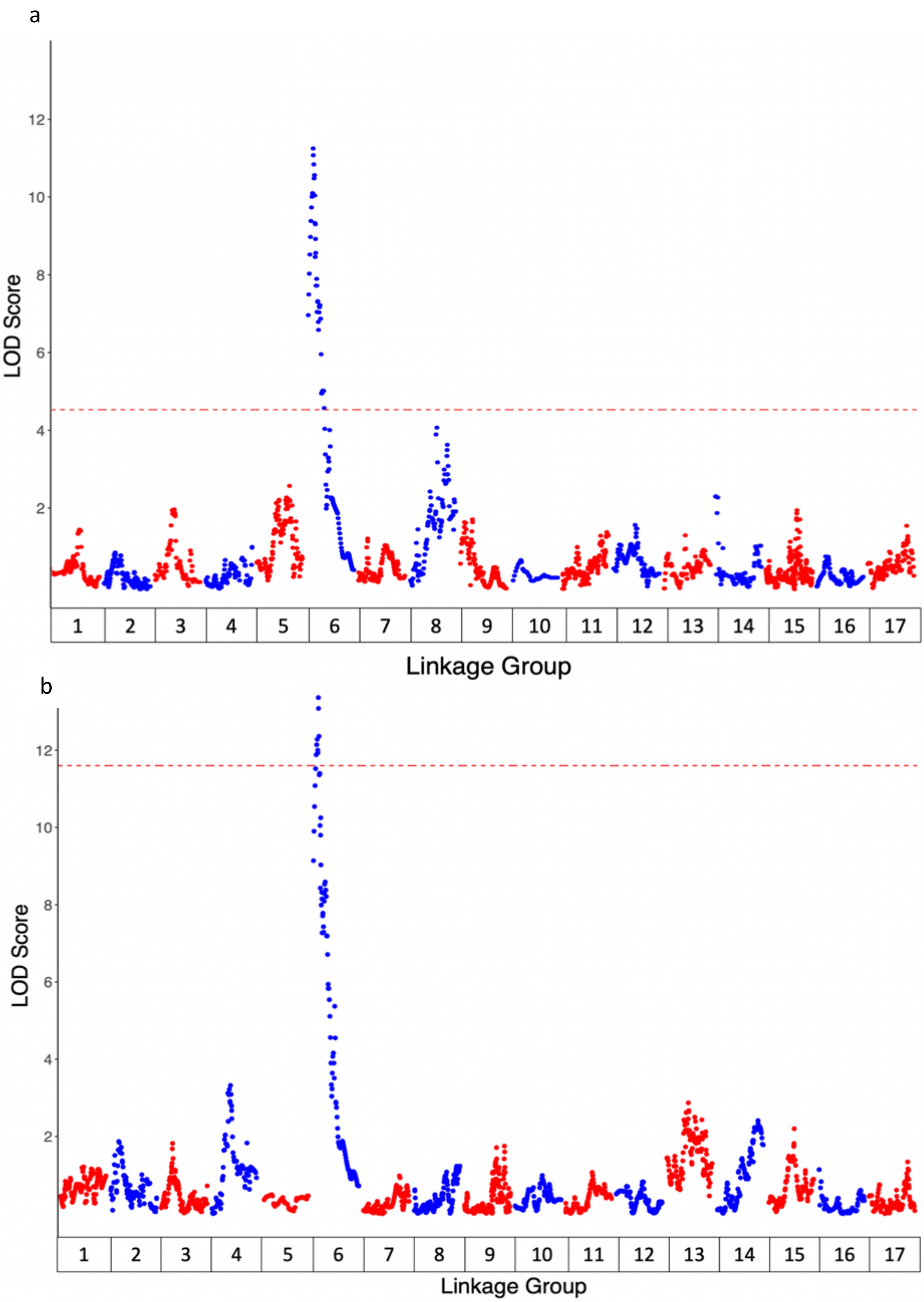


Figure 3.3. Major effect P*hytophthora cactorum* resistance quantitative trait locus (QTL) on chromosome six remains significant over multiple years in excised shoot assays.

Interval mapping (IM) of the 'M432' population for the years 2019 (a) and 2020 (b). A total of 61 and 86 individuals were phenotyped each year, respectively. The dots represent the logarithm of the odds (LOD) scores for the association of each of the single nucleotide polymorphism (SNP) markers present on each of the 17 linkage groups of the apple genome with resistance to *Phytophthora cactorum*. The horizontal red dotted line indicates the significance threshold for each year, 4.5 for 2019 and 11.6 for 2020 (*p-value* < 0.05) determined using a permutation test.

3.5.3 Resistance in the wider germplasm and preliminary genome-wide association study (GWAS)

The 99 apple accessions assessed in this study showed varying levels of susceptibility to *Pc* isolate R36/14. The most susceptible genotypes were the scion varieties ‘Delicious’ and ‘Duchess-of-Oldenburg’ and the rootstock varieties ‘Mac 24’ and ‘M.14’ (BLUE scores > 150 mm). In contrast, the most resistant apple varieties assessed in this study were the rootstock varieties ‘A469-4’, ‘Budagovsky 9’, ‘CG-11’, ‘M.24’ and ‘M.8’, as well as the scion variety ‘Grimes-Golden’, which showed no detectable infection symptoms. Distribution of BLUE scores is shown in Figure 3.4.

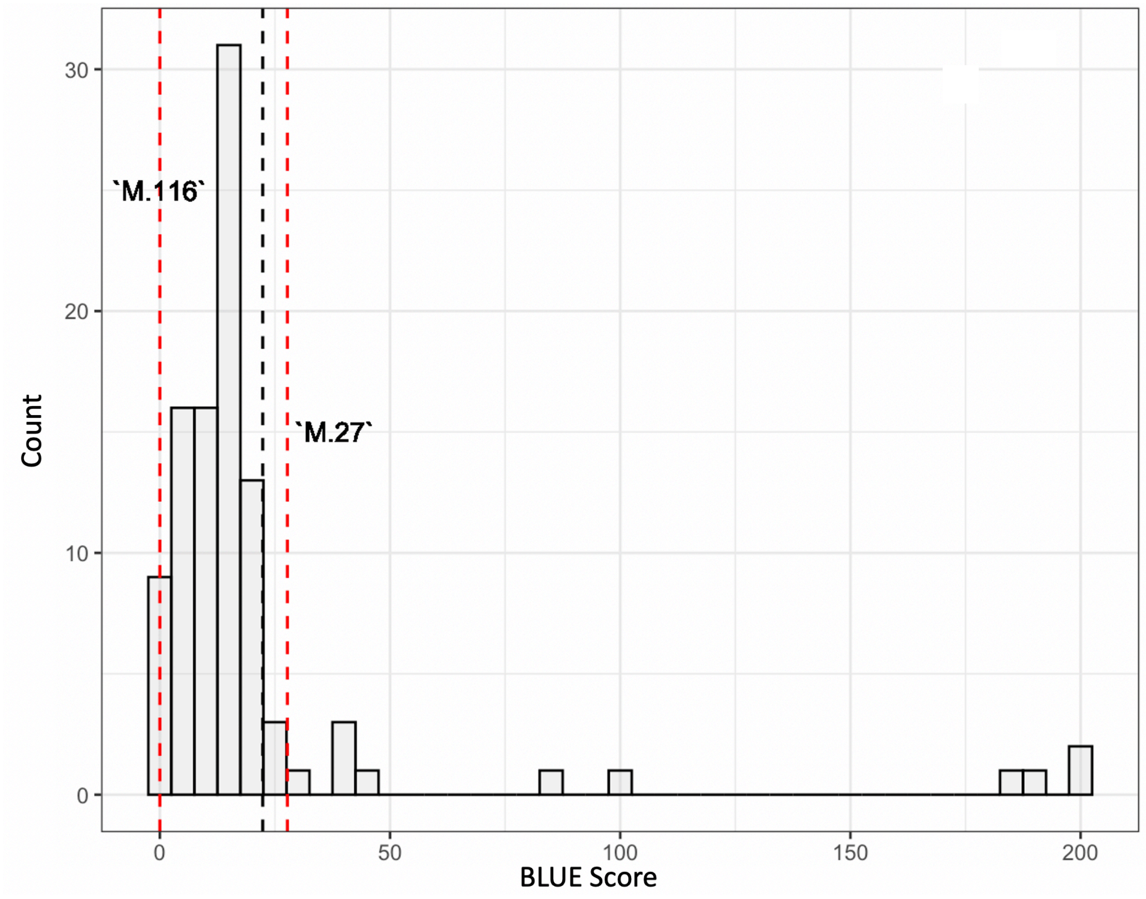


Figure 3.4. Distribution of the best linear estimator (BLUE) of the *Phytophthora cactorum* infection length scores for the individual apple accessions assessed during the course of this study. The black vertical dotted line indicates the median. The dotted red lines indicate the BLUE score of the parents.

The phenotypic data gathered for the 99 accessions of *Md* was employed to perform a preliminary GWAS, which identified three putative resistance loci on linkage groups 5, 6 and 15. Named *MdRPc2, MdRPc1* and *MdRPc3,* respectively. Notably the putative QTL on linkage group 6 is located within the same QTL region previously identified in the 'M432' mapping population (Figure 3.5).

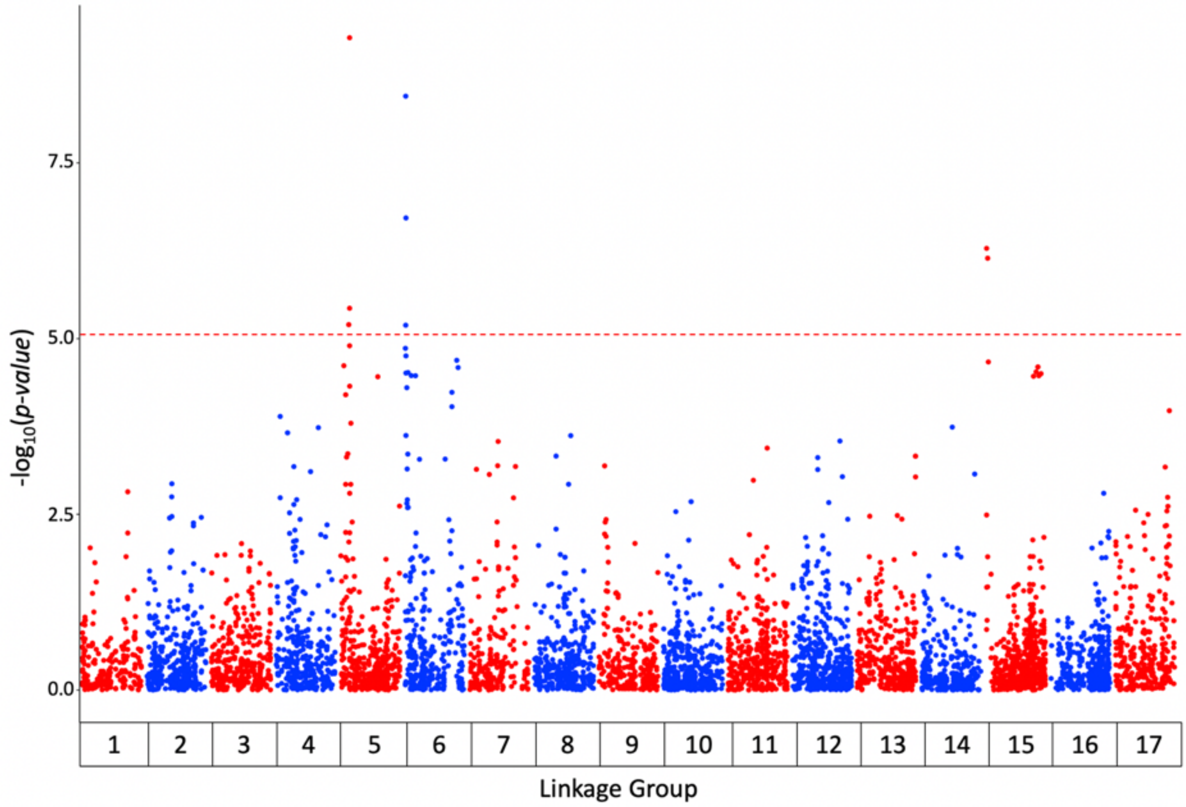


Figure 3.5. Preliminary genome-wide association study (GWAS) of the 99 apple accessions screened for resistance/susceptibility to *Phytophthora cactorum*. The dots indicate the scores for the association of each of the single nucleotide polymorphism markers present on each of the 17 linkage groups of the apple genome. The horizontal dotted line indicates the significance threshold ($-log_{10}(p) = 5.05$).

3.5.4 Excised apple shoot inoculation ('MCM007')

A total of 126 individuals belonging to a population generated from a 'M.27' X 'MM.106' cross was assessed for resistance to *Pc* using an excised shoot inoculation. The binomial distribution (p-value < 0.05; calculated using the parental genotypes as indicators of resistance thresholds) of the phenotypes observed is consistent with what was observed in the 'M432' population and suggests the presence of a major effect quantitative trait locus responsible for most of the observed variation (Figure 3.6).

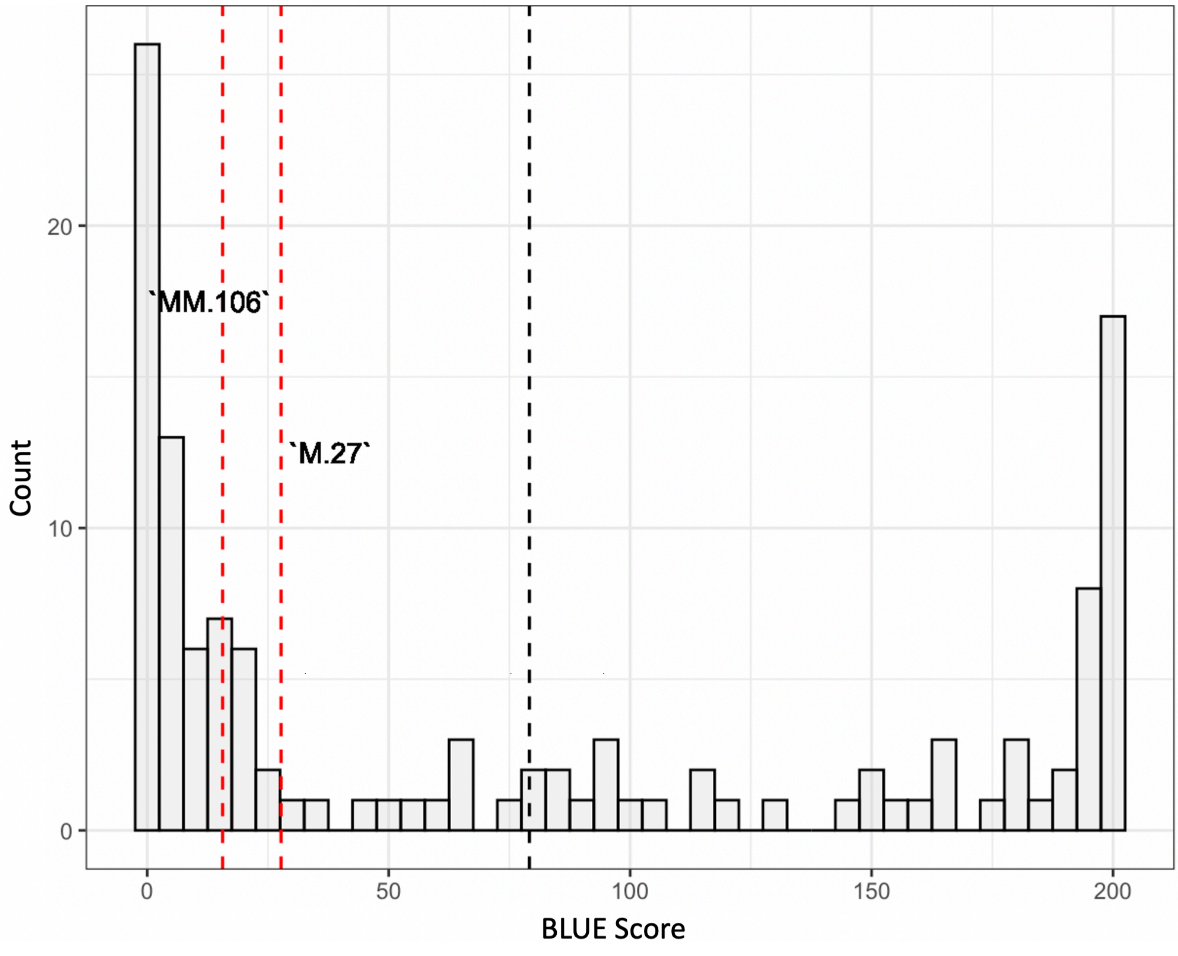


Figure 3.6. Distribution of the values for the best linear unbiased estimator (BLUE) of length of infection scores of each of the 126 individuals assessed in the phenotyping experiment of MCM007. The black dotted line indicates the median. The dotted red lines indicate the BLUE score of the parents.

3.5.5 KASP marker panel genotyping of MCM007

A panel of 50 KASP markers located within the QTL identified in the 'M432' population and preliminary GWAS, including significant ones from both experiments and additional markers from the 20k SNP array, were employed to genotype a representative subset of 'MCM007' individuals. Of the 50 markers selected for this screen, 34 were found to be informative in this population and were used in an attempt to fine map the resistance locus. Twenty-five markers remained significantly associated with resistance and the ten most highly associated markers have been selected for further validation on the rest of the 'MCM007' population, as well as individuals from the wider apple germplasm (Figure 3.7).

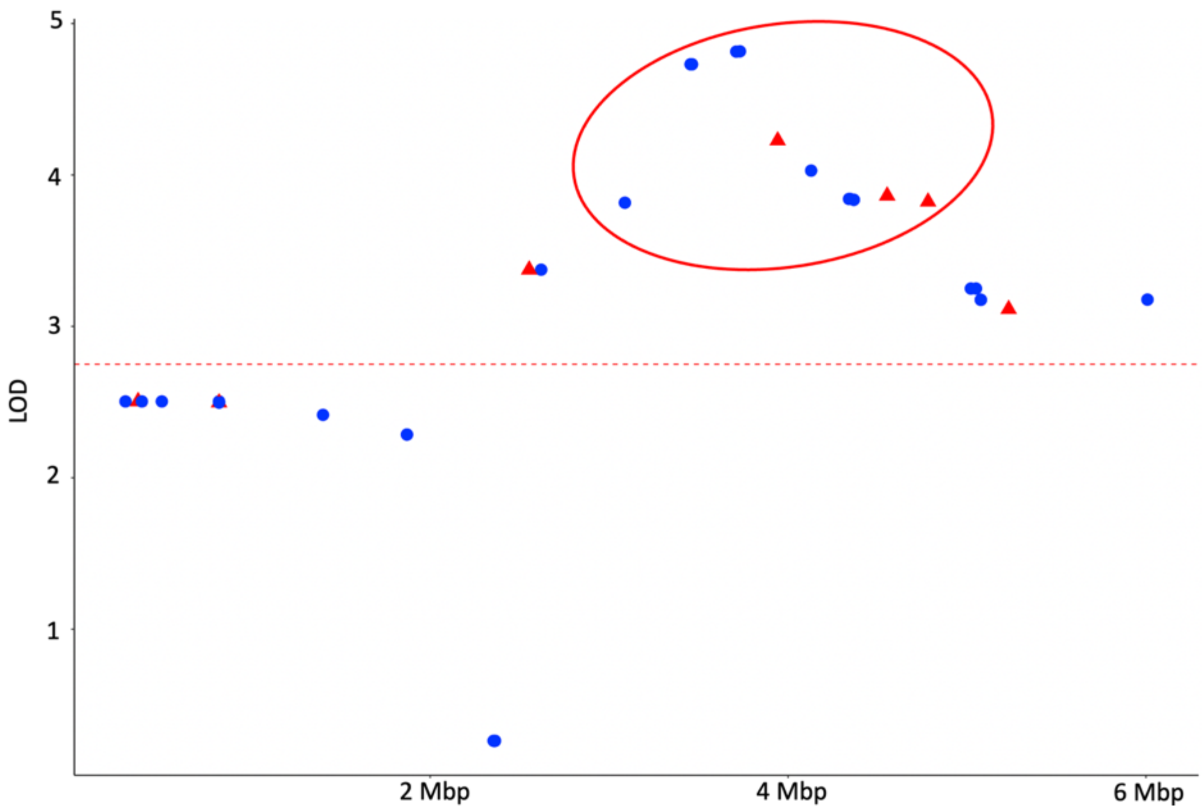


Figure 3.7. A selected panel of 50 single nucleotide polymorphism markers consisting both of markers previously found to be significant (red triangles) and not (blue dots) were used to map the bi-parental population 'MCM007' for resistance to *Phytophthora cactorum.* The logarithm of the odds (LOD) score for each marker are plotted on the y-axis, while the physical position on chromosome 6 (using the HFTH genome as reference) is plotted on the x-axis. The red dotted line indicates the significance threshold (*p-value* = 0.05), while the markers circled in red have been selected for further validation.

3.5.6 Putative QTL region annotation

3.5.6.1 Presence of known classes of resistance genes within the QTL region

The region 1.5 Mbp up- and down-stream of the most significant SNP for each of the putative QTL was further investigated to identify genes putative associated with pathogen recognition (Figure 3.8), signal transduction and transcriptional regulation. We identified a total 792 predicted gene models in the QTL regions. The 3 Mbp region around *MdRPc1* was found to contain 194 putative genes. *MdRPc2* was found to contain 370 putative genes; 28 were putatively annotated as TFs, while 27 were found to contain RAMs putatively associated with resistance. Fifteen of them were annotated as putative TFs, while nine were found to contain RAMs. Finally, the region surrounding *MdRPc3* was found to contain 228 putative genes. Among those, 14 were putatively annotated as TFs and 8 were found to contain RAMs (Supplementary Table 4).

3.5.6.2 SNP position within the QTL regions

The locations of each of the SNP markers identified as putatively associated with resistance in this study were verified using the NCBI nucleotide BLAST tool. SNPs located within putative gene models were assessed to determine whether they caused an amino acid change or premature termination. Nine of the SNP markers found within the *MdRPc1* locus were located within putative genes, with seven of them found within introns. Of the two that were within the gene's coding sequence, one was located within the putative G2-like TF *HF37175-RA* and resulted in a synonymous mutation. The other, found within the AT-hook motif-containing *HF37232-RA* gene, resulted in an arginine/tryptophan substitution. Only one of the SNPs significantly associated with resistance within the *MdRPc2* locus was located within a gene model. This was found to result in a premature stop codon being introduced within the apoptosis inhibitor 5-like protein (API5)-coding gene *HF11985-RA*'s coding sequence.

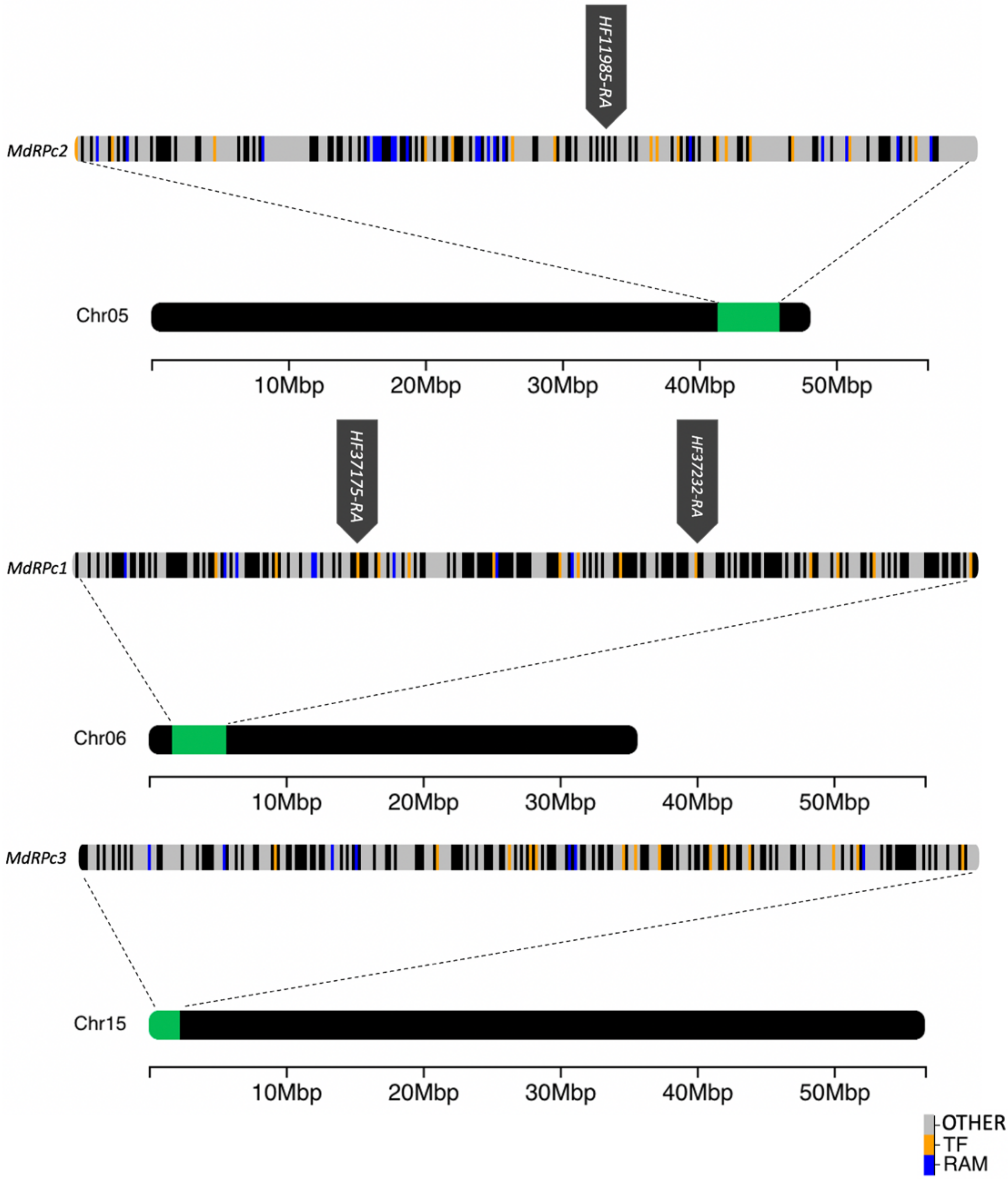


Figure 3.8. The three putative quantitative trait loci (QTL) regions contain a number of resistance-associated genes.

The three genomic regions found to be significantly associated with *Phytophthora cactorum* resistance in this study are highlighted in green on each respective chromosome. Genes containing a resistance associated motif (RAM) or annotated as a putative transcription factor (TF) are highlighted in blue and orange respectively. The names and locations of the three genes containing SNPs within their coding regions are indicated by the grey tags.

3.6 Discussion

During the course of this study, we tentatively identified the presence of a major effect QTL on chromosome 6 of the *Malus* x *domestica* genome. This putative resistance QTL was found to explain 54.6-58.2% of the phenotypic variation, observed in two consecutive rounds of phenotyping performed in 2019 and 2020. The same putative QTL region was found to be significantly associated with resistance in a preliminary GWAS performed on 99 *Md* accessions from apple rootstock varieties and the wider germplasm. This supports the previous result from the bi-parental cross mapping experiment and suggests the locus may be conserved across the germplasm. The GWAS also identified two additional loci on chromosomes 5 and 15, indicating the potential for additional sources of resistance present in the wider germplasm to be introgressed in rootstock breeding programs. Notably, while the locus identified on chromosome 6 explained a large proportion of the phenotypic variation observed across both years when the 'M432' population was phenotyped, it is likely that other smaller effect loci were not detected in our study. The reduced number of genotypes that were available and the potential environmental effects related to both the plants' physiological conditions as well as variation within the artificial inoculation method employed, may have limited the statistical power of detection of this analysis. Therefore, to assess the robustness of the markers associated with resistance, the related bi-parental population 'MCM007' was phenotyped for resistance to *Pc* and a subset of individuals were genotyped using a panel of 50 KASP SNP markers covering the original QTL area. This identified a number of markers significantly associated with resistance in this population, allowing for the selection of a ten-marker panel to be brought forward for further assessment. Testing of this marker panel on the rest of the MCM007 population and on more genetically distant crosses will determine the possibility of their future deployment in apple rootstock breeding programs. Moreover, while the isolate utilised in this study was the most virulent available in our collection, it would be advantageous to test the efficacy of this putative resistance locus against other isolates from different UK sites and countries of the world.

3.6.1 The putative QTL regions contain candidate resistance genes

Resistance genes are often found in clusters within plant genomes (Michelmore and Meyers, 1998; Ma *et al.,* 2019; van Wersch and Li, 2019; Lee and Chae, 2020; Yan *et al.,* 2021). All three of the putative resistance loci described in this study contained several resistance gene candidates. Resistance to *Phytophthora* species has been associated with several known classes of genes (Stewart *et al.,* 2004; Huang *et al.,* 2006; Du *et al.,* 2021a). These include nucleotide binding site–leucine-rich repeat receptor (NLR) genes, receptor-like kinases (RLKs) and receptor-like proteins (RLPs). A number of genes putatively coding for classes of resistance-associated proteins were identified in the region surrounding the most significant SNP marker from each of the putative loci identified in this study. The *MdRPc2* locus was found to contain eight leucine-rich repeat (LRR) containing genes, as well as ten RLKs and five RLPs, while the *MdRPc1* and *MdRPc3* loci were found to contain comparatively less RLK and RLP genes. Interestingly, two of the genes (*HF37158-RA* and *HF37161-RA*) found within the *MdRPc1* were putatively annotated as homologues of the *A. thaliana RPM-1* gene, which has been associated with resistance to a range of pathogens including *Pseudomonas syringae* in Arabidopsis and the fungal pathogen *Magnaporthe oryzae* and the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* in rice (Mackey *et al.,* 2002; Du *et al.* 2021b). Moreover, one of the genes near the *MdRPc3* locus was putatively annotated as a homologue of the *A. thaliana CLAVATA1* gene, a regulator of *Ralstonia solanacearum* resistance (Hanemian *et al.,* 2016; Zhang *et al.,* 2019a).

Several notable classes of TF were also identified in the putative QTL regions. Two putative WRKY-family transcription factors were found to be located near *MdRPc2.* Two putative NAC-family TFs were also found near both *MdRPc2* and *MdRPc1.* Notably, a homologue of the bHLH-family TF MYC2, which has been associated with pathogen resistance in *Arabidopsis* and tomato (Pozo *et al.,* 2008; Hiruma *et al.,* 2011; Du *et al.,* 2017), was found near *MdRPc1.*

3.6.2 Two SNP markers in loci *MdRPc1* and *MdRPc2* are associated with non-synonymous mutations

One of the markers (F_0990003) associated with resistance to *Pc* located on chromosome 5 of the apple genome was found to fall within the coding sequence of *HF11985-RA,* a API5-like gene. The mutation resulted in a premature stop codon being introduced in the gene's coding sequence, and thus it is likely to affect gene function. API5-like genes have not been extensively studied in plants and their functions remains unclear. A study in rice (*Oryza sativa*) has found API5 to be involved with programmed cell death regulation (Li *et al.,* 2011b), while transgenic expression of an insect apoptosis inhibitor gene in tomato and tobacco plants was found to enhance resistance to *Alternaria alternata*, potentially through modulation of ethylene signalling (Li *et al.,* 2010).

The RBbinsnp0265 marker, located on chromosome 6 and significantly associated with resistance in the bi-parental cross M432, was found to be located in the coding sequence on the AT-hook motif-containing putative TF gene *HF37232-RA.* The heterozygous allele carried by the resistant parent 'M.116' resulted in an arginine/tryptophan substitution. Though not found within the DNA-binding site of the putative TF, the change in polarity caused by the amino acid substitution is likely to affect protein stability and function (Sanders *et al.,* 2017; Degtyareva *et al.,* 2021). It is possible that two functional copies of the gene are required for full susceptibility, thus explaining the increased resistance to *Pc* observed in 'M.116'.

3.7 Conclusions

This study reports the identification of the first apple QTL putatively associated with resistance to *Pc.* A ~3 Mbp region of chromosome 6 was found to be associated with resistance in a QTL mapping analysis performed on a bi-parental cross of apple rootstock varieties 'M.27' and 'M.116'. The same region was also significant in a preliminary GWAS of the wider apple germplasm, along with two other loci on chromosomes 5 and 15. Moreover, this study has identified two candidate genes within the QTL on chromosome 5 and 6 that may be involved in resistance to *Pc*. Though limited by the reduced number of genotyped individuals and accessions available for this study, the results reported here warrant further investigation. The preliminary panel of markers selected for further validation may help inform future apple rootstock breeding choices, though future validation work will be needed to test their robustness. In particular, testing of the markers on larger and more genetically diverse apple populations, an expanded range of phenotyping techniques such as whole plant inoculation at different growth stages, as well as the use of multiple *Pc* isolates will be needed to determine the applicability of these results to commercial apple growing settings.

Chapter 4: Transcriptional response of *Malus* x *domestica* varieties to *Phytophthora cactorum* inoculation reveals differences in salicylic acid-mediated, systemic acquired resistance regulation

4.1 Abstract

The cultivated apple (*Malus* x *domestica)* is one of the most economically important fruits in the temperate regions of the world, with major efforts having been put into both scion and rootstock breeding programs. *Phytophthora cactorum* (*Pc*) can have a severe economic impact on commercial apple cultivation. It can affect trees from nursery to field. With oospores able to persist in the soil for decades and few commercially viable methods of control, the identification of sources of robust resistance has become increasingly important. This study represents the first report of a whole-transcriptome analysis of *Malus* x *domestica* root tissue response to *Pc* challenge. Differential gene expression and pathway enrichment analysis of the transcriptome changes of *Pc* resistant 'M.116' and moderately susceptible 'M.27' during infection has revealed differential regulation in salicylic acid-mediated signalling and systemic acquired resistance pathway activation. Moreover, several candidate genes with putative regulatory functions of these pathways were identified. Two homologues of the non-expressor of pathogenesis-related genes 1 (*NPR1*) gene as well as putative transcription factors belonging to the WRKY 22 and 40 families were found to be differentially expressed between varieties and are put forward for further study.

4.2 Introduction

The great increase in genomics resources available for horticultural species that has been seen in the past decade has resulted in tremendous improvements in our understanding of crop traits. From yield to fruit quality, to pest and pathogen resistance, these advancements have resulted in major improvements in all areas of horticultural production. Apple is one of the most culturally and economically important fruit varieties in the world, thus it is no surprise that there has been a substantial amount of work put towards understanding key crop traits. The first apple (*Malus* x *domestica*) genome was sequenced in 2010, and at the time it was only the tenth plant genome to be sequenced (Velasco *et al.,* 2010; Bolger *et al.,* 2014b). Since then, the quality of apple genomes published has steadily increased, generating more and better resources to study relevant crop traits. The 2017 double haploid 'Golden Delicious' genome (GDDH13) produced by Daccord and collaborators represented a substantial leap in assembly quality and completeness, thanks to the use of long read sequencing integrated by higher depth short read sequencing approach which resulted in a 10-fold genome coverage increase (Daccord *et al.,* 2017). It was soon followed by the sequencing of the apple anther-derived homozygous line HFTH1 in 2019, which represented a further improvement of the GDDH13 genome in both fold-coverage and estimated completeness (Zhang *et al.,* 2019b). In 2020, the 'Gala' genome, along with the *M. sieversii* and *M. sylvestris* genomes, was sequenced in an effort to identify contributions to the modern apple genome and investigate important crop traits associated with domestication (Sun *et al.,* 2020).

The same study also reported the transcriptome analysis of 'Gala' fruits at 13 developmental stages, identifying several genes associated with fruit quality (Sun *et al.,* 2020). Studies investigating transcriptional changes occurring during development and in response to environmental stresses have greatly benefited from the availability of better-quality genome assemblies and annotation. In recent years, many processes underlying key crop traits have been investigated in apple using transcriptomics. The regulation of the anthocyanin biosynthetic pathway has a well-established role in fruit ripening and colouration , as well as the response to both biotic and abiotic stresses (Lev-Yadun *et al.,* 2008; Landi *et al.,* 2015). Light-induced and temperature-induced transcriptional changes in this biosynthetic pathway have both been explored in several varieties (Vimolmangkang *et al.,* 2014; Song *et al.,* 2019). The transcriptional changes associated with other abiotic stresses have also received significant attention in recent years, with nitrogen, phosphorus, drought and salinity related stresses all having been investigated using comparative transcriptomics (Li *et al.,* 2019; Liu *et al.,* 2019a; Gao *et al.,* 2020; Sun *et al.,* 2021a, Sun *et al.,* 2021b).

Pests and pathogens represent a major threat to any plant crop. Thus, it is not surprising that many of the major threats to apple production have been investigated using transcriptomic approaches. RNA sequencing analysis (RNAseq) of apple plants challenged with *Podosphaera leucotricha, Botryosphaeria dothidea, Gymnosporangium yamadae, Valsa mali* and *Penicillum expansum* have been performed to explore above-ground and fruit disease development and resistance (Ke *et al.,* 2014; Liu *et al.,* 2019a; Shen *et al.,* 2019; Tian *et al.,* 2019; Tao; 2020; Zinati *et al.,* 2022). While the transcriptional changes in apple root systems following infection by the necrotrophic fungus *Pythium ultimum* and the hemi-biotrophic *Fusarium solani* and *Fusarium proliferatum* have been analysed to explore below-ground interactions (Shin *et al.,* 2016; Xiang *et al.,* 2021; Duan *et al.,* 2022). No studies relating to apple-oomycetes pathosystems have so far been published, with only one report from 1969 indicating the potential for a major resistance gene in the cultivar 'Northern Spy' (Knight and Alston, 1969).

Chapter 3 describes the discovery of a putative major effect resistance quantitative trait locus on chromosome 6 (*MdRPc1*), identified in the M432 population generated from the susceptible cultivar, 'M.27' X the resistant cultivar 'M.116'. 'M.27' was selected in 1934 from a 'M.13' x 'M.9' cross at East Malling Research station and demonstrates moderate susceptibility to *Pc*. 'M.116' was generated from a 'M.27' x 'M.M.106' cross and has demonstrated improved resistance to *Pc* compared to its parental genotypes (Luberti *et al.,* 2021). In this study, these two widely used rootstock varieties were employed to investigate the early response to *Pc* infection, with the aim of elucidating the mechanisms underpinning the early recognition of *Pc*, the identification of key regulatory genes, leading to resistance to *Pc*.

4.3 Materials and methods

4.3.1 Plant material and inoculation

'M.27' and 'M.116' plants were propagated in sterile tissue culture containers (cylindrical, clear glass honey jars, ø = 9 cm; medium thickness = ~4 cm) on Driver and Kuniyuki Walnut (DKW)/Juglans substrate (0.44% DKW, 0.9% agar, 4.5 µM BAP, 5 nM IBA, 3% sucrose, pH 5.6) and rooted on a modified DKW medium (0.44% DKW, 0.75% agar, 5 nM IBA, 0.35 nM GA3, 3% sucrose, pH 5.6), in a controlled environment chamber with average 68 µmols/m^2/s^{-1} light intensity, at 21 ˚C ±2 ˚C and 16/8 h, day/night light cycle (Driver and Kuniyuki, 1984). Successfully rooted plants were transferred to fresh medium 24 hours prior to inoculation with *Pc*, with the root system laid flat on the medium surface (Supplementary Figure 4.1).

Pc isolate, R36/14 (Nellist *et al*., 2021), isolated at the NIAB East Malling site (UK) in June 2014, was maintained on V8 agar medium at 20℃ ±1℃ in the dark. R36/14 zoospores were produced as described in Nellist *et al.* (2021), using compost extract medium to induce sporangia production and cold shocking to release the zoospores. Each plant was inoculated with 1 mL of 2×10^4 R36/14 zoospore suspension, distributed homogeneously over the root system and incubated in a controlled environment chamber with average 68 µmols/m^2/s^{-1} light intensity, at 21 ˚C ±2 ˚C and 16/8 h, day/night light cycle for up to 48 h. Three independent samples of the whole root system were taken at 0 (mock inoculated), 6, 9, 12, 24, 36 and 48 hours post inoculation (hpi) starting at 8am (during the light cycle), washed in sterile deionised water to remove any traces of medium and immediately frozen in liquid nitrogen (N_2) and stored at −80°C. Complete plant collapse was confirmed at 7 days post inoculation, with visible root lesions appearing between 24 and 36 hpi (data not shown).

Total RNA from all samples was extracted using the RNAqueous™ Total RNA Isolation Kit following the manufacturer's protocol for plant tissue, and sample quality and quantity was measured using NanoDrop™ 2000 and Agilent 4200 TapeStation. Only samples that met the sequencing provider's specifications of at least ≥ 200 ng, RNA Integrity Number (Agilent 2100) ≥ 4.0 and minimum purity measured with NanoDrop (A260/280 = 1.8-2.2; A260/230 ≥ 1.8) were kept. The marker gene PITG_11766 was employed to confirm the presence of *Pc* (Yan and Liou, 2006). All RNA samples were reverse transcribed using the QuantiTect Reverse

Transcription Kit (Qiagen), PCR was performed using MyTaq™ Red Mix with an annealing temperature of 60 ˚C and extension time of 10 seconds. The primers used were PITG_11766_F (CTCCGACGCAATCTTGGTAC) and PITG_11766_R (GTCTGACTAAGGGCAAGAAG). After presence was confirmed by PCR and samples 0, 6, 12 and 24 hpi were selected for sequencing, with the aim of elucidating the early stages of infection in both host and pathogen (see Chapter 5). RNA samples were sent to Novogene and sequenced to an average depth of 40 million reads per sample.

4.3.2 Data quality control and analysis

Read quality control was performed with FastQC (Andrews S., 2010; version 0.11.9) and the reads were trimmed using Trimmomatic (Bolger *et al.*, 2014a; version 0.32). The adapter sequences were removed, and low-quality bases (phred quality score below 3) were deleted from both ends of the reads. The reads were also scanned with a 4-base sliding window and cut if the average quality per base dropped below 20. Reads shorter than 36 bases were discarded.

Salmon v0.9.1 (Patro *et al*., 2017) was employed to quantify transcript abundance. The apple genome (*Malus* x *domestica* HFTH1 Whole Genome v1.0 - https://www.rosaceae.org/species/malus_x_domestica_HFTH1/genome_v1.0; Zhang *et al.,* 2019b) was used to build the mapping index, using a *k*-mer value of 31. Differential gene expression during infection was investigated using DESeq2 (Love *et al*., 2014). Genes were designated as differentially expressed if they had a DeSeq2 adjusted *p*-value (using the Benjamini and Hochberg method; *p*-adj) <0.05 and the $\log_2$(Fold change) (LFC) was > |1|. Genes are referred in the text as differentially expressed if there is a significant (LFC > |1|, *p*-adj < 0.05) difference between comparisons, constitutively expressed if the difference between timepoints was not significant; and constitutively differentially expressed between varieties if the difference between timepoints was not significant, but the difference between varieties was.

4.3.3 Gene annotation and enrichment analysis

Gene ontology (GO) annotation performed using eggNOG (Huerta-Cepas *et al,* 2019) and PANNZER2 (Törönen *et al.*, 2018) using the default settings. Custom gene matrix tables (GMTs) were constructed for gene ontology (GO) annotations and enrichment analysis were performed using g:Profiler online tool (Raudvere *et al.*, 2019). Sets of DEGs between 0 and 6 hpi, 12 hpi and 24 hpi in both varieties, as well as the genes DE between varieties at each timepoint were all employed for this analysis. The custom g:Profiler correction algorithm was applied, and pathways with an adjusted *p*-value < 0.05 were considered significantly enriched (Raudvere *et al*., 2019).

The 'plant resistance gene database' DRAGO2 (Osuna-cruz *et al.,* 2017) annotation tool was used to identify DEGs with resistance-associated motifs (RAMs). Transcription factor prediction was performed using the annotation tool provided by the Plant Transcription factor database (Tian *et al.,* 2020).

4.4 Results

4.4.1 Transcriptome sequencing of resistant and susceptible apple cultivars infected by *Pc*

The *Md* root transcriptome sequencing yielded a total of 1,045,763,293 raw reads (or 313.6 Gbp) of which 1,006,839,249 (97.9%, or 301.9 Gbp) were kept after quality checks. Only clean reads were employed for the subsequent alignment and quantification steps (Supplementary Table 5). The average mapping rate for the *Md* samples was 93.59% (Supplementary Table 6), with the rest being aligned to *Pc* genome (see Chapter 6) or inconclusive.

4.4.2 Differentially expressed gene analysis

Out of the 44,677 gene models predicted to be in the apple genome by Zhang *et al.* (2019), a total of 33,775 (75.60%) were found to be expressed during this experiment. Of those, 7,814 (23.13%) were found to be differentially expressed (DE) in at least one comparison between cultivars and/or timepoints, following inoculation with *Pc* isolate R36/14. A steady increase in the number of DE genes was observed in both cultivars during infection, with the greatest transcriptional difference from the inoculation (0 hpi) observed at 24 hpi. A noticeably greater number of DE genes were detected in 'M.116' (2,784 genes) compared to 'M.27' (1,664 genes) at that timepoint. The number of DEGs between cultivars followed a similar pattern, with almost double the number of genes being DE between 'M.27' and 'M.116' at 24 hpi than 0 hpi (Figure 4.1 and Figure 4.2).

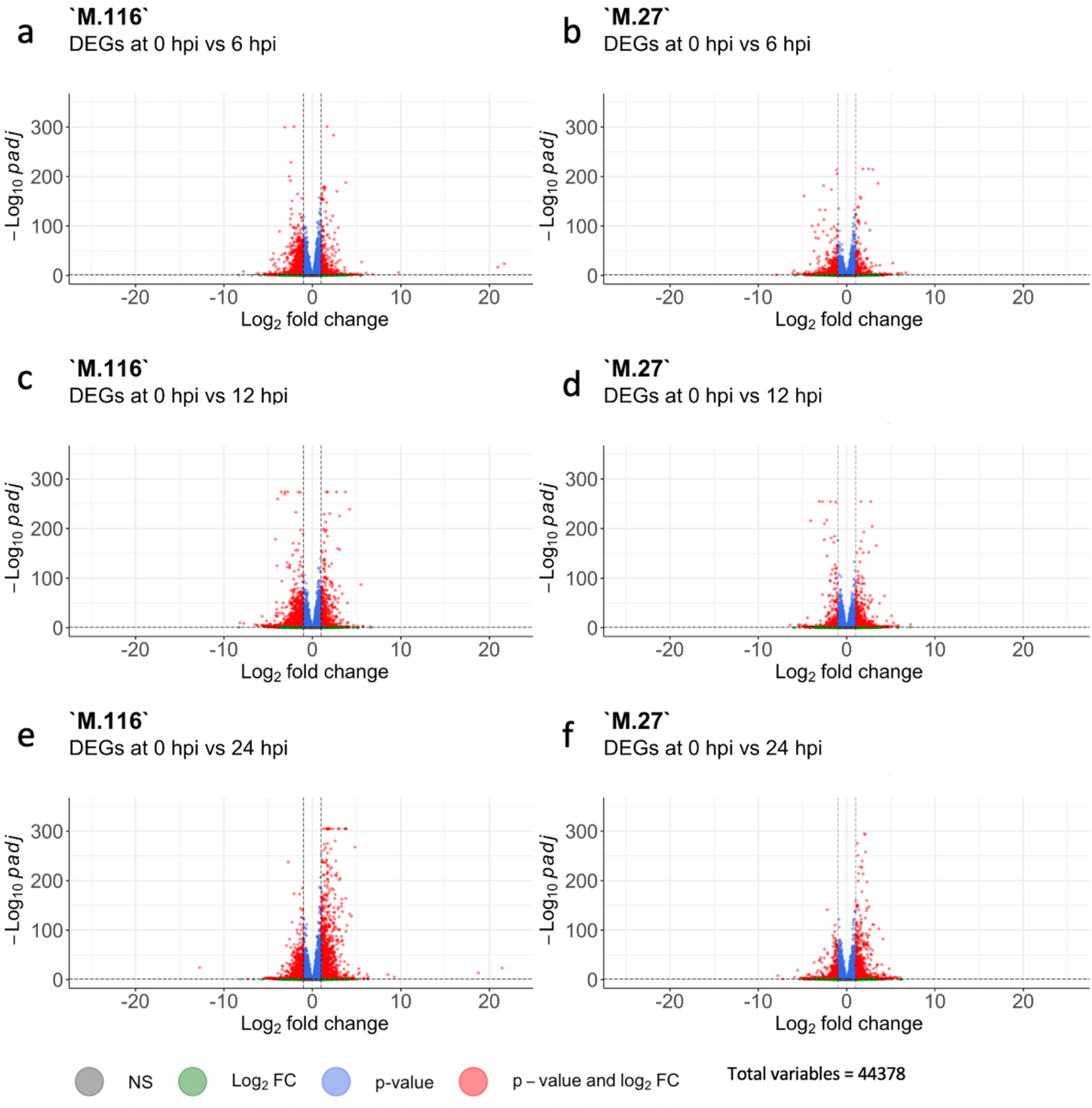


Figure 4.1. Volcano plots highlight a difference in the number of differentially expressed genes (DEGs) in response to *Phytophthora cactorum* infection between the two apple varieties at the sampled timepoints. The greatest response was observed at 24 hours post inoculation (hpi).

Genes are plotted according to their adjusted *p*-value (y-axis) on a $-Log_{10}$ scale and their change in expression (x-axis) presented on a Log_2 scale. Genes are coloured according to whether the differential expression is significant by *p*-value (blue), fold change (Log_2(FC); green), both (p-value and log_2 (FC); red) or is not significant (NS; grey).

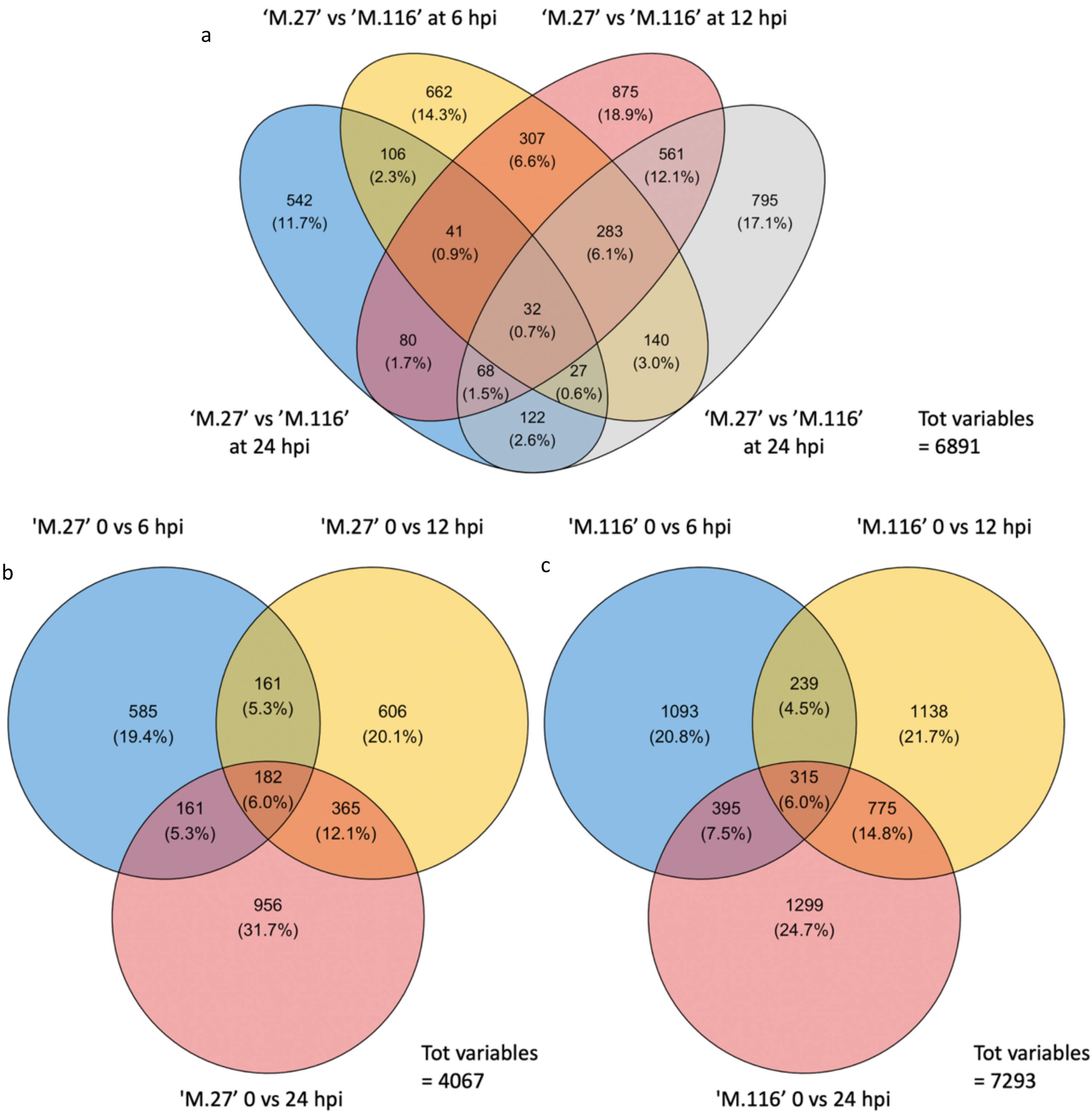


Figure 4.2. Venn diagram of the differentially expressed genes (DEGs) between the *Malus* x *domestica* cultivars 'M.116' and 'M.27' and timepoints at 0, 6, 12 and 24 hours post inoculation (hpi).

a) Genes DE between the two varieties at each timepoint, 0 and 6, 12, or 24 hpi. b) Genes DE between timepoints in 'M.27' and c) 'M.116' are encircled in blue, yellow, and red, respectively. The percentage of genes is shown below the number of genes in each group.

4.4.3 Functional annotation of differentially expressed genes

The annotation software eggNOG was used to annotate the 44,677 predicted gene models in the *Md* genome. A total of 36,804 were given a putative annotation, 31,126 of which were found to be expressed in this study and 7,428 DE between one or more of the comparisons analysed. The 'plant resistance gene database' DRAGO2 annotation tool (Osuna-cruz *et al.,* 2017) reported a total of 2,501 genes with putative RAMs in the *Md* genome. Of those, 580 were DE in our experiment with a total of 17 different classes of RAMs assigned to them; these included putative kinases, receptor-like proteins, and receptor-like kinases. Additional classes of RAMs identified amongst the sets of DEGs included CC, NBS, ARC and LRR motifs (Supplementary Table 7). The 'Plant Transcription factor database' (Tian *et al.,* 2020) annotation tool was employed to identify 2,339 putative TFs in the *Md* genome. A total of 683 were DE in this study, assigned to 49 different classes of TFs including AP2, ERF, NAC, MYB, bHLH, bZIP and 39 putative WRKY TFs (Supplementary Table 8).

4.4.4 Gene set enrichment analysis reveals differential regulation of salicylic acid-mediated signalling and systemic acquired resistance pathway activation

The sets of DEGs between 0 hpi and the other sampled timepoints, as well as between cultivars at each timepoint, were used to perform gene enrichment analysis to characterise the different responses associated with *Pc* infection in each cultivar (Supplementary Table 9; Supplementary Table 10). At 6 hpi, both cultivars showed significant enrichment in DEGs putatively associated with the regulation of pathways related oxidation-reduction processes (GO:0055114), 'M.116' was enriched for genes related to photosynthesis and light harvesting, while 'M.27' was enriched for the response to oxidative stress pathway (GO:0006979). The set of genes DE between the two cultivars at 6 hpi showed significant enrichment of genes putatively associated with the salicylic acid (SA) signalling pathway, lignin catabolism and the regulation of defence responses. At 12 hpi, growth related metabolic processes, such as cell wall biogenesis, phloem development and carbohydrate metabolic processes (GO:0042546, GO:0010088, GO:0005975) were enriched for in the DEGs sets of 'M.116', but not 'M.27', and were enriched for in the set of DEGs between the two cultivars at 12 hpi. The systemic acquired resistance (SAR) pathway and lignin catabolic pathway are also enriched between the two cultivars at 12 hpi. A similar pattern of differential regulation was observed at 24 hpi with the SAR pathway and lignin catabolic pathway being enriched for in sets of DEGs both between cultivars and in ‘M.116’ between 0 and 24 hpi. Several metabolic processes also show continued differential regulation, including the carbohydrate metabolic process pathway (Figure 4.3).

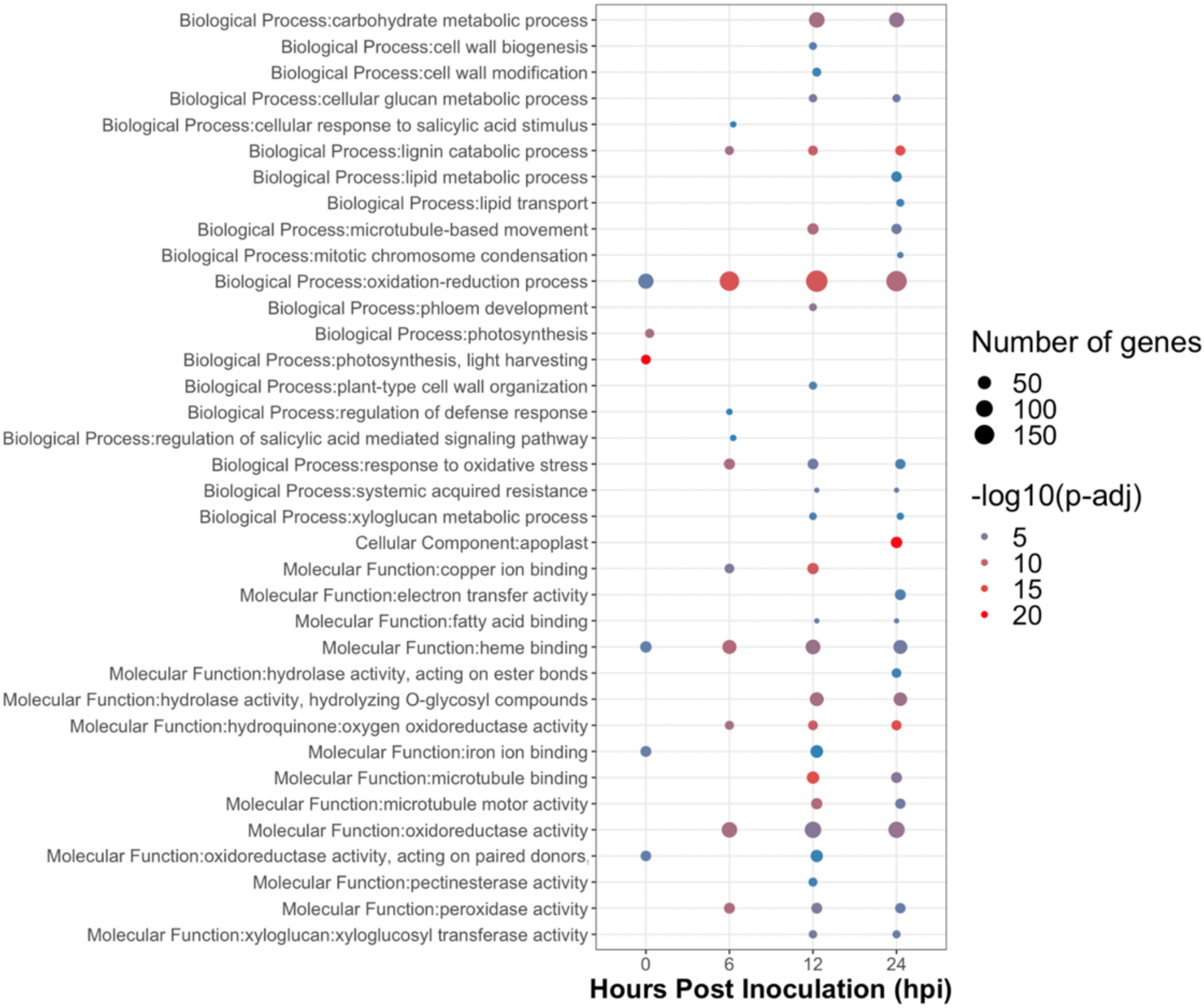


Figure 4.3. Gene enrichment analysis of the differentially expressed genes (DEGs) between varieties 'M.27' and 'M.116' highlights differential regulation of resistance-related pathways.

The circle diameter indicates the number of DEGs in the pathway at each timepoint, with the colour indicating the significance level reported as the -log10 of the adjusted *p-value* (-log10(p-adj)). Several resistance related pathways show differential regulation between the two varieties. At 6 hpi, DEGs between the two varieties annotated to the regulation of the salicylic acid-mediated signalling and of the defense response GO term show significant enrichment, with DEGs annotated to the systemic acquired resistance GO term becoming enriched at 12 and 24 hpi.

4.4.5 The most highly differentially regulated genes upon *Pc* inoculation contain several resistance-associated genes

The top 100 DEGs between cultivars at each timepoint ranked by LFC were selected for further analysis. At 6 hpi, the five most highly DEGs were *HF13819-RA, HF34510-RA, HF10313-RA,* *HF19362-RA, HF04202-RA*; these are putatively annotated as a 1,4-beta-D-glucanase-like gene, a *NRT1-PTR* family 4.3-like gene, a ferric reduction oxidase, a polyketide cyclase dehydrase and lipid transport superfamily protein gene, and a NAC domain-containing gene, respectively. Eight of the top 100 DEGs at 6 hpi were found to contain RAMs, including three receptor-like kinases and two TMV resistance protein N-like coding genes, while seven of the top 100 DEGs at 6 hpi were putatively annotated as TFs. At 12 hpi, the five most highly DEGs were *HF17632-RA, HF22827-RA, HF30101-RA, HF23722-RA, HF10313-RA*; these are putatively annotated as a *RETICULATA*-related gene, a NAC domain-containing gene, a cytochrome p450, a cadmium zinc-transporting ATPase, and a ferric reduction oxidase, respectively. Six of the top 100 DEGs at 12 hpi were found to contain RAMs, including four receptor-like kinases and a TMV resistance protein N-like coding gene, while nine of the top 100 DEGs at 12 hpi were putatively annotated as TFs. Finally, at 24 hpi the five most highly DEGs were *HF42338-RA, HF14227-RA, HF10978-RA, HF11892-RA, HF20393-RA*; putatively annotated as a methylesterase 11, a stigma-specific Stig1 family gene, a WD repeat containing gene, a leucine-rich repeat extensin-like protein, and a zinc finger A20 and AN1 domain-containing stress-associated protein, respectively. Seven of the top 100 DEGs at 24 hpi were found to contain RAMs, including five receptor-like kinases and a NB-ARC gene, while seven of the top 100 DEGs at 24 hpi were putatively annotated as TFs (Supplementary Table 11; Figure 4.4).

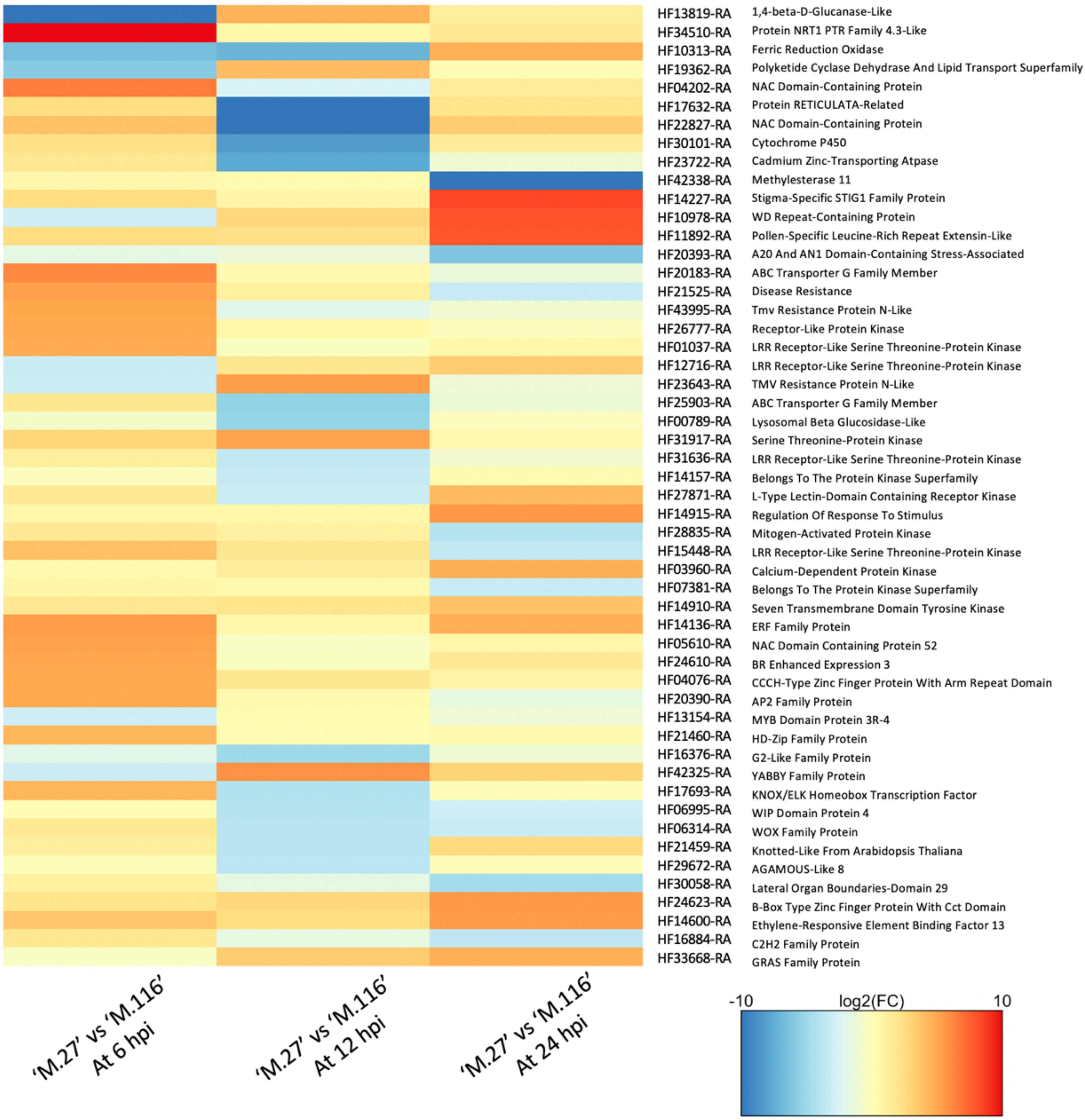


Figure 4.4. Heatmap of notable genes in the top 100 most highly differentially expressed genes (DEGs) ranked by $\log_2$(Fold change; LFC), shows transcription regulators and resistance-associated genes are highly represented.

The expression profiles of the top 5 most differentially regulated genes at each timepoint sampled that were also differentially expressed (DE) between 0 and one or more of the timepoints sampled in either or both varieties are plotted in a heatmap of the LFCs for each comparison. Descriptions of the putative function of the protein encoded by the gene are given on the righthand side of each.

4.4.6 Several DEGs were identified within the putative resistance QTL region on chromosome 6

A total of 63 DEGs were located within the putative QTL region located on the distal end of chromosome 6 (*MdRPc1*). Five of those contained putative RAMs, including four receptor-like kinases and an NLR gene. Three of the receptor-like kinases (*HF37180-RA, HF37137-RA, HF10080-RA*) were significantly more expressed in 'M.116' at 6 hpi ($|\log_2(\text{Fold change})| > 1$; p-adj < 0.05) while the fourth (*HF37203-RA*) was more highly expressed in 'M.27' at 12 hpi. Notably, the disease resistance-associated NB-ARC-LRR family gene *HF37158-RA* was up-regulated at 24 hpi in 'M.116' but not in 'M.27' ($\log_2$(Fold change) = 1.43; p-adj = 2.18×10^{-61}). Additionally, six putative TFs located within the QTL region were found to be DE. A NAC domain-containing gene was found to be up-regulated in 'M.116' at 6 hpi, while a putative MYB-family TF was up-regulated by 'M.27' at 24 hpi ($\log_2$(Fold change) = 1.89; p-adj = 0.03). Two TFs (*HF10078-RA,* a putative *NIN*-like family gene; *HF37132-RA* a putative trihelix family gene) were both down-regulated ($|\log_2(\text{Fold change})| < 1$; p-adj < 0.05) in 'M.27' at 24 hpi, with *HF37132-RA* also being down-regulated in 'M.116' at 24 hpi. Finally, a C2H2 zinc finger family gene (*HF37229-RA*) and an *LSD1* zinc finger-like gene (*HF37220-RA*) were up-regulated by 'M.27' at 6 and 12 hpi, respectively. Other notable DEGs withing the QTL region were a glycine-rich protein-coding gene (*HF37245-RA*) significantly more highly expressed in 'M.27' at 24 hpi ($\log_2$(Fold change) = 2.00; p-adj = 1.53×10^{-9}), two RING-H2 finger genes (*HF37256-RA, HF10047-RA*) and a F-box kelch-repeat gene (*HF10173-RA*) significantly more highly expressed in 'M.27' at 12 hpi ($|\log_2(\text{Fold change})| > 1$; p-adj < 0.05) (Figure 4.5; Supplementary Table 12). Two genes (*HF11985-RA* and *HF37232-RA*), that had been found to contain non-synonymous SNP mutations (see Chapter 3) were found to be expressed *in planta* during *Pc* infection of apple root tissue of 'M.27' and 'M.116', though neither was significantly differentially expressed either between varieties or sampled timepoints.

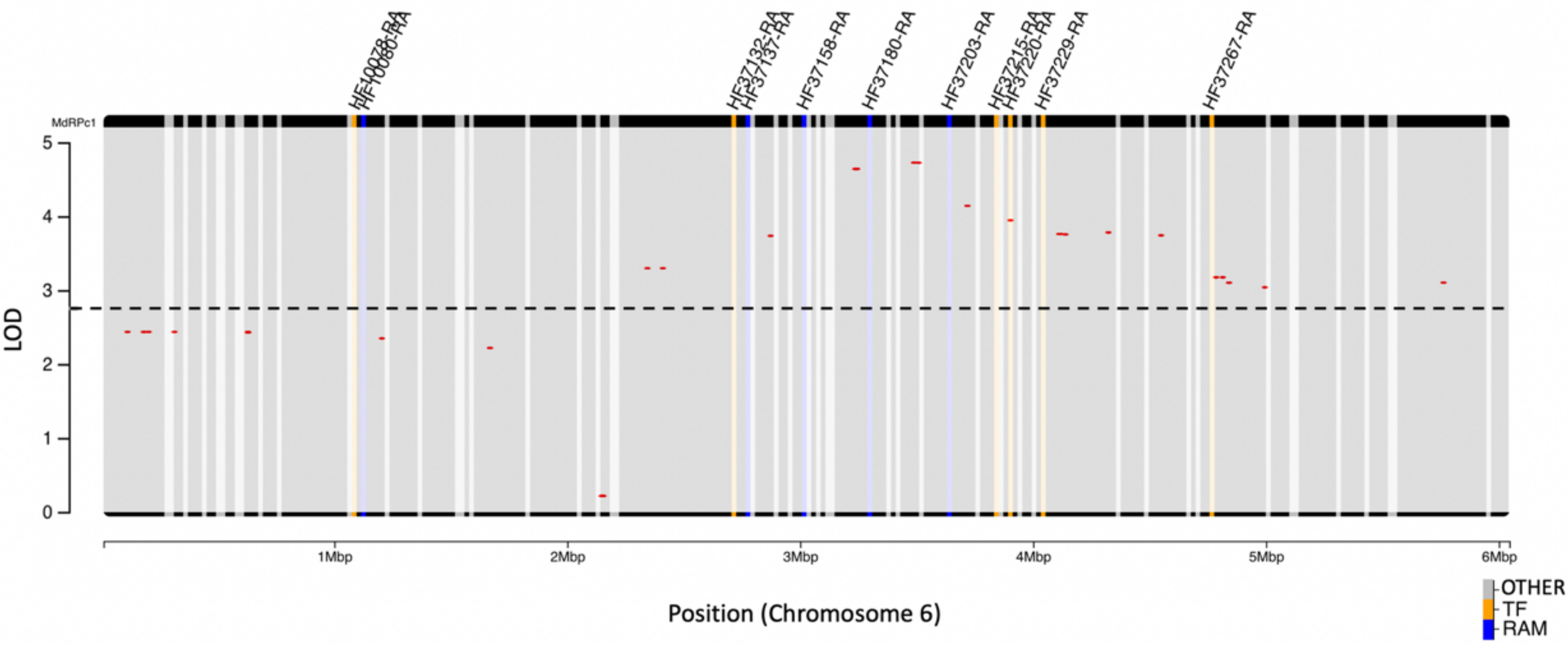


Figure 4.5. Differentially expressed genes in the *Phytophthora cactorum* putative quantitative trait loci (QTL) region identified in the bi-parental 'M.27'x'M.116' cross.

Each vertical line represents the genomic region annotated to a gene DE in this study. Genes highlighted in yellow, or blue have been putatively annotated as transcription factors (TF) or as containing resistance associated motifs (RAM). Each of the marker positions on the genome and LOD score is represented by a red dot. The dotted line indicates the significance threshold for the markers.

4.5 Discussion

The two apple dwarfing root stock cultivars employed in this study ('M.27' and 'M.116') are well established and widely used in commercial apple production systems. They are moreover highly related to founders of current lines being selected within the rootstock breeding programmes at NIAB at East Malling. The differences in the early transcriptional responses to *Pc* inoculation exhibited by the two cultivars employed in this study highlight several putative factors underlying the improved resistance of 'M.116'.

4.5.1 Gene set enrichment analysis revealed early differences in systemic acquired resistance pathway activation

Upon inoculation with *Pc,* both apple cultivars showed a defined transcriptional response. The number of genes DE between the control samples and each timepoint became increases as the infection progressed, with 'M.116' having a noticeably greater number of DEGs than 'M.27'. Likewise, the number of genes DE between cultivars also increased as time progressed, indicating an increasingly distinct response to *Pc* infection. Gene set enrichment analysis supported this by highlighting an increasingly divergent response of the two apple cultivars to inoculation. Both the 'M.27' and 'M.116' sets of DEGs between 0 and 6 hpi were enriched for oxidation-reduction related processes (GO:0055114); 'M.27' was also enriched for the response to the oxidative stress pathway, while 'M.116' showed differences in the regulation of photosynthetic processes which indicates a stress response in the plant host (Xie *et al.,* 2019). Notably, the set of genes DE between cultivars at this early timepoint showed significant enrichment for genes related to the regulation of the SA-mediated defence response (GO:0071446, GO:0031347, GO:2000031). At 12 hpi, the differences between cultivars became more accentuated, with a number of processes being differentially regulated between cultivars. The DEGs between 0 and 12 hpi in 'M.116' were enriched for a number of growth-related pathways, including cell wall biogenesis, phloem development and the metabolism of carbohydrates such as glucans and xyloglucans (GO:0042546, GO:0010088, GO:0005975, GO:0006073, GO:0010411), with them also being enriched in the DEG set between varieties. This is in line with what has been observed in other plant pathosystems, including *F. vesca,* and suggests a redirection of metabolic resources towards defence (Rojas *et al.,* 2014; Toljamo *et al.,* 2016; Toljamo *et al.,* 2021) Genes involved in lignin catabolism and SAR were also significantly enriched in 'M.116' at this timepoint (GO:0009627, GO:0046274). 'M.27' showed a more moderate response at this stage, with differential regulation of cell wall organisation and transmembrane related genes (GO:0009664, GO:0055085). A very similar pattern of differential regulation was observed at 24 hpi, with significant differences in the enrichment of genes associated with metabolic and resistance-associated pathways. Notably, the SAR pathway continued to be significantly enriched in the 'M.116' DE gene set between 0 and 24 hpi, but not in 'M.27'. In contrast, lignin catabolism was significantly enriched for in both cultivars between 0 and 24 hpi, as well as between cultivars at 24 hpi, thus suggesting differential regulation of the pathway. Lignin is known to act as a barrier to infection in plants, therefore it can be postulated that the differential expression of genes associated to this pathway between the two varieties studied could affect resistance to *Pc* (Xu *et al.,* 2011; Lee *et al.,* 2019; Cao *et al.,* 2021; Xiao *et al.,* 2021). Which has also been observed in soybean resistance to *P. sojae* and pepper resistance to *P. capsici* (Li *et al.,* 2020d; Wang *et al.,* 2020).

The results of the gene set enrichment analysis indicated that 'M.116' was able to recognise *Pc* and activate immune response pathways within hours of *Pc* infection, while 'M.27' showed a markedly lower transcriptional response. The regulation of the SA-mediated signalling pathway has been extensively associated with plant immune response to a range of pathogens (Klessig *et al.,* 2018; Jia *et al.,* 2018), including several examples of *Phytophthora* species (Shibata *et al.,* 2010; Deenamo *et al.,* 2018; Cui *et al.,* 2019a; Li *et al.,* 2019; Coles *et al.,* 2022). In this study, the early enrichment for SA-associated genes was followed by enrichment for genes putatively belonging to the SAR pathway. SA-mediated signalling has been established as an essential underlying component of SAR for many years (Shine *et al.,* 2019; Tripathi *et al.,* 2019; Kamle *et al.,* 2020), and is essential to resistance in plant-biotroph pathosystems (Yang *et al.,* 2015; Ullah *et al.,* 2019; Kou *et al.,* 2021; Islam *et al.,* 2021), including to the initial biotrophic phase of *Phytophthora* pathogens (Saiz-Fernández *et al.,* 2020; Soliman *et al.,* 2021). Thus, it is possible to postulate that the observed enrichment for genes associated with SA-signalling and SAR in the cultivar 'M.116' may be contributing to resistance by impeding pathogen colonisation of the plant tissue through the initial biotrophic phase of infection. Several of the DEGs annotated to the SAR pathway have putative functions related to plant pathogen resistance and are interesting candidates for further study. Of the genes up-regulated in 'M.116' between 0 and 24 hpi (LFC > 1, *p*-adj < 0.05), *HF03818-RA* and *HF08478-RA* have been annotated as protein kinase genes, putatively coding for a L-type lectin-domain containing receptor kinase and a mitogen-activated protein kinase. Both protein families are extensively associated with pathogen recognition and signalling leading to plant resistance (Huang *et al.,* 2013; Wang *et al.,* 2015; Wang *et al.,* 2017; Bi *et al., 2018;* Wang *et al.,* 2019b; Cheng *et al.,* 2020; Niraula *et al.,* 2020; Woo *et al.,* 2020; Zhang *et al.,* 2020); thus, it is possible that these two genes serve a role in the initial recognition of *Pc* and activation of the SAR response. The non-expressor of pathogenesis-related genes 1 (*NPR1*) like genes *HF30742-RA* and *HF18939-RA* were similarly found to be up-regulated in 'M.116' but not in 'M.27' at 24 hpi. *NPR1* is the central regulator of SAR (Cao *et al.,* 1994; Delaney *et al.,* 1995; Backer *et al.,* 2019), and is a receptor for SA (Wu *et al.,* 2012). Overexpression has been shown to improve resistance to a wide range of pathogens both biotrophic and necrotrophic in several plant hosts (Wally *et al.,* 2009; Zhang *et al.,* 2010; Le Henanff *et al.,* 2011; Matthews *et al.,* 2014; Son *et al.,* 2021), including domesticated apple (Malnoy *et al.,* 2007; Chen *et al.,* 2012). It is therefore plausible that the higher expression levels of the *NPR1*-like *HF30742-RA* and *HF18939-RA* genes could play a role in the increased resistance displayed by 'M.116'. Finally, two genes putatively annotated to the GDSL lipolytic enzyme family show differential regulation between the two cultivars after inoculation. Both *HF28942-RA* and *HF25148-RA* are down-regulated between 0 and 24 hpi in 'M.116', while being up-regulated by 'M.27' in the same comparison (LFC > |1|, p-adj < 0.05). A third GDSL lipolytic enzyme family gene (*HF24161-RA*) shows early up-regulation in 'M.27' at 12 hpi, while the inverse pattern is observed in 'M.116'. A recent study performed in rice found the down-regulation of *OsGLIP1* and *OsGLIP2*, two lipase genes of the GDSL family, to be induced by both pathogen infection and SA. Moreover, overexpression of the two genes increased susceptibility to the pathogen *Xanthomonas oryzae* pv. *oryzae* (Gao *et al.,* 2017), thus indicating these two genes could function as susceptibility factors in *Md.*

4.5.2 Regulation of receptor genes and transcription factors upon *Pc* inoculation

Out of the 2,501 genes with putative RAMs, 581 (23.23%) were found to be DE in this study. A total of 323 of those DEGs have been putatively annotated as receptor-like genes, including 19 wall-associated receptor kinase-like genes, with 5 of them DE between cultivars at 6 hpi, 5 of them DE between cultivars at 12 hpi, and 2 DE between cultivars at 24 hpi. This class of transmembrane receptor genes is involved in the first stages of plant-pathogen interactions and has a well-documented role in pathogen detection, with their protein product serving as the outermost recognition site for pathogen invasion and aiding resistance (Li *et al.,* 2020a; Liu *et al.,* 2021b; Yu *et al.,* 2022). The rest of the DEGs with RAMs have been annotated as a range of protein kinase-coding genes classes, including mitogen-activated protein kinase, calcium-dependent protein kinase, serine threonine-protein kinase, L-type lectin-domain containing receptor kinase, and LRR receptor-like kinase. Members of these gene families serve a great variety of roles within plant systems (Lehti-Shiu and Shiu, 2012; Dufayard *et al.,* 2017; Yip Delormel and Boudsocq, 2019). They are involved in pathogen effector recognition and are often necessary for effective pathogen resistance in a number of plant-microbe pathosystems such as tomato-*Botrytis cinerea*, Arabidopsis-*Pseudomonas syringae* and *Triticum aestivum-Rhizoctonia cerealis* (Zhang *et al.,* 2018; Guerra *et al.,* 2020; Wang *et al.,* 2021). Their regulatory role is dynamic and complex, in plant-*Phytophthora* pathosystems they have been reportedly associated with both negative (Arabidopsis-*Phytophthora parasitica*; Li *et al.,* 2022; Qiuang *et al.,* 2021) and positive (potato-*P. infestans;* Zhang *et al.,* 2021) regulation of resistance. Of the five RAM-containing DEGs present in the putative resistance QTL region, three encoded putative protein kinases, one was a putative LLR-kinase, and the last contained an NB-LRR domain. Two of the protein kinase-coding genes (*HF10080-RA, HF37137-RA*) were significantly more highly expressed in 'M.116' at 6 hpi, while the NB-LRR domain containing putative resistance gene *HF37158-RA* was up-regulated by 'M.116' between 0 and 24 hpi. NLR (NB-LLR) class genes have been previously associated with resistance in plant-*Phytophthora* pathosystems (Saunders *et al.,* 2012; Cui *et al.,* 2017; Jiang *et al.,* 2018). This suggests that they may be potential resistance gene candidates and should be subject to further analysis.

As mentioned above, the regulatory elements that govern plant immunity play complex and dynamic roles during infection. This is reflected in the transcriptional profiles of both RAMs-containing genes and the putative transcription factors DE during this experiment. Of the 2,339 genes with putative TF annotation, 683 (29.20%) were found to be DE in this study. Several notable classes of transcription factors were present, including 40 WRKYs. WRKY transcription factors serve a great variety of functions in plant stress responses, being involved in both positive and negative regulation of resistance (Pandey *et al.,* 2007; Bakshi and Oelmüller, 2014; Jiang *et al.,* 2017). For instance, the two putative WRKY40-family genes *HF32511-RA* and *HF00040-RA* are significantly up-regulated in 'M.116' between 0 and 24 hpi. The *Arabidopsis thaliana* WRKY genes *AtWRKY18* and *-40* can act both as negative regulators of resistance to *Pseudomonas syringe,* and as positive regulators of resistance to *Botrytis cinerea,* while also suggesting they may have antagonistic interactions in *Arabidopsis* pathosystems (Xu *et al.,* 2006). Notably, a recent study in *soybean* identified *GmWRKY40* as a positive regulator of resistance to *Phytophthora sojae* suggesting a similar functions for the two apple WRKY40 TFs putatively identified in this study (Cui *et al.,* 2019b). Similarly, the putative WRKY22 gene *HF10274-RA* was found to be up-regulated between 0 and 24 hpi in 'M.116' (LFC = 1.29; *p*-adj = 1.45×10^{-24}). This TF family has been associated with positive regulation of resistance in *Arabidopsis,* rice, and pepper pathosystems (Abbruscato *et al.,* 2012; Hsu *et al.,* 2013; Hussain *et al.,* 2018). In pepper, the authors of the study demonstrate that expression of *CaWRKY22* was induced by both *Ralstonia solanacearum* inoculation as well as exogenous application of SA. Moreover, it has been shown that overexpression of *CaWRKY22* positively regulates both *CaWRKY40* and *CaWRKY6*. In turn, overexpression of *CaWRKY40* and *CaWRKY6* was shown to positively regulate *CaWRKY22* (Hussain *et al.,* 2018). As the putative WRKY22 gene (*HF10274-RA*) and the two putative WRKY6 genes (*HF12290-RA*, *HF17708-RA*) DE in this experiment are also up-regulated by 'M.116' between 0 and 24 hpi, it appears plausible that these three genes may serve a similar function in *Md.*

4.6 Conclusions

This study details the first report of *Md* whole-genome transcriptional response to *Pc* challenge. The analysis performed has revealed elements of the immune response putatively underlying the resistant phenotype observed in 'M.116'. These results tentatively suggest that 'M.116' can recognise and mount a salicylic acid-mediated immune response within hours of *Pc* infection. Five differentially expressed genes putatively annotated as belonging to transcription factor families WRKY6, WRKY22 and WRKY40 have been identified as candidates for the regulation of the salicylic acid-mediated immune response. Moreover, it is suggested that the *NPR1*-like genes *HF30742-RA* and *HF18939-RA* up-regulated in 'M.116' upon *Pc* infection may have a role in modulating the systemic acquired resistance response. Lastly, it is proposed that the DE NB-LRR gene *HF37158-RA,* located in the putative resistance QTL, may play a crucial role in pathogen recognition and immune response activation. Future work will be needed to functionally characterise the roles of the candidate genes proposed here. Assessment of expression profiles via qRT-PCR at later timepoints after inoculation, as well as in different infected tissues and plant development stages will help further elucidate their role in resistance. As the assessment of plants in the QTL study was performed after four weeks, this will help relate those results to the transcriptome analysis data explored in this chapter.

Chapter 5: RNAseq reveals pathways for resistance to *Phytophthora cactorum* in *Fragaria* x *ananassa*

5.1 Abstract

Cultivated strawberry (*Fragaria* x *ananassa*) is an important horticultural crop in the UK, and the world. Crown rot [*Phytophthora cactorum* (*Pc*)] disease affects commercial strawberry production at all stages, from nursery to field, in polytunnel and glasshouse production systems. There are currently few effective and commercially viable means to control *Pc* infection. Thus, the use of resistant cultivars is key to sustainable commercial strawberry production. In this study, the root system of a susceptible ('Emily') and moderately resistant ('Fenella') cultivars were challenged with *Pc* to determine key factors underlying the resistant phenotype. The two cultivars' responses to inoculation were dissected using an integrated approach of gene differential expression analysis, enrichment analysis and co-expression network analysis. Differences in phytohormone signalling pathways regulation, as well as regulation of pathogen-induced cell death emerged as potentially important determinants of resistance. Additionally, several candidate resistance genes, including putative transcription factors and receptor genes, showing differential regulation between the two cultivars are put forward for further characterisation.

5.2 Introduction

The cultivated strawberry (*Fragaria* x *ananassa*) is an outcrossing species of herb-like perennials mostly cultivated in the northern hemisphere (Hummer and Hancock, 2009). Pests and disease are amongst the biggest constraints in strawberry production. In northern Europe, crown rot (*Phytophthora cactorum; Lebert & Cohn J. Schröt*), grey mould (*Botrytis cinerea*) and powdery mildew (*Podosphaera aphanis*) are the major diseases impacting cultivation (Parikka and Tuovinen, 2014). In 2020, world strawberry production was estimated at 8.8 million tonnes with a market value in the UK of over £508 million (FAOSTAT - https://www.fao.org/faostat - accessed 21/04/2022). As the majority of commercial strawberry cultivation in the UK is done in polytunnels, glasshouses and in an increasingly large portion in soilless tabletop systems, *Pc* remains a substantial threat to production (Boyer *et al.,* 2016). Tabletop production systems, which use soil-alternative substrates such as coconut husk fibre (coir), are particularly vulnerable to *Pc* infection due to its motile, asexual zoospores' ability to spread through irrigation systems.

Pc can cause leather rot and crown rot in strawberry, which can affect production at all stages and lead to substantial yield reductions (Erwin and Ribeiro, 1996). It can remain latent for several months with plants only showing symptoms under stress conditions, resulting in sudden disease outbreaks (Pettitt and Pegg, 1994). Crown rot infection was first identified in 1952 in Germany (Deutschmann, 1954). It causes wilting of the plant, usually beginning from the youngest leaves, and red-brown lesions within the crown. Reportedly, up to 40% of total strawberry crops were lost in Norway during one outbreak (Stensvand *et al.*, 1999). *Pc* is a hemi-biotrophic oomycete, switching from an initial biotrophic colonisation of the host tissue to a later necrotrophic lifestyle (Erwin and Ribeiro, 1996). It was considered to have a broad host range; however, a recent study has provided evidence that it should be considered a species complex and not a single species (Nellist *et al.,* 2021).

F. x *ananassa (Fxa)* is an allo-octoploid (2n = 8x = 56), which originated as an accidental hybrid of two wild species, *Fragaria virginiana* and *Fragaria chiloensis*, it is generally dioecious and native to the American continent (Edger *et al.*, 2019). The complex nature of the cultivated strawberry genome has made determining its evolutionary history a significant challenge. A recent study has produced the first chromosome-scale assembly of an octoploid-strawberry genome (Edger *et al.* 2019). This has shed light on the identities of the diploid progenitors that represent each of the four sub-genomes of the cultivated strawberry. Edger *et al.* (2019) suggest the existence of a tetraploid intermediate progenitor comprising *Fragaria nipponica* and *Fragaria iinumae* and of a hexaploid intermediate progenitor produced by the subsequent incorporation of the *Fragaria viridis* genome. *Fragaria vesca* (*Fv*) subsp. *bracheata* is proposed to be the last parental contributor. This thesis is supported by the geographical distribution of octoploid strawberry species, which is restricted to the north American continent with the exception of *F. chiloensis* populations found in the Hawaiian Islands and Chile (Johnson *et al.*, 2014).

Resistance to *Pc* in strawberry is polygenic in nature (Eikemo *et al*., 2003; Denoyes-Rothan *et al.,* 2004; Shaw *et al.,* 2006; Shaw *et al.,* 2008; Schafleitner *et al*., 2013; Nellist *et al*., 2019), with several genomic loci associated with resistance to *Pc* having been identified in the past decade. A study performed on *Fv* identified a major resistance-gene locus on linkage group 6 that explained 74.4% of the phenotypic variation observed in the study. The locus was named Resistance to *Phytophthora cactorum* 1 (*RPc-1*) and was found to span 3.3 Mb (Davik *et al.*, 2015). An analysis of the transcriptional response to *Pc* in *Fv* identified 26 differentially expressed genes (DEGs) in the *RPc-1* locus, including two L-type-lectin-receptor-like-kinases (RLKs), two G-type-lectin-RLKs and one receptor like protein (RLP) that were significantly up-regulated within the *RPc-1* upon *Pc* infection (Toljamo *et al.,* 2016).

A recent study performed on octoploid strawberry in a bi-parental cross between cultivars 'Emily' and 'Fenella' identified three major loci associated with resistance. These are located on linkage groups, LG6C, LG6D and LG7D and together account for 36.5% of the phenotypic variation observed in the experiment (Nellist *et al.*, 2019). Mangandi *et al.*, 2017 also found a major resistance locus to *Pc* on LG7D. While a recent metabolomics study investigated compatible *Pc-Fxa* interactions, finding 45 different metabolites to be associated with *Pc* inoculation (Toljamo *et al.,* 2021). The most highly represented class of metabolites were triterpenoids, as well as lysophospholipids, linoleic and linolenic acid (Toljamo *et al.,* 2021). These fatty acids are involved in elicitor-triggered signalling events during plant immune response (Léon *et al.,* 2002; Yaeno *et al.,* 2004; Ongena *et al.,* 2004; Viehweger *et al.,* 2006).

They are known to promote cell death in tobacco, as well as increasing susceptibility to *Phytophthora parasitica* var. *nicotianae* (Wi *et al.,* 2014). Thus, they are postulated by Toljamo *et al*. (2021) to play a role in *Pc* pathogenesis in strawberry. To date, no reports have been published on the transcriptional response of *Fxa* to challenge with *Pc*. RNA sequencing (RNAseq) is a useful technique for studying disease resistance in plants. This study focused on the *Pc* susceptible ('Emily') and a moderately resistant ('Fenella') *Fxa* cultivars to investigate resistance to *Pc*. RNAseq was utilised to examine the gene expression profiles of roots from both cultivars at 0, 12 and 48 hours post inoculation (hpi) with *Pc*. Differentially expressed genes (DEGs) regulated in both cultivars during the infection process were analysed to identify enriched classes of regulatory genes associated with *Pc* challenge, with the aim of identifying resistance gene candidates. Moreover, the pathway enrichment analysis allowed for the elucidation of the biological processes regulated during *Pc* infection, and the differences between the two cultivars. These results provide the foundation for elucidating the underpinning mechanisms of the resistance response of strawberry to *Pc.* In addition, they provide a valuable resource for the future development of *Pc*-resistant strawberry plants and future elite cultivars.

5.3 Materials and methods

5.3.1 Plant material and inoculation

In vitro root inoculation of *Fxa* cultivars 'Emily' (susceptible) and 'Fenella' (moderately resistant), as well as total RNA extraction and sequencing was carried out by and fully described in Nellist *et al.* (2021). Both cultivars were micropropagated by GenTech Propagation Ltd. and plants transferred on *Arabidopsis thaliana* salts (ATS) media (prepared as described in Taylor *et al.,* 2016) under sterile conditions. The root system was laid flat on the ATS media in individual to 120 × 120 × 15 mm, four vent, petri dishes (Corning, Gosselin) and inoculated with 1 mL of 2x10^4 zoospores of *Pc* isolate P414 suspended in compost extract. Mock inoculated plants were inoculated with 1 mL of compost extract alone. The root system was kept flat for 2 hours to allow for the zoospores to encyst before the plants were placed in a growth cabinet (Panasonic MLR-325H) at 22°C, on a 16/8 h, day/night light cycle with a photosynthetic photon flux (PPF) of 150 µmol m^{-2} s^{-1} provided by fluorescent lamps (FL40SSENW37). Three independent samples of the whole root system were collected after 0 (mock inoculation), 12 and 48 hpi starting at 8am (during the light cycle) and immediately placed in liquid nitrogen (N_2). Complete plant collapse was confirmed at 7 days post inoculation, with visible root lesions appearing between 24 and 48 hpi (data not shown).Total RNA was extracted as described in Nellist *et al.* (2021), following a modified version of the protocol described in Yu *et al.* (2012). Briefly, homogenised, frozen root material and PVPP (10% of root material weight) were added to pre-warmed (65°C) 3% CTAB extraction buffer for cell lysis. After centrifuging, an equal amount of chloroform:isoamyl alcohol (24:1) was added to the supernatant and the upper phase was transferred to a new tube after centrifugation. The same procedure was repeated for the addition of chloroform and 4M LiCl was used to precipitate the RNA. The sample was then washed with 70% EtOH and resuspended in DEPC-treated water. Sample quality was checked, and samples were sent for sequencing to Novogene to a depth of 50 million reads per sample for strawberry samples.

5.3.2 Data quality control and alignment

Read quality control was performed with FastQC (Andrews S., 2010; version 0.11.9) and the reads were trimmed using Trimmomatic (Bolger *et al.*, 2014a; version 0.32). The adapter sequences were removed, and low-quality bases (Phred quality score below 3) were deleted from both ends of the reads. The reads were also scanned with a 4-base sliding window and cut if the average quality per base dropped below 20. Reads shorter than 36 bases were discarded.

Salmon v0.9.1 (Patro *et al*., 2017) was employed to quantify transcript abundance. The latest available assembly of the *Fxa* genome (*Fragaria* x *ananassa* Camarosa Genome v1.0.a2 - Re-annotation of v1.0.a1 - https://www.rosaceae.org/Analysis/9642085; Liu *et al.,* 2021a) was used to build the mapping index, using a *k*-mer value of 31. Differential gene expression during infection was investigated using DESeq2 (Love *et al*., 2014). Genes were designated as differentially expressed if they had a DeSeq2 adjusted *p*-value (using the Benjamini and Hochberg method; *p*-adj) <0.05 and $\log_2$(Fold change) (LFC) was >|2|. Genes are referred in the text as differentially expressed if there is a significant (LFC > 2, *p*-adj < 0.05) difference between comparisons, constitutively expressed if the difference between timepoints is not significant; and constitutively differentially expressed between cultivars if the difference between timepoints is not significant, but the difference between cultivars is.

5.3.3 Gene annotation and enrichment analysis

Gene ontology (GO) and Kyoto encyclopaedia of genes and genomes pathways (KEGG) annotation were performed using eggNOG (Huerta-Cepas *et al,* 2019) and PANNZER2 (Törönen *et al.*, 2018) using the default settings. Custom gene matrix tables (GMTs) were constructed for both KEGG and GO annotations. GO and KEGG enrichment analysis were performed using g:Profiler online tool (Raudvere *et al.*, 2019). Sets of DEGs between 0 and 48 hpi, and 0 and 12 hpi in both cultivars were employed for this analysis. Sets of up and down regulated genes within those DEGs sets were also investigated to find enriched GO terms and KEGG pathways. The custom g:Profiler correction algorithm was applied, and pathways with an adjusted $p < 0.05$ were considered significantly enriched (Raudvere *et al*., 2019).

REVIGO (Supek *et al.,* 2011) was employed to reduce redundant GO terms within the up and down regulated enrichment sets and cluster them together based on semantic relationships to identify related biological processes that are co-regulated in response to infection in the two cultivars.

The plant resistance gene database's DRAGO2 (Osuna-cruz *et al.,* 2017) annotation tool was used to identify DEGs with resistance-associated motifs (RAMs). Transcription factor prediction was performed using the annotation tool provided by the Plant Transcription factor database (Tian *et al.,* 2020).

5.3.4 Co-expression network analysis

DEGs in both 'Emily' and 'Fenella' were analysed to identify co-expression networks using the WGCNA R package (Langfelder and Horvat*,* 2008; v1.70-3). DEGs between 0 and 48 hpi in either 'Emily' or 'Fenella' were selected for the generation of the co-expression network. Counts for each gene were transformed to correct for library size and, after filtering out low expressed genes, 10,324 DEGs were used in the analysis. A beta-softpower was chosen using the pickSoftThreshold function and the value that best fit the signed-network to a scale-free topology for each set (expression data belonging to 'Emily' or 'Fenella') was selected. Topological Overlap Matrix (TOM) was used to construct a hierarchical clustering tree with the hclust function ("average" method). A threshold of 0.15 (correlation > 85%) was selected and excluded modules with less than 50 genes (Langfelder and Horvath, 2008). A module eigengene (ME), or summary profile, was calculated for each module by performing principal component analysis and retaining the first one as representative for the whole module. To determine each ME's specific correlation to cultivar and infection stages, a binary indicator (stage of interest = 1, all other samples = 0) was used as described in Downs *et al.* (2013). A positive correlation indicates the genes in the module are more highly expressed in that sample set than in the rest, for that module, while a negative correlation indicates the opposite. Gene enrichment analysis for both GO terms and KEGG pathways was performed on all modules identified in the co-expression network analysis.

5.4 Results

5.4.1 Transcriptome sequencing of resistant and susceptible strawberry cultivars infected by *Pc*

The *Fxa* root transcriptome sequencing yielded a total of 788,131,018 raw reads (or 236.2 Gbp) of which 767,044,267 (97.3%, or 229.9 Gbp) were kept after quality checks. Only clean reads were employed for the subsequent alignment and quantification steps (Supplementary Table 13). The average mapping rate for the *Fxa* samples was 76.72% (Supplementary Table 14), with the rest being aligned to *Pc* genome (Nellist *et al.,* 2021) or inconclusive.

5.4.2 Identification of DEGs in moderately resistant and susceptible strawberry cultivars during the infection process

Of the 120,401 predicted *Fragaria* gene models, 82,699 (68.69%) were found to be expressed in this experiment. A total of 16,530 genes (19.99% of all expressed genes) were differentially expressed (DE) in at least one comparison between cultivars and or timepoints during infection with *Pc* isolate P414. A total of 3,940, 3,835 and 3,206 genes were found to be DE between cultivars at 0, 12 and 48 hpi, respectively, with 1,899 of those genes being constitutively DE at all timepoints (Figure 5.1). Between 0 and 12 hpi, 378 genes were found to be DE. Of those, 224 were uniquely DE in 'Emily' and 127 uniquely DE in 'Fenella' with 27 genes DE in both cultivars. A noticeably greater transcriptional change was observed between 0 and 48 hpi in both cultivars. Between 0 and 48 hpi, 4,565 and 2,445 DEGs unique to 'Emily' and 'Fenella' were observed respectively. While 3,283 DEGs were shared between the two cultivars. A set of 2,525 constitutively expressed genes, whose expression levels did not change between 0 and 48 hpi, were found to have significantly different expression levels between the two cultivars at 48 hpi, while 479 and 277 genes were DE between 0 and 48 hpi in 'Emily' and 'Fenella' and at 48 hpi between cultivars, respectively. Of those, 75 genes were DE in both cultivars as well as between the two at 48 hpi (Figure 5.1).

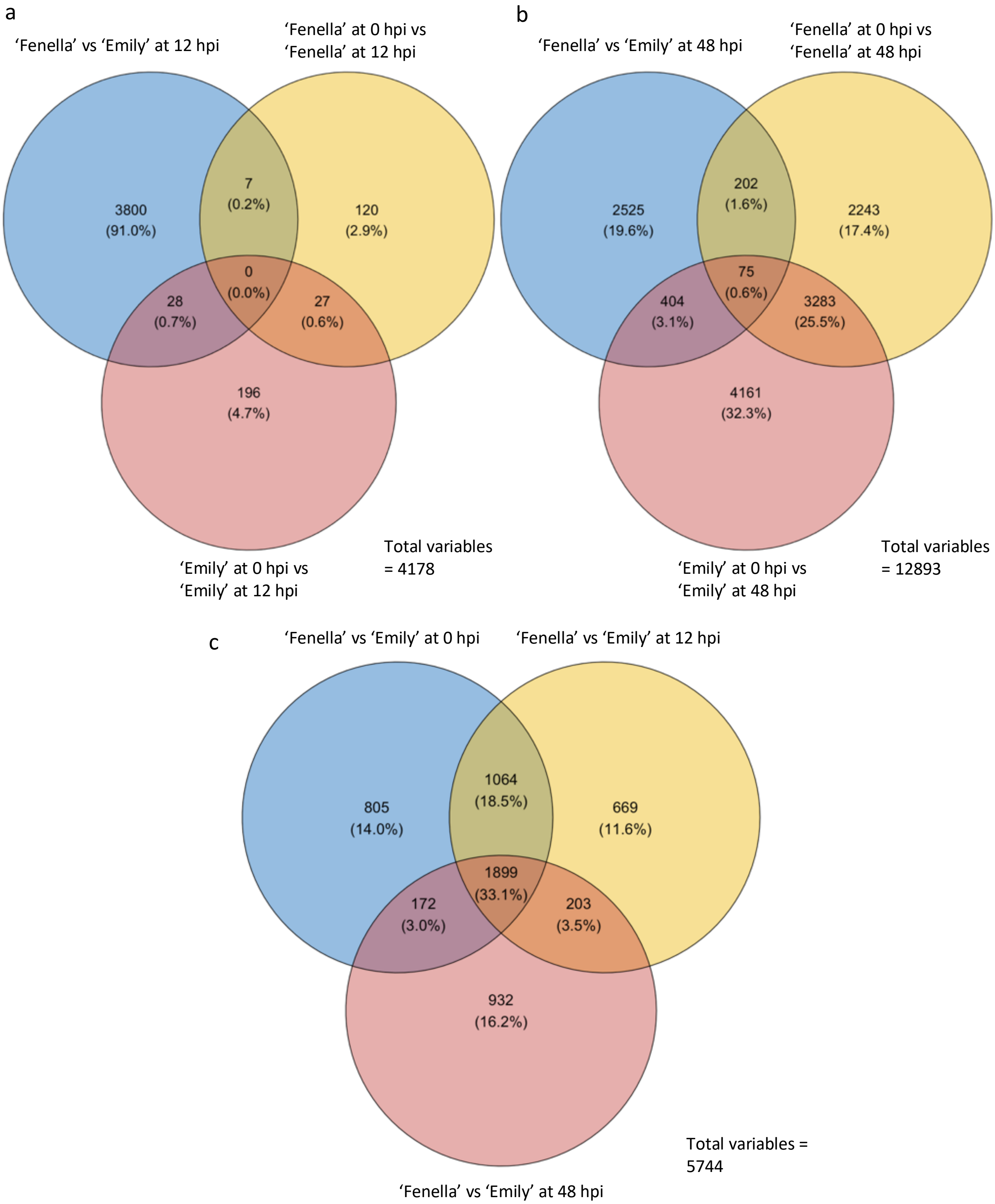


Figure 5.1. *Phytophthora cactorum* infection leads to diverse transcriptional changes in *Fragaria* x *ananassa*. Venn diagram of the differentially expressed genes (DEGs) between the varieties 'Emily' and 'Fenella', 0, 12 and 48 hours post inoculation (hpi).

Genes differentially expressed (DE) between the two varieties at each timepoint are shown in the blue circle (a and b). Genes DE between timepoints in 'Emily' and 'Fenella' are encircled in red and yellow respectively (a and b). Panel c represents the genes DE between the two varieties at each timepoint. The percentage of genes is shown below the number of genes in each group.

In ‘Emily’, 184 genes were down-regulated (LFC < -2) between 0 and 12 hpi, while 67 were up-regulated (LFC > 2) (Figure 5.2a). By 48 hpi, 3,955 and 3,968 genes were down-regulated and up-regulated compared to 0 hpi, respectively (Figure 5.2c). In ‘Fenella’, 71 and 83 genes were down-regulated and up-regulated between 0 and 12 hpi, respectively (Figure 5.2b). While 2,441 and 3,362 genes were down-regulated and up-regulated between 0 and 48 hpi, respectively (Figure 5.2d).

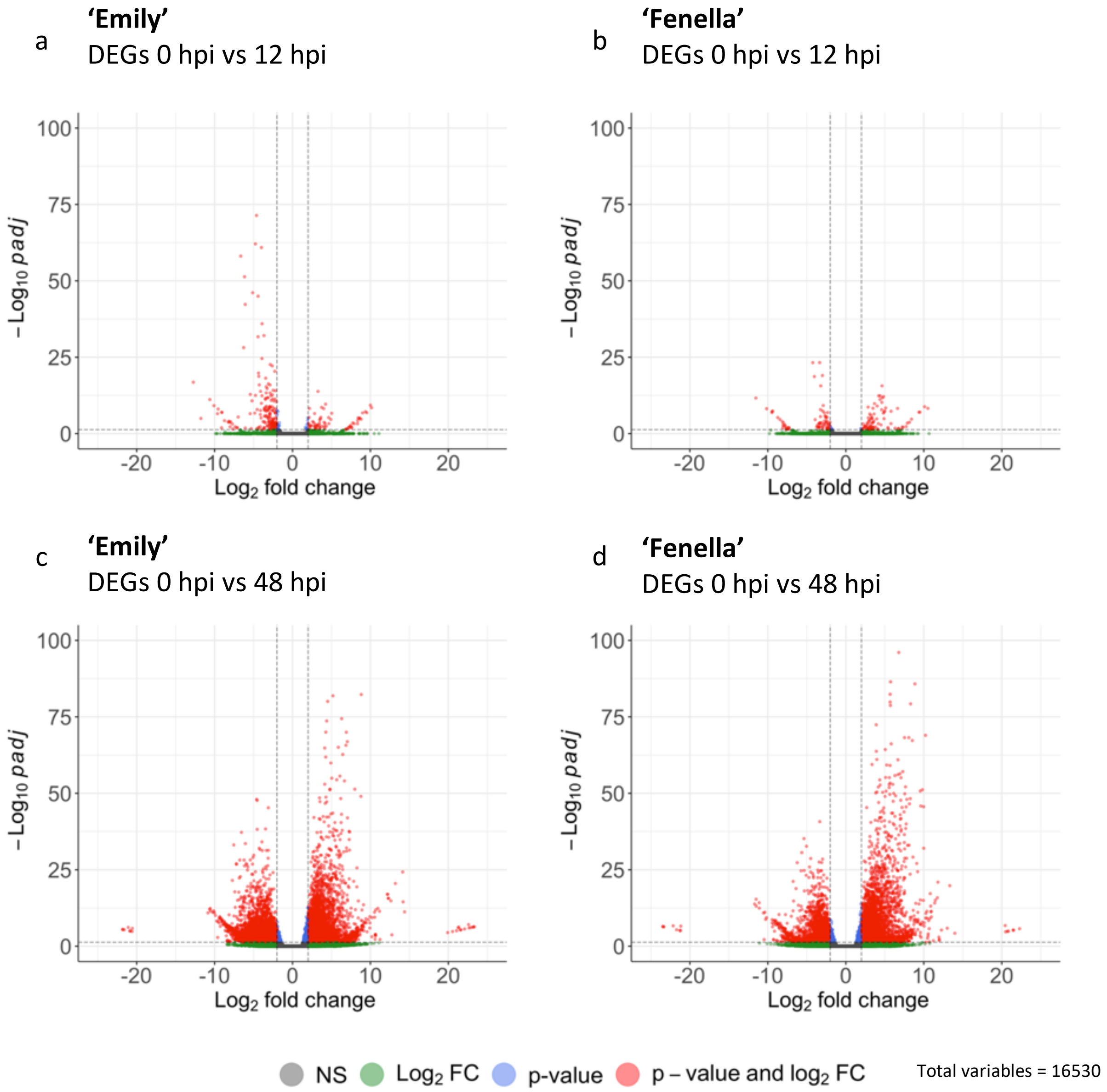


Figure 5.2. Volcano plots show a marked difference in the number of differentially expressed genes (DEGs) in response to *Phytophthora cactorum* infection between timepoints, with the greatest response observed at 48 hours post inoculation (hpi).

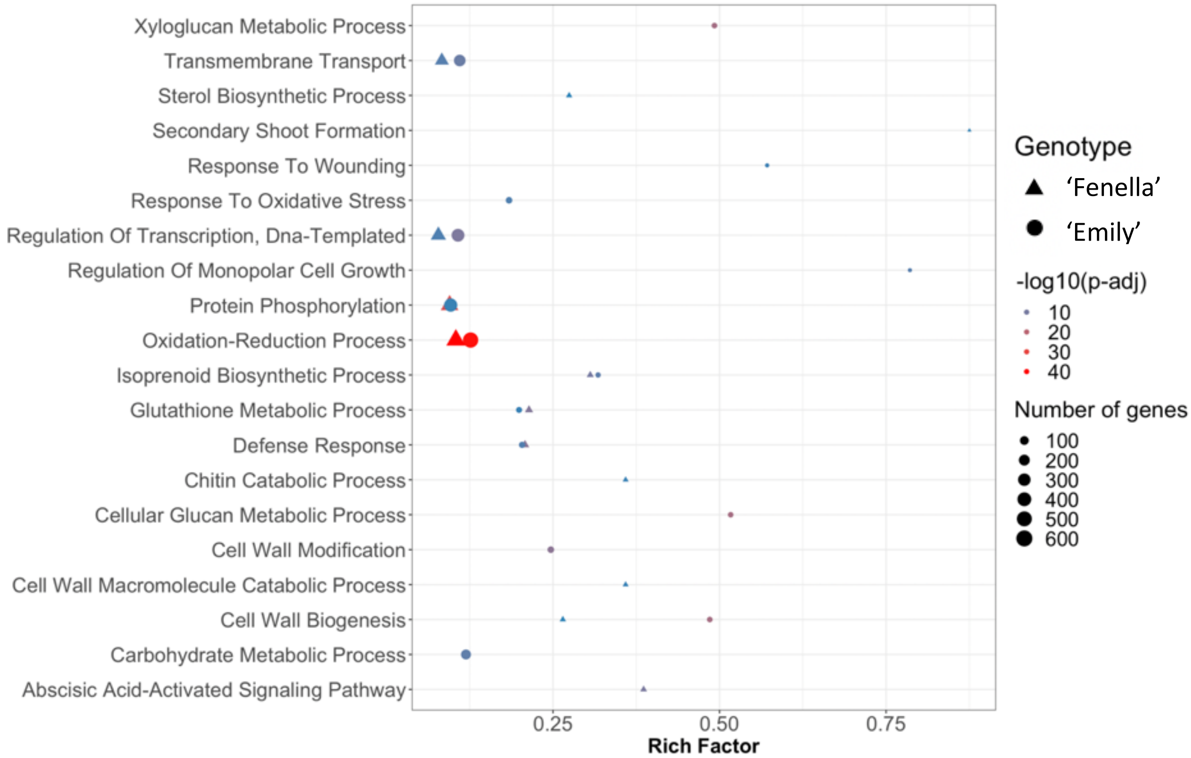
Genes are plotted according to their adjusted *p*-value (y-axis) on a -Log_{10} scale and their change in expression (x-axis) presented on a Log_2 scale. Genes are coloured according to whether the differential expression is significant by *p*-value (blue), fold change (Log_2(FC); green), both (p-value and log_2(FC); red) or is not significant (NS; grey).

5.4.3 Gene set enrichment analysis reveals differential regulation of pathways associated with resistance

DEGs in both 'Emily' and 'Fenella' were investigated using gene set enrichment analysis to identify KEGG pathways and GO terms associated with *Pc* infection. Between 0 and 12 hpi, no GO terms were found to be significantly enriched in the DEGs sets from either cultivar. However, both cultivars showed significant enrichment for the KEGG pathways 'Photosynthesis - antenna proteins' and 'Circadian rhythm – plant' (ko00196 and ko04712), while only 'Fenella' showed significant enrichment for the 'Sesquiterpenoid and triterpenoid biosynthesis' KEGG pathway. Between 0 and 48 hpi, a total of 23 KEGG pathways and 66 GO terms were found to be significantly enriched in 'Emily', while 24 KEGG pathways and 68 GO terms were significantly enriched in 'Fenella' (Figure 5.3 and Supplementary Table 15). In both 'Emily' and 'Fenella', the most significantly enriched GO biological process (BP) is 'oxidation-reduction process' (GO:0055114), followed by several growth-related processes including 'cell wall biogenesis' (GO:0042546), cell-wall components metabolism, as well as processes related to the development of other growth-related structures and transmembrane transport. Both sets of DEGs were enriched for pathogen response-related GO BPs, including 'defence response' (GO:0006952), 'response to oxidative stress' (GO:0006979) and BPs linked to the production secondary metabolites related to plant immune responses. Notably, 'abscisic acid-activated signalling pathway' (GO:0009738), 'auxin-activated signalling pathway' (GO:0009734) and 'chitin catabolic process' (GO:0006032) were enriched in 'Fenella' but not in 'Emily', while the opposite is true for 'response to wounding' (GO:0009611; Figure 5.3a and Supplementary Table 15). Several KEGG pathways related to plant immune response were enriched in both sets of DEGs, such as 'Plant-pathogen interaction' (ko04626) and 'MAPK signalling pathway – plant' (ko04016), as well as a number of pathways associated with the production of secondary metabolites (Figure 5.3b and Supplementary Table 16).

To further explore the differences in transcriptional response to *Pc* challenge, GO enrichment analysis was also performed in the up- and down-regulated gene sets for both cultivars and the significantly enriched GO terms were clustered based on semantic relationships using the web-tool REVIGO (Supplementary Table 17). A total of 13 down-regulated and seven up-regulated clusters were identified. Both cultivars show a marked down-regulation of metabolic processes associated with growth and proliferation. The most significantly enriched GO term in the up-regulated gene sets for both cultivars was 'oxidation-reduction process' (GO:0055114), with both cultivars sharing several defence-associated processes including 'defence response', 'response to wounding', 'abscisic acid-activated signalling pathway' and 'chitin catabolic processes' (GO:0006952, GO:0009611, GO:0009738 and GO:0006032, respectively). Additionally, several catabolic processes relating to cell-wall and metabolic processes of several defence-associated secondary metabolites are also up-regulated. Notably, the 'negative regulation of cell death' (GO:0060548) term is enriched in 'Fenella' but not in 'Emily'. The number of DEGs in the 'abscisic acid-activated signalling pathway' up-regulated in 'Fenella' is close to double the number of DEGs in 'Emily', while the opposite is true for the 'response to wounding' GO term (Supplementary Table 18).

a



b

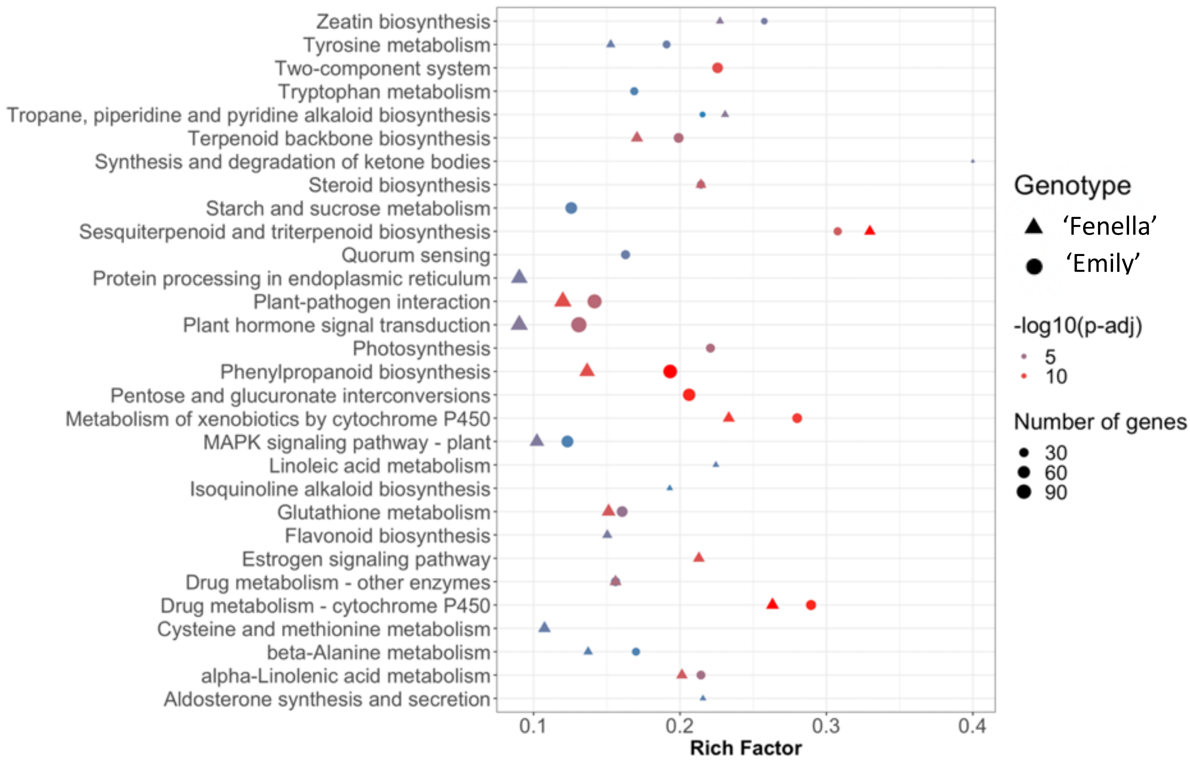


Figure 5.3. Gene ontology (GO) and Kyoto encyclopaedia of genes and genomes (KEGG) pathway enrichment analysis outlines a complex physiological response in *Fragaria* x *ananassa* 'Emily' and 'Fenella' to *Phytophthora cactorum* (*Pc)* inoculation.

Differentially expressed genes (DEGs) in both 'Emily' and 'Fenella' between 0 and 48 hours post inoculation (hpi) were further investigated using gene set enrichment analysis to identify molecular pathways associated with *Pc* infection, utilising GO term (a) annotations and KEGG pathway, (b) the 20 most significant GO terms for biological processes and 20 most significant KEGG pathways from each were plotted.

5.4.4 Gene co-expression analysis identifies 11 co-expression modules

The full set of DEGs between 0 and 48 hpi in both cultivars was employed to construct gene co-expression network modules, based on normalised expression values for each gene. The analysis identified a total of 11 co-expression modules ranging from 70 to 5,013, with 20 genes not being assigned to any module (Figure 5.4). Gene enrichment analysis was performed on all modules, using both GO and KEGG annotations. Module 1 (turquoise) contains almost half of the genes analysed and is enriched for several infection related processes including the ‘defence response’, ‘response to wounding’, ‘abscisic acid-activated signalling pathway’ and ‘chitin catabolic processes’ pathways. It is further enriched for oxidation-reduction processes, protein modification, calcium-ion transport, as well as the synthesis of several secondary metabolites. Module 2 (blue) is enriched for several metabolic processes such as ‘photosynthesis’ (GO:0015979), ‘cell wall biogenesis’ (GO:0042546) and ‘regulation of monopolar cell growth’ (GO:0051513), as well as plant hormone signal transduction and the synthesis of several secondary metabolites. Module 3 (brown) is principally enriched for hormone signalling and signal transduction processes, ‘auxin-activated signalling pathway’ (GO:0009734), ‘response to hormone’ (GO:0009725) and ‘transmembrane transport’ (GO:0055085) are all significantly enriched for. Modules 4, 5, 6 and 10 (yellow, green, red and purple, respectively) are enriched for processes related to plant growth, cell-wall biogenesis and modification. Interestingly, Module 11 (lime green) was found to be enriched for ‘systemic acquired resistance’ (GO:0009627) and ‘fatty acid binding’ (GO:0005504; Supplementary Table 19 and 20).

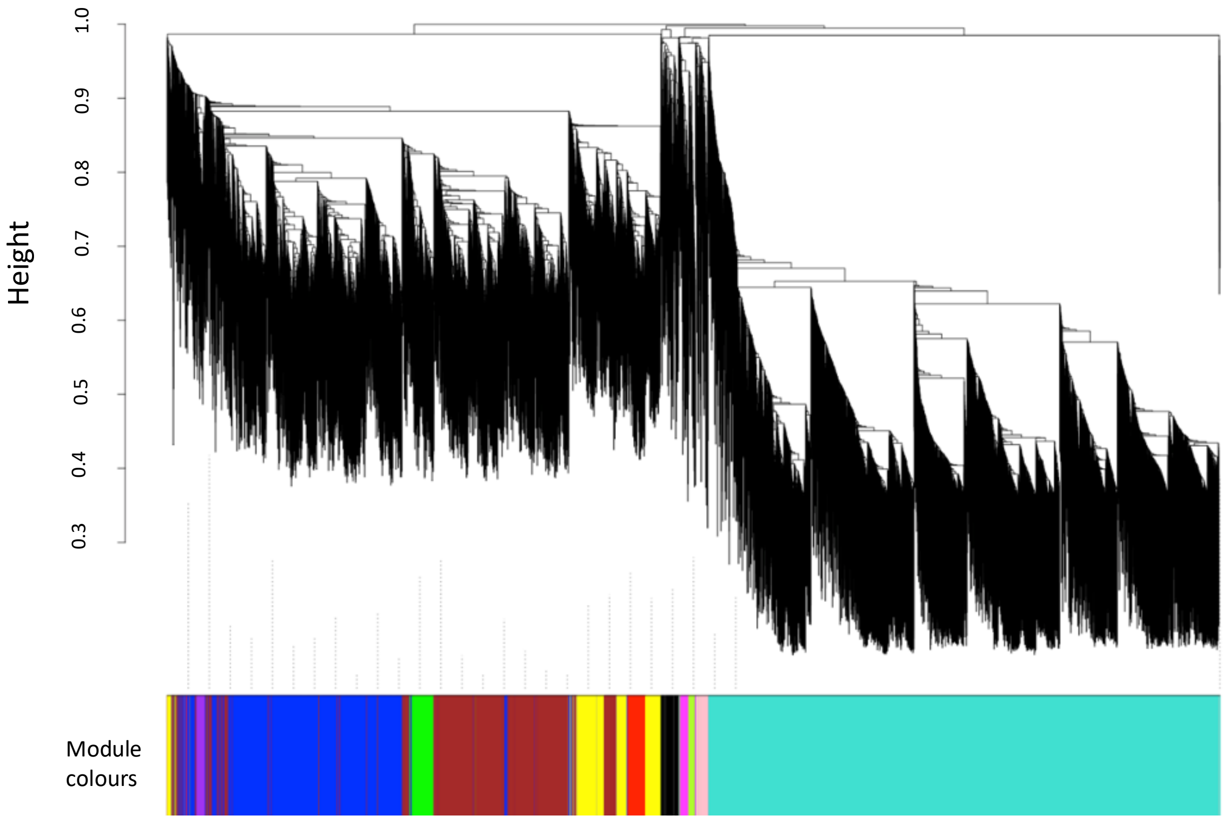


Figure 5.4. Differentially expressed genes (DEGs) between 0 and 48 hpi in 'Emily' and 'Fenella' group into eleven distinct co-expression networks.

The y-axis indicates the co-expression distance between genes, the x-axis represents the genes included in the analysis, each colour indicates a separate module.

The MEs generated for the 11 modules identified by co-expression analysis were employed to investigate the correlation between each module and the samples from each timepoint and cultivar. At 48 hpi, Module 1 was significantly positively correlated to samples from both 'Emily' and 'Fenella'. The opposite was true for Modules 2, 3, 5 and 6. Modules 4 and 10 showed a significant negative correlation in 'Emily', but not in 'Fenella'. Module 7 was found to have a positive correlation to 'Emily' and a negative correlation to 'Fenella', while Modules 8 and 9 showed the opposite pattern of correlation. Finally, Module 11 is significantly correlated to 'Fenella' (Figure 5.5).

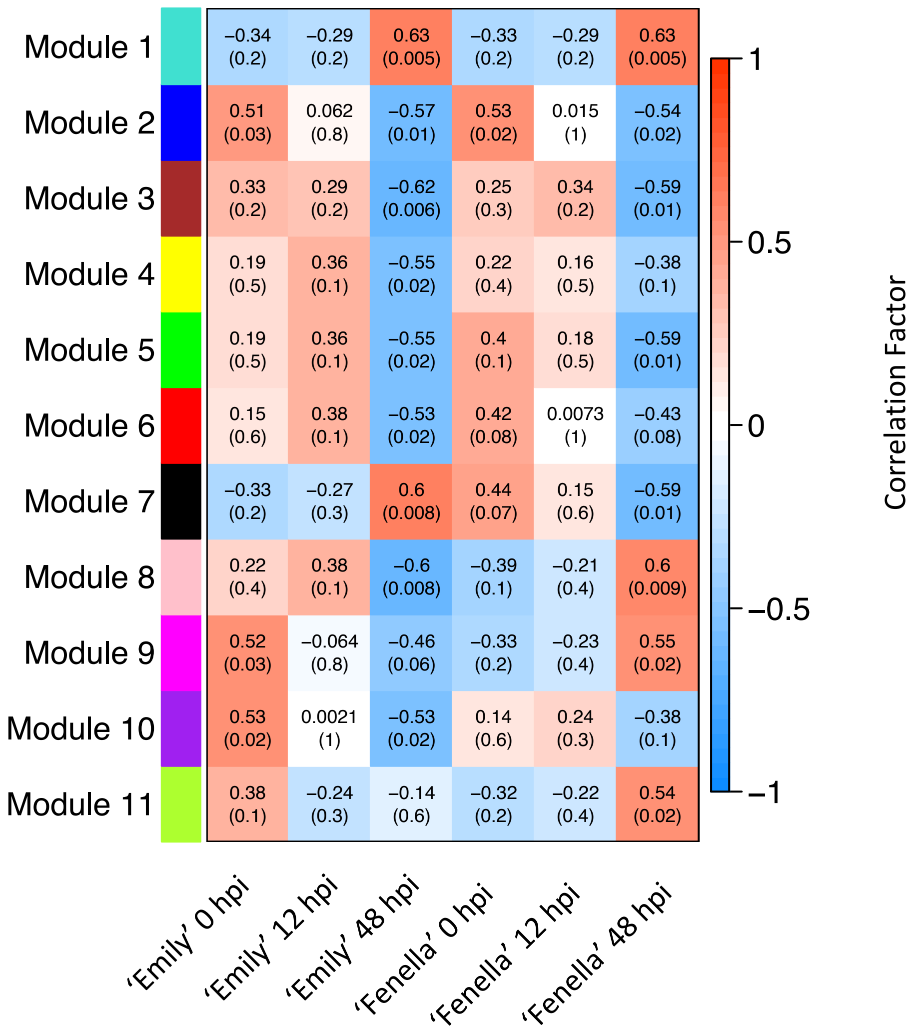


Figure 5.5. Modules show different levels of correlation to timepoints and varieties.

The heatmap shows the level of correlation between a module and the variety at each timepoint, statistical significance is shown underneath the correlation factors as *p*-values in brackets. The larger the |value| of the correlation factor, the greater the correlation is between the modules and the samples. Positive numbers indicate higher or preferential gene expression in that sample set, while the opposite applies to negative numbers.

5.4.5 Regulation of transcription factors and RAM-containing genes

Using the ‘Plant Transcription factor database’ (Tian *et al.,* 2020), a total of 6,378 putative transcription factors (TFs) were found to be present in the *Fxa* genome. Of those, 1,167 were either constitutively differentially expressed between the two *Fxa* cultivars or between 0 hpi and one or more of the time points sampled during the experiment. Within the set of DEGs annotated, genes were putatively assigned to 46 TF classes (Supplementary Table 21), including AP2, ERF, NAC, MYB, bHLH, WRKY and bZIP. Ninety-seven of the genes belonging to the WRKY TF family were found to be DE. *FxaC_17g43140.t1*, which encodes a putative WRKY9 transcription factor, and was found to be significantly down-regulated between 0 and 48 hpi (LFC = -2.84, *p*-adj = 0.003) in 'Fenella' while remaining constitutively expressed at higher levels in 'Emily'. Another gene (*FxaC_28g25912.t1*), putatively assigned to the WRKY30 transcription factor family, was constitutively expressed in 'Fenella' at significantly higher levels (LFC = 4.53, *p*-adj = 7.03 x 10^{-9}). Notably two genes putatively annotated as WRKY family were assigned as homologues of the *A. thaliana* gene *AT5G45050.1*, which encodes a TF-like gene involved in defence-related signal transduction. One of them (*FxaC_25g28300.t1*), was found to be constitutively expressed in the moderately resistant cultivar 'Fenella', while it had significantly lower expression (LFC = 5.53, *p*-adj = 2.18 x 10^{-17}) in the susceptible cultivar 'Emily'.

The 'plant resistance gene database' DRAGO2 annotation tool (Osuna-cruz *et al.,* 2017), found a total of 6,904 genes with putative RAMs in the *Fxa* genome. Of those, 1,337 were DE in our experiment, with a total of 27 different RAMs classes assigned to them; they comprised a total of 602 putative protein kinases, 259 receptor-like kinases and 131 receptor-like proteins. The remaining classes of RAMs identified in DEGs included CC, NBS, ARC and LRR motifs (Supplementary Table 22).

5.4.6 The most highly DEGs between varieties upon *Pc* inoculation

The top 100 genes DE between cultivars at 48 hpi ranked by LFC, that were also significantly regulated during the experiment, were selected for further analysis. Notable genes are highlighted in Figure 5.6. *FxaC_21g06310.t1,* a zinc finger AN1 domain-containing stress-associated gene, was the most up regulated gene in 'Fenella' between 0 and 48 hpi (LFC = 11.39, *p*-adj = 7.74 x 10^{-15}), followed by *FxaC_10g13200.t1, FxaC_13g13200.t1, FxaC_19g09450.t2* and *FxaC_27g25630.t1.* These genes putatively code for a ribosomal protein in the eL27 family , a cytochrome p450 family protein , a mitochondrial carrier TC 2.A.29 family protein and an oxidoreductase , respectively. The most down-regulated between 0 and 48 hpi was a serine-arginine repetitive matrix gene (*FxaC_21g06310.t1;* LFC = -23.42, *p*-adj = 4.03 x 10^{-7}), followed by *FxaC_5g13320.t2, FxaC_11g35780.t2,* *FxaC_24g12100.t1* and *FxaC_13g00320.t2*. These genes putatively code for a heat stress TF , a protein kinase superfamily protein , a *Rtr1/RPAP2* family protein and an enhancer of *AG-4* protein , respectively. In 'Emily' the most up-regulated gene between 0 and 48 hpi was *FxaC_13g11560.t1*, a NIM1-interacting protein-coding gene (LFC = 9.20, *p*-adj = 1.26 x 10^{-8}), followed by *FxaC_13g01540.t1, FxaC_17g16930.t1, FxaC_9g48110.t2* and *FxaC_1g11480.t1*. These genes putatively code for a protein involved in the accumulation and replication of chloroplasts , a receptor-like protein , a GDSL lipolytic enzyme family protein and a phosphoserine aminotransferase , respectively. The most down-regulated gene between 0 and 48 hpi was *FxaC_18g40860.t5*, a peroxisomal membrane protein (LFC = -10.03, *p*-adj = 3.80 x 10^{-7}), followed by *FxaC_13g53340.t2, FxaC_10g14950.t1, FxaC_26g24660.t1* and *FxaC_12g42490.t2.* These genes putatively code for a zinc knuckle CCHC-type family protein , a methyladenine glycosylase , a sulfotransferase 1 family protein and a protein of unknown function, respectively.

Out of the top 100 DEGs, eleven were found to putatively contain RAMs domains. The most DE RAM-containing gene between the two cultivars at 48 hpi was the G-type lectin S-receptor-like serine threonine-protein kinase gene *FxaC_15g11860.t1* (LFC = 10.11, *p*-adj = 2.5 x 10^{-11}). Three other genes containing putative kinase domains were amongst the top 100 DEGs, as well as two LRR-receptor-like protein-coding genes homologous to the *CLAVATA2* gene. The pathogenesis related protein 1-like gene (*FxaC_7g01820.t1)* was found to be down-regulated in 'Emily' upon infection (LFC = -6.62, *p*-adj = 0.1 x 10^{-1}). Only two putative TF genes were found in the top 100 DEGs. *FxaC_13g11630.t4*, a MADS-box-like putative TF, was found to be down-regulated in 'Fenella' between 0 and 48 hpi (LFC = -6.47, *p*-adj = 0.2 x 10^{-1}). While the WRKY domain containing gene (*FxaC_25g22040.t1*) was up-regulated in 'Emily' (LFC = 2.02, *p*-adj = 0.2 x 10^{-2}).

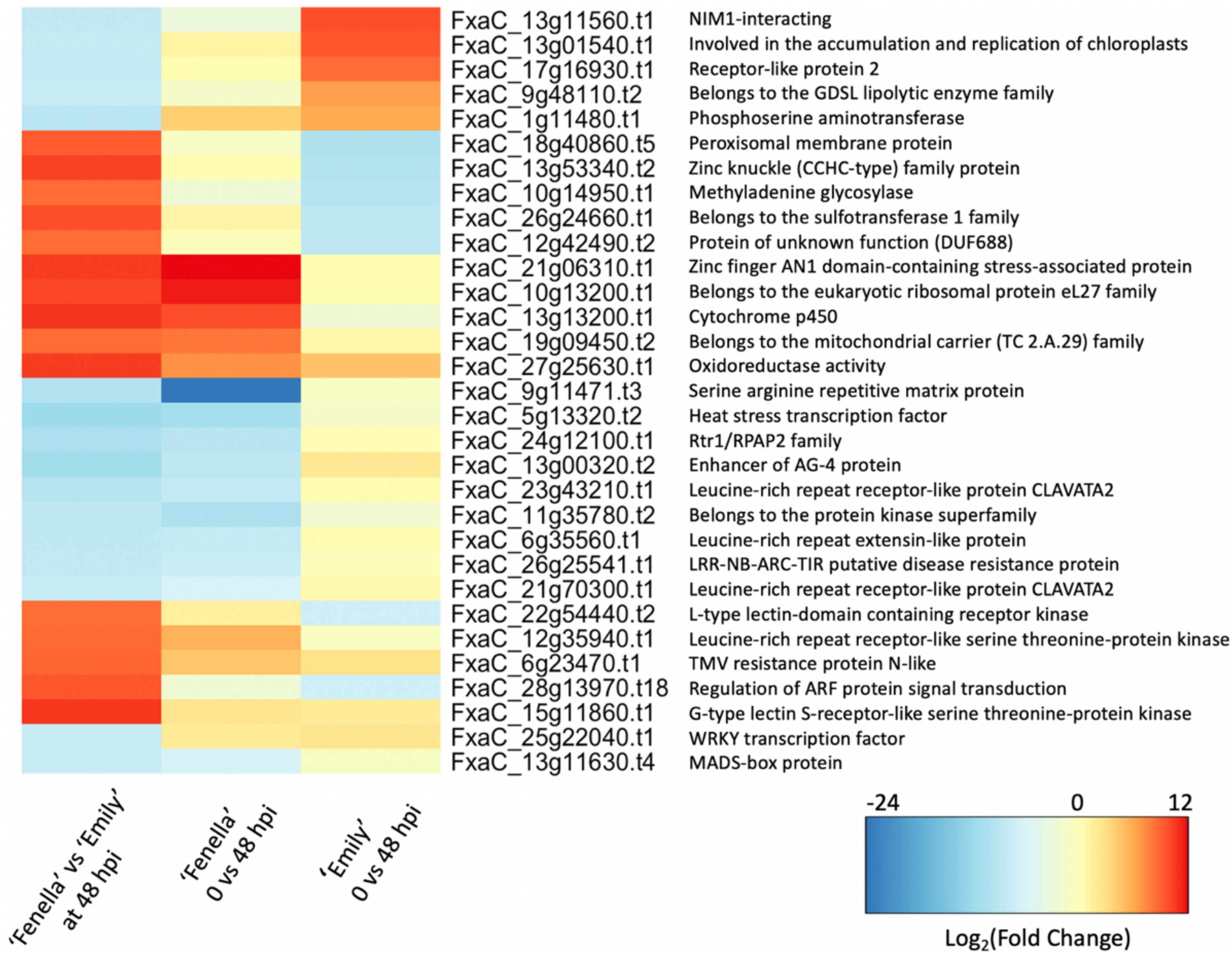


Figure 5.6. Heatmap of notable genes in the top 100 most highly differentially expressed genes (DEGs) ranked by $\log_2$(Fold change) (LFC).

The expression profiles of the top 5 most up- and down-regulated genes between 0 and 48 hours post inoculation (hpi) in both 'Emily' and 'Fenella' that were also differentially expressed (DE) between varieties, as well as the resistance-associated motif containing genes and putative transcription factors most highly DE between 0 and 48 hpi and between varieties, are plotted in a heatmap of the LFCs for each comparison. Descriptions of the putative function of the protein encoded by the gene is given on the righthand side of each.

5.4.7 DEGs found within previously identified putative resistance QTLs

Four large-effect QTL have previously been putatively associated with resistance to *Pc* in *Fxa* *FaRPc2* (Mangandi *et al.,* 2017), *FaRPc6C*, *FaRPc6D* and *FaRPc7D* (Nellist *et al.,* 2019). DEGs within 1.5Mb of the most significant marker for each locus were selected for further analysis (Figure 5.7). A total of 265 DEGs were found in the four QTLs. Of the three major effect QTLs reported by Nellist *et al.* (2019); 36, 62 and 114 DEGs were located near loci *FaRPc6C* (chromosome 6-4), *FaRPc6D* (chromosome 6-2) and *FaRPc7D* (chromosome 7-3), respectively. The region surrounding the QTL reported by Mangandi *et al.* in 2017 (*FaRPc2*, chromosome 7-3) contained 148 DEGs. The *FaRPc2* and *FaRPc7D* QTL regions are located on the same chromosome and 95 DEGs are located within the overlapping region between the two (Supplementary Table 23).

Several DEGs with putative functions associated with plant immune response were found in the regions surrounding the known QTLs. Two protein kinase genes located within the *FaRPc6C* region were found to be DE. *FxaC_24g33150.t1*, encoding a calcium-dependent kinase, was up-regulated between 0 and 48 hpi in both 'Emily' (LFC = 2.00, *p*-adj = 0.13×10^{-2}) and 'Fenella' (LFC = 2.08, *p*-adj = 7.50×10^{-6}). The L-type lectin-domain containing receptor kinase gene (*FxaC_24g34031.t1*) was similarly up-regulated in both cultivars (LFC = 2.11, *p*-adj = 0.15×10^{-1}, in 'Emily'; LFC = 2.43, *p*-adj = 0.64×10^{-2}, in 'Fenella'). Four DE putative TF genes were also found in the region, as well as two F-box and F-box related genes. *FxaC_24g35720.t4*, one of the two F-box related genes, was found to be up-regulated in 'Fenella' between 0 and 48 hpi (LFC = 2.14, *p*-adj = 7.99×10^{-6}), but not in 'Emily'. A glucan endo-1,3-beta-glucosidase-like gene (*FxaC_24g34700.t1*) was found to be down-regulated in 'Emily' (LFC = -3.45, *p*-adj = 0.28×10^{-1}), but not in 'Fenella' (Figure 5.7 and Figure 5.8).

The genomic region surrounding the most significant SNP within *FaRPc6D* was found to contain three DE protein kinase genes. *FxaC_23g57060.t1*, a serine threonine-protein kinase WNK8-like gene, was up-regulated in both cultivars between 0 and 48 hpi (LFC = 6.28, *p*-adj = 2.89×10^{-11}, in 'Emily'; LFC = 4.65, *p*-adj = 0.93×10^{-2}, in 'Fenella'). A wall-associated receptor kinase gene (*FxaC_23g60890.t1*) was constitutively more highly expressed in 'Emily' than 'Fenella' (LFC = |3.82|, *p*-adj = 1.54×10^{-10} at 48 hpi). Two Cu-oxidase genes (*FxaC_23g59800.t1* and *FxaC_23g59990.t1*) involved in lignin degradation, were up-regulated in 'Emily' between 0 and 48 hpi (LFC = 5.12, *p*-adj = 3.42×10^{-14}; LFC = 3.20, *p*-adj = 0.19×10^{-3}, respectively). Notably, a resistance to powdery mildew 8 (*RPW8*) encoding putative resistance gene (*FxaC_23g57270.t1*) was found to be constitutively more highly expressed in 'Fenella' than in 'Emily' (LFC = 8.32, *p*-adj = 1.39 x 10^{-6} at 48 hpi; Figure 5.7 and Figure 5.8).

The region of chromosome 7-3 (7D) that encompasses the two QTLs (*FaRPc2* and *FaRPc7D*) was found to contain 13 DE putative protein kinase genes. Four of these were located within the *FaRPc2* locus, one within the *FaRPc7D* locus and eight were found on the overlap region. Five of the putative protein kinase domain-containing genes (*FxaC_26g08080.t1*, *FxaC_26g06560.t1*, *FxaC_26g06510.t1*, *FxaC_26g07721.t1* and *FxaC_26g03040.t1*) were constitutively DE between the two cultivars at 48 hpi (LFC > |2|, *p*-adj < 0.05; see Supplementary Table 23), all of them were expressed higher in 'Emily' than in 'Fenella'. A total of 12 putative TF genes were found to be DE. Two of these were located within the *FaRPc2* locus, two within the *FaRPc7D* locus and eight were found in the overlapping region. Three of them (*FxaC_26g07730.t1*, *FxaC_26g06360.t1* and *FxaC_26g06040.t1*) were found to be constitutively DE between the two cultivars at 48 hpi (LFC > |2|, *p*-adj < 0.05; see Supplementary Table 23), all of them were expressed higher in 'Emily' than in 'Fenella'. *FxaC_26g05440.t1*, a putative TF gene belonging to the WRKY30 TF family found in the overlapping region of the two QTL, was DE in both cultivars upon *Pc* infection (LFC = 2.80, *p*-adj = 0.48 x 10^{-1}, in 'Emily'; LFC = 4.87, *p*-adj = 2.08 x 10^{-9}, in 'Fenella'). Finally, a DEG (*FxaC_26g07110.t1*) containing both a F-box an LRR-repeat motif and located within the *FaRPc2* locus was found to be constitutively expressed at higher levels in 'Emily' compared to 'Fenella' (LFC = 5.45, *p*-adj = 0.59 x 10^{-2}; Figure 5.7 and Figure 5.8).

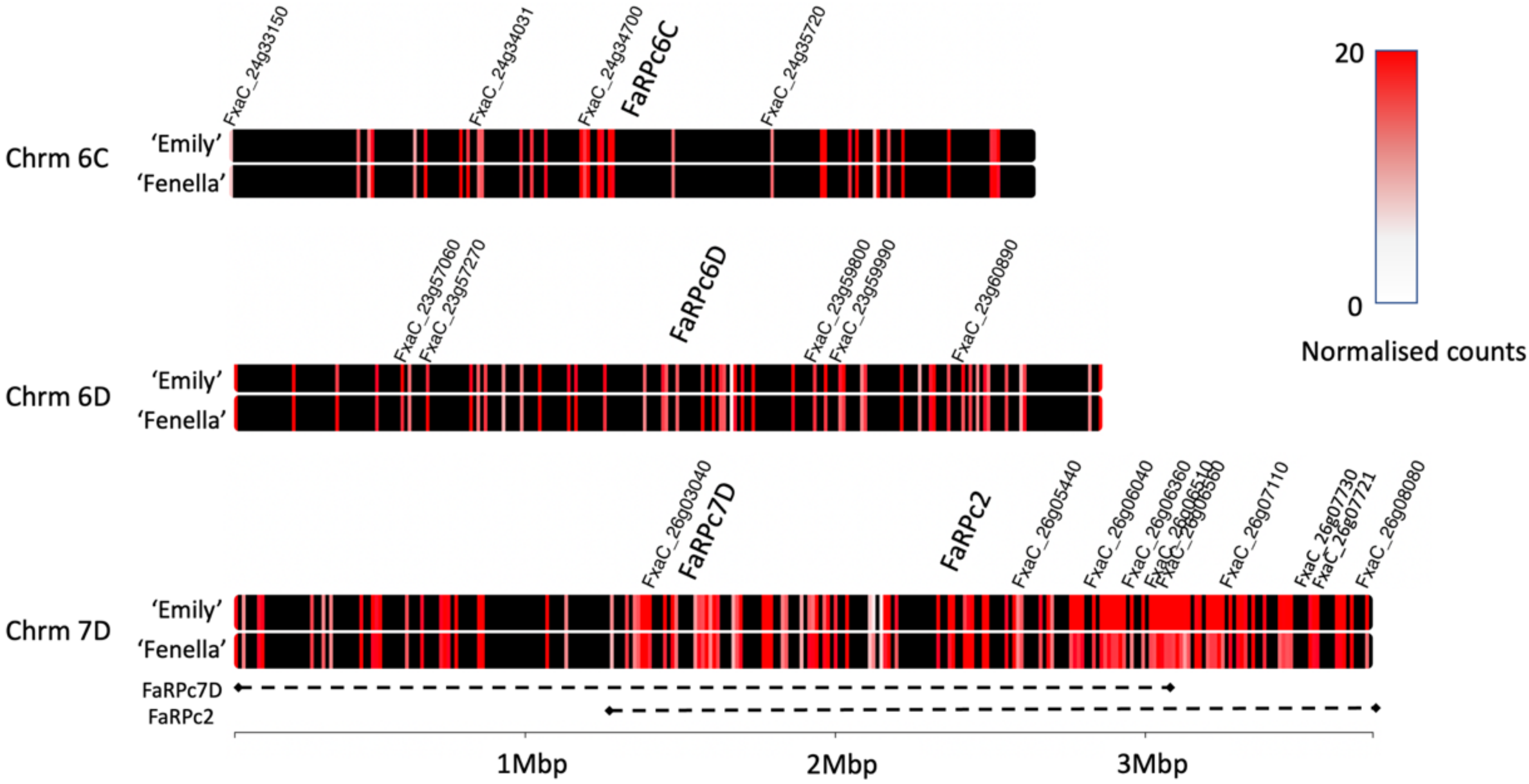


Figure 5.7. Heat-map showing the differentially expressed genes (DEGs) near the known resistance QTL.

Coloured regions represent genes that were found to be differentially expressed upon *Phytophthora cactorum* inoculation within 1.5 Mbp of the most significant marker (indicated by the name of the quantitative trait loci, QTL) for each of the known putative resistance QTLs identified in *Fragaria x ananassa.* The colour denotes how highly expressed each gene is at 48 hours post inoculation. Notable DEGs are named.

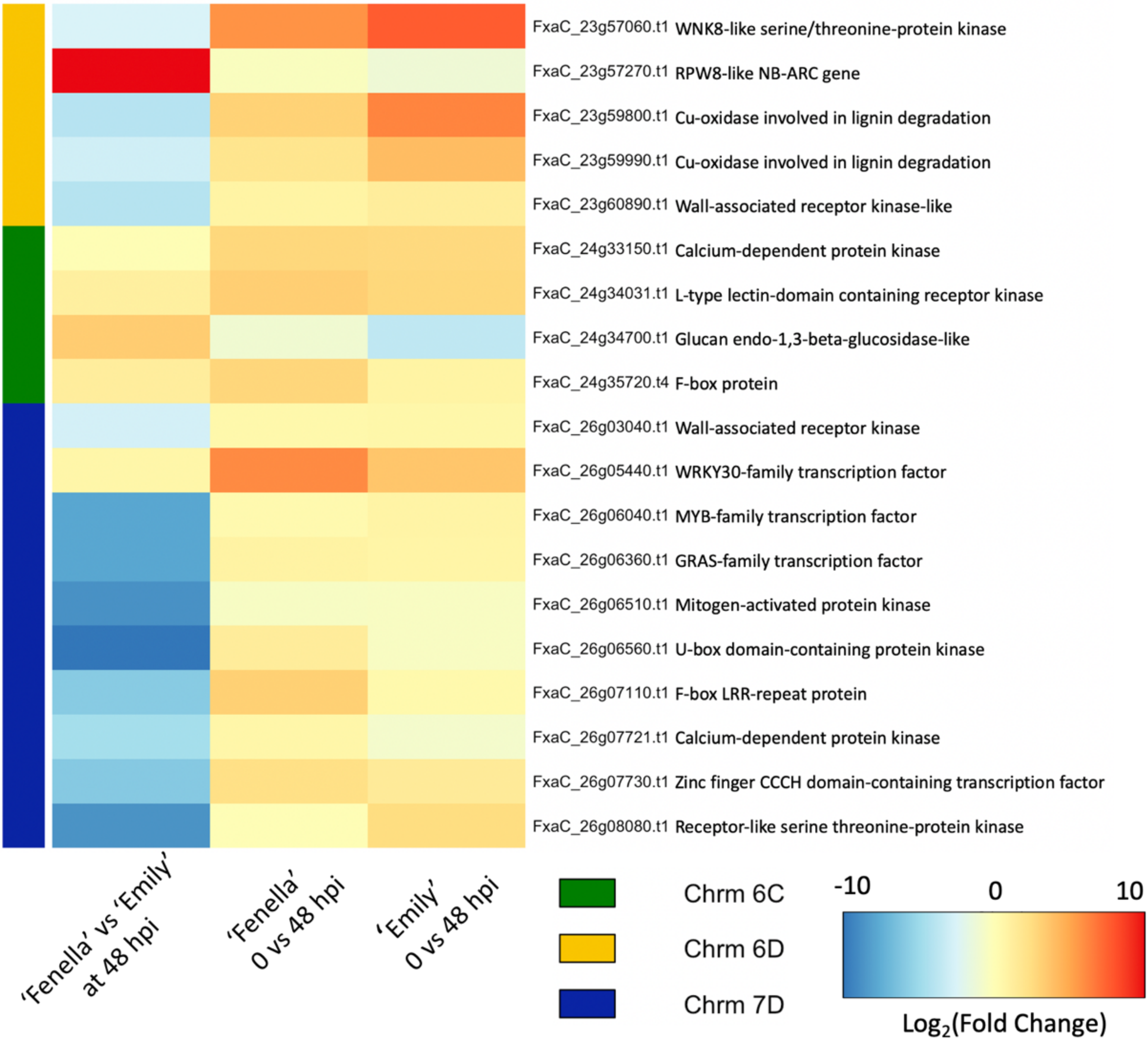


Figure 5.8. Heatmap of notable differentially expressed genes (DEGs) located within the known putative resistance QTL regions.

The heatmap shows the expression patterns of selected DEGs. Log_2(Fold change) values are provided for each gene. Descriptions of the putative functions of the protein encoded are shown next to each gene. The row-side coloured box indicates which chromosome the DEG is located on.

5.5 Discussion

The strawberry cultivars 'Emily' and 'Fenella' were released by NIAB EMR in 1995 and 2009, respectively. 'Emily' is susceptible to *Pc,* while 'Fenella' displays moderate resistance. A large-scale transcriptional response was observed upon *Pc* inoculation, with almost a fifth of all expressed genes being differentially regulated. The patterns of transcriptional reprogramming observed during *Pc* infection of the *Fxa* cultivars analysed in this study highlight the negative regulation of programmed cell-death as a potential determinant of resistance.

5.5.1 Cultivars show similarities as well as key transcriptional differences in pathway activation in response to inoculation

Both cultivars showed a notable down-regulation of genes associated with plant growth and metabolic processes. Gene enrichment analysis of down-regulated DEGs in both cultivars showed enrichment of several processes associated with plant growth mechanisms. Cell wall biogenesis, cellulose synthesis, monopolar cell growth, secondary root formation and cell wall synthesis associated GO terms and KEGG pathways were all enriched for in down-regulated gene sets at 48 hpi with *Pc.* Moreover, of the 11 co-expression modules identified, five (Modules 2, 3, 4, 5, 6) were significantly ($p < 0.05$) enriched for plant growth-related processes. The significant negative correlation between samples at 48 hpi in both cultivars and the modules indicate the genes in these modules were preferentially negatively regulated compared to the rest of the samples. Taken together, the results of the co-expression analysis and gene set enrichment analysis support the idea that *Fxa* down-regulates growth-related processes in response to infection. This is in line with the biotic stress response observed in other plant species, including *F. vesca* (Rojas *et al.,* 2014; Toljamo *et al.,* 2016; Toljamo *et al.,* 2021).

Likewise, both cultivars display similar up-regulation of many GO terms and KEGG pathways representing biological processes related to the plant immune response. 'defense response', 'response to wounding', several signal transduction-related processes, 'chitin catabolic processes' and 'cell wall macromolecule catabolic processes' are enriched in both 'Emily' and

‘Fenella’ up-regulated genes set, as well as calcium ion transportation. All these pathways are also enriched for in Module 1 of the gene co-expression network, which is positively correlated to both cultivars at 48 hpi. This supports the idea that both cultivars show a strong immune response to *Pc* inoculation. Though some notable pathways were found to be uniquely enriched for in a cultivar-specific manner. Up-regulation of the ‘negative regulation of cell death’ pathway was observed in ‘Fenella’ at 48 hpi, while the ‘lignin catabolic process’ and the ‘cell wall modification’ pathways are up-regulated in ‘Emily’, but not in ‘Fenella’ at 48 hpi. Lignin accumulation is known to enhance resistance to pathogens (Xu *et al.,* 2011; Lee *et al.,* 2019; Li *et al.,* 2020d; Wang *et al.,* 2020; Cao *et al.,* 2021; Xiao *et al.,* 2021); thus, lignin catabolism could help accelerate pathogen tissue colonisation of *Fxa* (Fujimoto *et al.,* 2015; Xu *et al.,* 2011). As *Pc* is known to have a hemi-biotrophic lifestyle, it is possible that inducing cell death in the host could serve as a means to facilitate tissue colonisation during the necrotrophic life phase of the oomycete (Midgley *et al.,* 2022).

5.5.2 Gene set enrichment analysis reveals the complex role of phytohormones in response to *Pc* challenge

Several pathways associated with phytohormone regulation were enriched in this study. The ‘Brassinosteroid biosynthesis’ pathway was enriched in both cultivars’ down-regulated gene sets at 48 hpi. While both up-regulated gene sets are enriched for the ‘Terpenoid backbone biosynthesis’ pathway at 48 hpi. ‘Fenella’ shows early up-regulation of the ‘sesquiterpenoid and triterpenoid biosynthesis’ pathway at 12 hpi, which is then found to similarly up-regulated in both cultivars at 48 hpi. This is in accordance with what was observed in *F. vesca* (Toljamo *et al.,* 2016; Toljamo *et al.,* 2021), and as postulated by the authors of that study it is reasonable to assume that it would be beneficial for *Fxa,* like *F. vesca,* to also redirect isoprenoid metabolites to terpenoid biosynthesis instead of brassinosteroid synthesis (Toljamo *et al.,* 2016). The α-linoleic acid metabolism pathway is enriched in both sets of up-regulated genes at 48 hpi, with the ‘Fenella’ gene set also being enriched for the ‘Linoleic acid metabolism’ pathway. Linoleic acid is a precursor in the synthesis of jasmonic acid (JA), a class of hormones heavily involved in the immune response, and which has been extensively linked to broad spectrum resistance (Chauvin *et al.,* 2013; Fragoso *et al.,* 2014; Grebner *et al.,* 2013; Mousavi *et al.,* 2013; Li *et al.,* 2020b). In Arabidopsis and *N. benthamiana*, a negative regulator of resistance to *P. parasitica* was recently shown to suppress endogenous JA biosynthesis, reinforcing the possibility that JA may play a significant role in plant-*Phytophthora* interactions (Li *et al.,* 2020c). The abscisic acid (ABA) signalling pathway is enriched in both cultivars, though almost twice as many genes belonging to this pathway are DE in 'Fenella' than they are in 'Emily'. ABA is known to play a key role in plant-microbe interactions, promoting both resistance and susceptibility in different plant pathosystems, being employed by pathogenic microbes as an effector to suppress plant immune responses, as well as having a role in mutualistic interactions (Cao *et al.,* 2011; Lievens *et al.,* 2017). Several examples of negative regulation of immunity by ABA have been characterised (Mohr and Cahill*.,* 2007; Fan *et al.,* 2009; Sánchez-Vallet *et al.,* 2012; Liu *et al.,* 2018), as well as an increasing number of cases where ABA positively regulates resistance to a number of plant pathogens (Ton and Mauch-Mani*,* 2004; Adie *et al.,* 2007; Hernández-Blanco *et al.,* 2007; García-Andrade *et al.,* 2011; García-Andrade *et al.,* 2020), including to pathogenic oomycete *Hyaloperonospora arabidopsidis* (Escudero *et al.,* 2017). Moreover, the *Raf*-like kinase *Raf36* was recently shown to negatively regulate *Arabidopsis thaliana* (hereinafter referred to as Arabidopsis) resistance against *P. parasitica* (Li *et al.,* 2022). As *Raf36* is a negative regulator of ABA, it may be postulated that ABA regulation plays a role in *P. parasitica* virulence in Arabidopsis (Kamiyama *et al.,* 2021). The difference in the magnitude of regulation of the ABA pathway observed in this study may therefore contribute to the different levels of susceptibility to *Pc* in *Fxa* cultivars*.* It was also observed that the auxin signalling pathway was enriched in the down-regulated gene set in 'Fenella' but not in 'Emily'. This phytohormone is known to play a complex role in the regulation of pathogenesis and can enhance disease symptoms (Fu, 2011). It has been extensively associated with enhanced pathogen susceptibility in a number of plant pathosystems (Mutka *et al.,* 2013; Liu *et al.,* 2016; French *et al.,* 2018; Zou *et al.,* 2019b; Su *et al.,* 2020), including in soybean-*P. sojae* and Arabidopsis-*P. parasitica* interactions (Evangelisti *et al.,* 2013; Stasko *et al.,* 2020). Thus, it is possible that the down-regulation of the auxin signalling pathway contributes to the moderately resistant phenotype exhibited by 'Fenella'.

5.5.3 Regulation of receptor genes and transcription factors upon *Pc* inoculation

Plants can detect invading organisms using specialised classes of RAM-containing receptor genes. These include transmembrane proteins able to detect PAMPs as well as receptors located in the cytoplasm that can detect pathogen-secreted effectors; both can trigger plant immune responses (Zipfel, 2014). In this study, 1,337 DE RAM genes (19.37% of the total RAM-containing genes in the *Fxa* genome) were identified, 19 of which were located within a previously identified QTL region (Supplementary Table 23). Fifty-two putative wall-associated receptor kinases were found to be DE. *FxaC_26g04640.t1,* a putative wall-associated receptor kinase, was significantly more highly expressed in 'Emily' at 12 hpi (LFC = |3.55|, *p*-adj = 4.11×10^{-19}). Nine other putative wall-associated receptor kinase were DE between cultivars at 48 hpi. These types of receptor genes are involved in the first stages of plant pathogen-interactions and have well documented roles in pathogen detection and resistance, serving as the outermost recognition site for pathogen invasion (Li *et al.,* 2020a; Liu *et al.,* 2021b; Yu *et al.,* 2022). A total of 878 putatively cytoplasmic protein kinases have also been found to be DE during the course of this study, including three of them DE between cultivars at 12 hpi and 388 of them DE between cultivars at 48 hpi. These receptor genes serve a great variety of roles within plant systems (Lehti-Shiu and Shiu, 2012; Yip Delormel and Boudsocq, 2019), including being involved in pathogen effector recognition and being necessary for pathogen resistance in a number of plant-microbe pathosystems (Guerra *et al.,* 2020; Zhang *et al.,* 2018; Wang *et al.,* 2021). In other plant-*Phytophthora* pathosystems they have been reportedly associated with both negative (Li *et al.,* 2022; Qiuang *et al.,* 2021) and positive (Zhang *et al.,* 2021) regulation of resistance. This is reflected in what was observed in this analysis of *Fxa*'s transcriptional response to *Pc* inoculation, with the *Fxa* cultivars having both up- and down-regulated sets of protein kinases. Notably, two putative CLAVATA2 receptor-like protein coding genes (*FxaC_21g70300.t1*, FxaC_23g43210.t1), containing a putative LRR domain, were among the most highly down-regulated genes in 'Fenella' between 0 and 48 hpi. Genes in the CLAVATA family have been associated with several biological processes in plants such as plant growth and development, and immunity (Pan *et al.,* 2016). CLAVATA1 and CLAVATA2 expression is necessary for both nematode and *Ralstonia solanacearum* susceptibility in Arabidopsis (Replogle *et al.,* 2011; Hanemian *et al.,* 2016), with knock-out of those genes having been shown to confer resistance to the pathogenic oomycete *H. arabidopsidis* as well as *R. solanacearum* (Hanemian *et al.,* 2016). It is therefore possible that negative regulation of these CLAVATA2-like genes upon *Pc* inoculation may lead to the enhanced resistance observed in 'Fenella'.

TFs are the master regulators of plant immune responses. In this study, 1,167 DE TF genes were identified (18.29% of the total TF genes in the *Fxa* genome). Ninety-seven of the TFs DE in this experiment were putative members of the WRKY family, a TF class strongly associated with plant defence responses (Birkenbihl *et al.,* 2018; Phukan *et al.,* 2016), including in the soybean-*P. sojae* pathosystem, where *GmWRKY40* was identified as a positive regulator of resistance (Cui *et al.,* 2019b). One of the most highly DE genes between cultivars at 48 hpi in this experiment, up-regulated in 'Emily' upon infection, was *FxaC_25g22040.t1;* a gene putatively assigned to the WRKY TF family. *FxaC_17g43140.t1* encodes a putative WRKY9 TF and was found to be significantly down-regulated (LFC = -2.84, *p*-adj = 0.003) in 'Fenella' while remaining constitutively expressed at higher levels in 'Emily'. The homologue of Arabidopsis *WRKY9* was found to trigger cell death in *Nicotiana benthamiana* upon phosphorylation by a MAPK (Adachi *et al.*, 2015). *FxaC_25g28300.t1,* another putative WRKY-family gene, was constitutively expressed at higher levels in 'Fenella' compared to 'Emily'. It was found to have putative LRR, NB-ARC, and TIR domains. Plant receptor genes sometimes encode unusual 'decoy' target domains able to bind pathogen-secreted effector proteins and in turn activate the immune response (Cesari *et al.,* 2013; Zhai *et al.,* 2014) in a proposed 'integrated decoy' model (Cesari *et al.,* 2014). The TIR-NLR gene-pair *RRS1/RPS4* has been shown to confer resistance to both bacterial and fungal pathogens in Arabidopsis though an integrated decoy WRKY domain (Narusaka *et al., 2009;* Le Roux *et al.,* 2015; Sarris *et al.,* 2015; Ma *et al.,* 2018; Mukhi *et al.,* 2021). Thus, *FxaC_25g28300.t1* chimeric domain composition could serve a similar function. A member of the WRKY30 TF family (*FxaC_26g05440.t1*) located near both *FaRPc2* and *FaRPc7D* was found to be significantly (LFC > 2, *p*-adj < 0.05) up-regulated in both cultivars between 0 and 48 hpi. Genes in the *WRKY30* family have been shown to enhance resistance to cucumber mosaic virus, by reducing reactive oxygen species damage (Zou *et al.,* 2019a).

Other notable TFs families identified among the DEGs were AP2, ERF, NAC, MYB, bHLH, bZIP and RAV; all have been extensively associated with biotic plant responses (Jisha *et al.,* 2015; Liu *et al.,* 2014; Buscaill and Rivas, 2014; Huang *et al.,* 2016; Noman *et al.,* 2017; Wang *et al.,* 2022b). *FxaC_26g08080.t1,* located near the *FaRPc2* locus, was constitutively differentially expressed between cultivars. This gene contains a putative bHLH domain as well as a protein kinase domain, indicating a potentially similar 'integrated decoy' function as the one proposed for *FxaC_25g28300.t1.* Moreover, a putative RAV-family transcription factor (*FxaC_23g54180.t1*) was found to be sharply down-regulated in 'Emily' at 48 hpi, while remaining expressed in 'Fenella' at significantly higher levels. RAV-family transcription factors have been associated with regulation of several plant processes, including defence response. In tomato, the overexpression of *SlRAV2* resulted in enhanced tolerance to bacterial wilt (Li *et al.,* 2011a). Ectopic expression of *CARAV1* in Arabidopsis confers enhanced resistance against infection by *Pseudomonas syringae,* additionally it was shown that *CARAV1* activation could be induced by *P. syringae* pv. *tabaci*, salicylic acid and abscisic acid (Sohn *et al., 2006)*. Therefore, *FxaC_23g54180.t1* could play a role in the resistance mechanism that leads to high tolerance to *Pc* infection in 'Fenella'.

5.5.4 Other notable disease resistance-associated DEGs with putative resistance/susceptibility roles

Several DEGs homologous to genes and gene families previously associated with plant resistance to microbes were identified in this study. The most highly up-regulated gene between 0 and 48 hpi in 'Fenella' (*FxaC_21g06310.t1*) is a putative zinc finger AN1 domain-containing stress-associated protein coding gene. Stress-associated genes have regulatory roles in plant responses to biotic and abiotic stresses (Giri *et al.,* 2013; Tyagi *et al.,* 2014). Tomato (*Solanum lycopersicum*) *SlSAP3* and *SlSAP4* genes have been reported as positive regulators of immunity against *P. syringae* pv. Tomato DC3000 and *Botrytis cinerea*, respectively (Liu *et al.,* 2019c; Liu *et al.,* 2019b). Notably, *SlSAP4* is shown to enhance resistance to *B. cinerea* through interactions with ethylene (ET)/JA signalling pathway (Liu *et al.,* 2019c). In this study, *FxaC_21g06310.t1* was found to be sharply up-regulated in the moderately resistant cultivar 'Fenella' upon *Pc* inoculation, while no regulation was observed in the susceptible 'Emily', hence suggesting a possible contribution to the tolerant phenotype observed in ‘Fenella’. A similar expression pattern was observed in the putative cytochrome p450 gene *FxaC_13g13200.t1.* Genes belonging to the cytochrome p450 superfamily are involved in a range of biological processes, including responses to biotic and abiotic stimuli (Jun *et al.,* 2015; Pandian *et al.,* 2020). The rice (*Oryza sativa* L.) cytochrome P450 protein 716A subfamily CYP716A16 was recently found to positively regulate resistance to the necrotrophic pathogen *Rhizoctonia solani* and the hemi-biotrophic pathogen *Xanthomonas oryzae* pv. *Oryzae* (Wang *et al.,* 2022a). In soybean (*Glycine max* L.), the cytochrome P450 family gene *GmCYP82A3* showed a similar positive regulation role enhancing pathogen resistance. It was consistently highly induced in resistant cultivars by *Phytophthora sojae* infection. Furthermore, transgenic tobacco plants expressing *GmCYP82A3* showed increased resistance to *P. parasitica* (Yan *et al.,* 2016). In this study, *FxaC_13g13200.t1* was one of the most highly up-regulated genes in moderately resistant cultivar ‘Fenella’ upon *Pc* infection. As cytochrome P450 genes are involved in the biosynthesis of a range of phytoalexins (Jun *et al.,* 2015; Pandian *et al.,* 2020), including sesquiterpenoids and triterpenoids (Sawai and Saito, 2011; Chen *et al.,* 2019a; Zheng *et al.,* 2019), and the biosynthetic pathway of these two antimicrobial compounds shows early regulation in ‘Fenella’, it could be hypothesised that *FxaC_13g13200.t1* contributes to enhancing resistance to *Pc* through positive regulation of phytoalexins biosynthesis. The RPW8-family gene *FxaC_23g57270.t1,* found near *FaRPc6D,* was constitutively more highly expressed in ‘Fenella’ than ‘Emily’ throughout the experiment. *RPW8*-family genes have been extensively associated with plant resistance, though the exact mechanism remains elusive (Xiao *et al,* 2001; Li *et al.,* 2017; Ma *et al.,* 2014). A recent publication by Zhao *et al.* (2021) has suggested that *RPW8* may first induce, then negatively regulate cell-death, thus, resulting in a localised cell-death response and pathogen resistance. On the other hand, a putative susceptibility factor was the most highly up-regulated gene in ‘Emily’, *FxaC_13g11560.t1,* annotated as a *NIM1*-interacting (*NIMIN*)-family gene. This family of genes interact with non-expressor of pathogenesis-related genes 1 (*NPR1*), the central regulator of systemic acquired resistance (Cao *et al.,* 1994; Delaney *et al.,* 1995). Overexpression studies of *NIMIN* and *NIMIN*-like genes in Arabidopsis and rice have shown their ability to suppress systemic acquired resistance and cause enhanced susceptibility to *X. oryzae* pv. *oryzae* and *P. syringae* pv. *tomato* (Chern *et al.,* 2005; Chern *et al.,* 2008; Weigel *et al.,* 2005). This suggests the possibility that *FxaC_13g11560.t1* may have a similar susceptibility effect in the response to *Pc* inoculation in ‘Emily’.

5.6 Conclusions

This study details the first report of *Fxa* whole-genome transcriptional response to *Pc* challenge. Resistance to *Pc* has been previously reported as quantitative in strawberry. Thus, it is not surprising to see a substantial overlap in the two cultivars' responses to *Pc* infection. However, this analysis has also highlighted critical differences in the responses of moderately resistant strawberry cultivar 'Fenella' and susceptible 'Emily' to *Pc* inoculation. While both varieties display transcriptional reprogramming of metabolic pathways, the cultivar-specific regulation of a vast array of transcription factor genes and other genes involved in defence signalling pathways were found to be regulated in both cultivars during infection, indicating a large-scale transcriptional reprogramming. The results presented here suggest complex phytohormone crosstalk may play a crucial role in resistance, with the roles of auxin and ABA signalling pathway being of particular interest, due to differential regulation between the two cultivars analysed. Future work on phytohormone quantification *in planta* during *Pc* infection will be needed to clarify their role, as well as the role of the other secondary metabolites described here. Moreover, the data indicates that regulation of cell-death may function as a determinant for effective resistance as it is tentatively suggested by these results that the hemi-biotrophic lifestyle of *Pc* may be benefited by an uncontrolled plant tissue necrosis. Several promising candidate resistance and susceptibility genes have emerged out of the large number of differentially regulated genes involved in pathogen recognition, signal transduction and transcriptional regulation that have been identified in the course of this work, including a number of genes within previously described resistance QTL. Future work will be needed to elucidate the functions and links to resistance that these genes may have in *Fxa,* as well as to identify markers for future resistance breeding efforts. The expression profiles of the candidate resistance genes identified within the QTL during later stages of infection should be investigated using qRT-PCR to further assess their function in later stages of infection. As the plant assessment in the QTL mapping study was performed after four weeks, it would be necessary to explore later data sampling points in order to better understand the temporal dynamics of regulation involved in the resistance mechanism. Ultimately, gene knock-outs of the most promising candidates will be needed to functionally characterise them.

Chapter 6: *Phytophthora cactorum* deploys a vast array of effector genes during infection of *Malus* x *domestica*

6.1 Abstract

The *Phytophthora* genus encompasses some of the most devastating plant pathogens in the world. *Phytophthora cactorum* (*Pc*) can cause disease in a wide range of host plant species, including economically important ones such as apple (*Malus* x *domestica*) and strawberry (*Fragaria* x *ananassa*). Despite the prevalence of the disease and the lack of effective chemical control, there has been very little research aiming to elucidate the factors underlying infection in the apple-*Pc* pathosystem. This study reports the first whole-transcriptome analysis of *Pc* during infection of *Malus* x *domestica* root tissue. We uncovered a large array of genes encoding putative secreted effectors regulated by *Pc,* including a number of candidate RxLR-motif containing cytoplasmic effectors, Crinklers (CRNs), and CAZy genes. Moreover, we investigated the expression of several homologues of known effectors from other *Phytophthora* species in an effort to identify promising candidates for future research into *Phytophthora* host range determinants.

6.2 Introduction

The genus *Phytophthora* comprises a number of pathogenic oomycete species responsible for substantial damages to crops worldwide (Erwin and Ribeiro, 1996). In the past century, there have been several instances in which epidemics of *Phytophthora* species have caused widespread damage to economically important crops (Rizzo *et al*., 2005). The extreme severity of *Phytophthora* out-breaks has sparked a great interest worldwide in finding sources of resistance, as well as improving the current understanding of resistance mechanisms to *Phytophthora* species in order to generate more durable resistance (Kamoun, 2001). *Phytophthora cactorum (Pc)* is a hemi-biotrophic oomycete with a host range of over 160 plant species (Erwin and Ribeiro, 1996). First described in cactus plants in 1870 (Blackwell, 1943), this pathogen can infect a number of commercially important horticultural crops such as strawberry (*Fragaria* x *ananassa; Fxa)*, apple (*Malus* x *domestica; Md*) and pear (*Pyrus* genus) (Erwin and Ribeiro, 1996). Although originally regarded as a single species, recent studies suggest that *Pc* should instead be considered a species complex (Nellist *et al.,* 2021).

Pathogenic oomycetes possess a vast array of secreted proteins used to manipulate the host's immune system and promote virulence (McGowan and Fitzpatrick, 2017; Wang *et al.,* 2019a; Wang and Jiao, 2019). One of the most studied classes of oomycete effectors is the RxLR-motif containing cytoplasmic effectors family (Morgan and Kamoun, 2007; Anderson *et al.,* 2015). This type of effector typically carries a signal peptide, which enables secretion of the protein, and an N-terminal RxLR motif often followed by an EER motif; while the C-terminal domain is highly variable and may contain WY domain repeats (Win *et al.,* 2012). The other major family of cytoplasmic effectors is the Crinklers (CRNs) gene family (Schornack *et al.,* 2010; Stam *et al.,* 2013). They take their name from the crinkling effect shown by plants in which are ectopically expressed (Torto *et al.,* 2003) and are characterised by the N-terminal LFLAK domain. This is followed by a DWL domain, as well as a DI domain often present between the two (Stam *et al.,* 2013). RxLR-motif containing genes (RxLRs) have been shown to suppress the host immune response by repressing cell death (Wang *et al.,* 2011), while CRNs are thought to induce pattern triggered immunity (Jupe *et al.,* 2013). The induction of opposing host immune responses, as well as contrasting patterns of expression during infection, suggest that they may be associated with the biotrophic and necrotrophic life stages of *Phytophthora* species, respectively (Wang *et al.,* 2011; Jupe *et al.,* 2013; Anderson *et al.,* 2015). Several major classes of secreted apoplastic effectors have also been characterised in *Phytophthora* species (Wang and Jiao, 2019). These are secreted by the pathogen in the surrounding environment and serve a number of different functions, from host invasion to nutrient acquisition (Wang and Jiao, 2019; DeVries *et al.,* 2020; Rafiei *et al.,* 2021). Carbohydrate-active enzymes (CAZy), including cell-wall degrading glycosyl hydrolases (GHs), are a the largest and most varied family of apoplastic effectors (Blackman *et al.,* 2014; Brouwer *at al.,* 2014; Rafiei *et al.,* 2021; Bradley *et al.,* 2022). There are also protease inhibitors, necrosis inducing proteins (NIPs) and a range of phytotoxins (Orsomando *et al.,* 2011; Feng *et al.,* 2014; La Spada *et al.,* 2020). Other non-effector secreted proteins are also known to trigger immune responses in the plant host. These are termed elicitins and serve an indispensable role as sterol binding and carrier proteins for *Phytophthora* species, as they cannot produce oxidosqualene themselves (Gottlieb *et al.,* 1978; Wood and Gottlieb, 1978). Due to the nature of their function, these genes are considered conserved microbe-associated molecular patterns (MAMPs) and are known to elicit the plant host's immune response (Du *et al.,* 2015).

Pc has one of the largest genomes in its genus with a size ranging from 59 Mb - 66 Mb for isolates from strawberry and apple, to 121.5 Mb for an isolate from ginseng (*Pananx* genus; Armitage *et al.,* 2018; Yang *et al*., 2018; Nellist *et al.,* 2021). It is second in size only to *Phytophthora infestans* at 240 Mb (Haas *et al.,* 2009), and genomic studies have indicated that it has undergone a recent whole genome duplication event (Yang *et al*., 2018). Phylogenetic studies have placed it in subclade 1a (Yang *et al.*, 2017) and it is most closely related to *P. infestans, P. parasitica* and *P. capsici (*Yang *et al.,* 2018*)*. The genome of *Pc* possesses an estimated 27,000-29,000 genes belonging to around 11,000 gene families, around 900 of which were found to be unique to *Pc* (Yang *et al*., 2018, Armitage *et al.,* 2018; Nellist *et al.,* 2021). Recent bioinformatic analysis of the genomes of a number of *Pc* isolates from both *Md* and *Fxa* revealed a large effector array is regulated upon infection (Armitage *et al.,* 2018; Nellist *et al.,* 2021). The number of putative secreted effectors containing the RxLR domain ranges from 132 to 199 depending on the isolate, between 70 and 127 putative CRNs were also identified, as well as numerous apoplastic effectors, CAZy genes, phytotoxins, elicitins and NIPs (Armitage *et al*., 2018; Nellist et al., 2021). Moreover, the bioinformatic analysis of 21 *Pc* isolates carried out by Nellist and collaborators (2021) has revealed a consistent pattern of expansion/contraction between phylogenetic clades, with the apple clade seeing the expansion of 119 orthogroups, totalling 241 genes. These include five RxLR, two CRNs and seven secreted proteins.

This study is the first report of whole-transcriptome changes in *Pc* during infection of *Md* root tissue. A virulent *Pc* isolate was inoculated on the root systems of two apple cultivars, one resistant ('M.116') and one moderately susceptible ('M.27'), in an effort to elucidate the early interactions between this pathogen and its host. Moreover, this dataset identifies virulence gene candidates and potential determinants for host-specificity in *Pc.*

6.3 Materials and methods

6.3.1 Plant material and inoculation

'M.27' and 'M.116' plants were propagated in sterile tissue culture containers (cylindrical, clear glass honey jars, ø = 9 cm; medium thickness = ~4 cm) on Driver and Kuniyuki Walnut (DKW)/Juglans substrate (0.44% DKW, 0.9% agar, 4.5 µM BAP, 5 nM IBA, 3% sucrose, pH 5.6) and rooted on a modified DKW medium (0.44% DKW, 0.75% agar, 5 nM IBA, 0.35 nM GA3, 3% sucrose, pH 5.6), in a controlled environment chamber at 21 ˚C ±2 ˚C with 16/8 h, day/night light cycle (Driver and Kuniyuki, 1984). Successfully rooted plants were transferred to fresh medium 24 hours prior to inoculation with *Pc*, with the root system laid flat on the medium surface (Supplementary Figure 1).

Pc isolate, R36/14 (Nellist *et al*., 2021), isolated at the NIAB East Malling site (UK) in June 2014, was maintained on V8 agar medium at 20℃ $\pm$1℃ in the dark. R36/14 zoospores were produced as described in Nellist *et al.* (2021), using compost extract medium to induce sporangia production and cold shocking to release the zoospores. Each plant was inoculated with 1 mL of 2×10^4 R36/14 zoospore suspension, distributed homogeneously over the root system and incubated in a controlled environment chamber at 21 ˚C ±2 ˚C with 16/8 h, day/night light cycle for up to 48 h. Samples of the whole root system were taken at 0 (mock inoculated), 6, 9, 12, 24, 36 and 48 hours post inoculation (hpi) starting at 8am, washed in sterile deionised water to remove any traces of medium and immediately frozen in liquid nitrogen (N_2) and stored at −80°C. Complete plant collapse was confirmed at 7 days post inoculation, with visible root lesions appearing between 24 and 36 hpi (data not shown).

Total RNA from all samples was extracted using the RNAqueous™ Total RNA Isolation Kit following the manufacturer's protocol for plant tissue, and sample quality and quantity was measured using NanoDrop™ 2000 and Agilent 4200 TapeStation. Only samples that met the sequencing provider's specifications of at least ≥ 200 ng, RNA Integrity Number (Agilent 2100) ≥ 4.0 and minimum purity measured with NanoDrop (A260/280 = 1.8-2.2; A260/230 ≥ 1.8) were kept. To confirm the presence of *Pc* all RNA samples were reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen), PCR was performed using MyTaq™ Red Mix with an annealing temperature of 60 ˚C and extension time of 10 seconds. The primers used were PITG_11766_F (CTCCGACGCAATCTTGGTAC) and PITG_11766_R (GTCTGACTAAGGGCAAGAAG). After presence was confirmed by PCR and samples 0, 6, 12 and 24 hpi were selected for sequencing, with the aim of elucidating the early stages of infection in both host (see Chapter 4) and pathogen. RNA samples were sent to Novogene and sequenced to an average depth of 40 million reads per sample.

6.3.2 Mycelia production

In order to increase comparability with Nellist *at al.* 2021 mycelia were used as control. Mycelia of the R36/14 *Pc* isolate were grown in clarified V8-juice broth with 500 µg/mL of ampicillin (Fisher) and 10 µg/mL of rifampicin (Fisher) in a shaker incubator set to 180 rotations per minute (rpm) at 21°C ±2°C under a natural light/dark cycle for seven days. The mycelia were washed in sterile dH_2O, vacuum filtered and immediately flash frozen in liquid N_2. All samples were stored at −80°C. Total RNA was extracted from three biological replicates of R36/14 mycelia using the RNAqueous™ Total RNA Isolation Kit, following the manufacturer's protocol. RNA sample quality was tested using NanoDrop™ 2000 and Agilent 4200 TapeStation. Only samples that met the sequencing provider's specifications of at least ≥ 200 ng, RNA Integrity Number (Agilent 2100) ≥ 4.0 and minimum purity measured with NanoDrop (A260/280 = 1.8-2.2; A260/230 ≥ 1.8) were kept. *Pc* mycelia samples were sent to Novogene and sequenced to an average depth of 20 million reads per sample.

6.3.3 Data quality control and analysis

Read quality control was performed with FastQC (Andrews S., 2010; version 0.11.9) and the reads were trimmed using Trimmomatic (Bolger *et al.*, 2014a; version 0.32). The adapter sequences were removed, and low-quality bases (phred quality score below 3) were deleted from both ends of the reads. The reads were also scanned with a 4-base sliding window and cut if the average quality per base dropped below 20. Reads shorter than 36 bases were discarded.

Reads were first filtered using the *Md* genome (Zhang *et al.,* 2019b), Salmon v0.9.1 (Patro *et al*., 2017) was then employed to quantify transcript abundance. The latest assembly available for the genome of *Pc* isolate R36/14 (https://www.ncbi.nlm.nih.gov/assembly/GCA_016906365.1) was used to build the mapping index, using a *k*-mer value of 31. Differential gene expression during infection was investigated using DESeq2 (Love *et al*., 2014). Genes were designated as differentially expressed if they had a DeSeq2 adjusted *p*-value (using the Benjamini and Hochberg method; *p*-adj) <0.05 and the $\log_2$(Fold change) (LFC) was > |1|. Genes are referred in the text as differentially expressed if there is a significant (LFC > |1|, *p*-adj < 0.05) difference between comparisons, constitutively expressed if the difference between timepoints was not significant; and constitutively differentially expressed between cultivars if the difference between timepoints was not significant, but the difference between cultivars was.

6.3.4 Gene functional annotation

Gene functional annotation was performed using eggNOG with the default settings (Huerta-Cepas *et al,* 2019). Custom gene matrix tables (GMTs) were constructed for gene ontology (GO) annotations and enrichment analysis were performed using g:Profiler online tool (Raudvere *et al.*, 2019). Sets of DEGs between 0 and 6 hpi, 12 hpi and 24 hpi in both cultivars, as well as the genes DE between cultivars at each timepoint were all employed for this analysis. The custom g:Profiler correction algorithm was applied, and pathways with an adjusted *p*-value < 0.05 were considered significantly enriched (Raudvere *et al*., 2019). Signal peptide prediction was performed using SignalP (v6.0), while subcellular localisation prediction was performed using DeepLoc (v2.0) (Teufel *et al.,* 2022; Thumuluri *et al.,* 2022).

To determine whether a putatively secreted protein was likely to be an effector, the output of SignalP 6.0 analysis was ran through EffectorP (v3.0) (Sperschneider and Dodds., 2022). To complement the annotation provided by eggNOG, putative RxLR effector genes were identified following the method described by Armitage *et al.* (2018).

6.4 Results

6.4.1 Transcriptome sequencing of resistant and susceptible apple cultivars infected by *Pc*

The *Md* root transcriptome sequencing yielded a total of 1,045,763,293 raw reads (or 313.6 Gbp) of which 1,006,839,249 (97.9%, or 301.9 Gbp) were kept after quality checks. Only clean reads were employed for the subsequent alignment and quantification steps (Supplementary Table 5). A total of 112,281,184 clean reads was kept after filtering out *Md* reads. The average mapping rate for the *Pc* samples was 91.9% for the mycelia and 22.94% for the inoculated *Md* root samples (Supplementary Table 24), with the rest being aligned to *Md* genome (see Chapter 4) or inconclusive.

6.4.2 Differential gene expression analysis

Out of the 29,124 gene models predicted to be in the R36/14 genome (Nellist *et al.,* 2021), a total of 17,156 (58.91%) were found to be expressed in this experiment. Of those, 6,634 (38.67%) were found to be differentially expressed (DE) in at least one comparison between treatments following inoculation of each *Md* cultivar with *Pc* isolate R36/14. A sharp increase in the number of DE genes was observed in R36/14 during infection of both *Md* cultivars was observed throughout the experiment, with the greatest transcriptional difference from the mycelial control observed at 24 hpi. A noticeably greater number of differentially regulated genes were detected in R36/14 during infection of 'M.116' (5,806 genes) compared to R36/14 during infection of 'M.27' (3,311 genes) at that timepoint (Figure 6.1 and Figure 6.2).

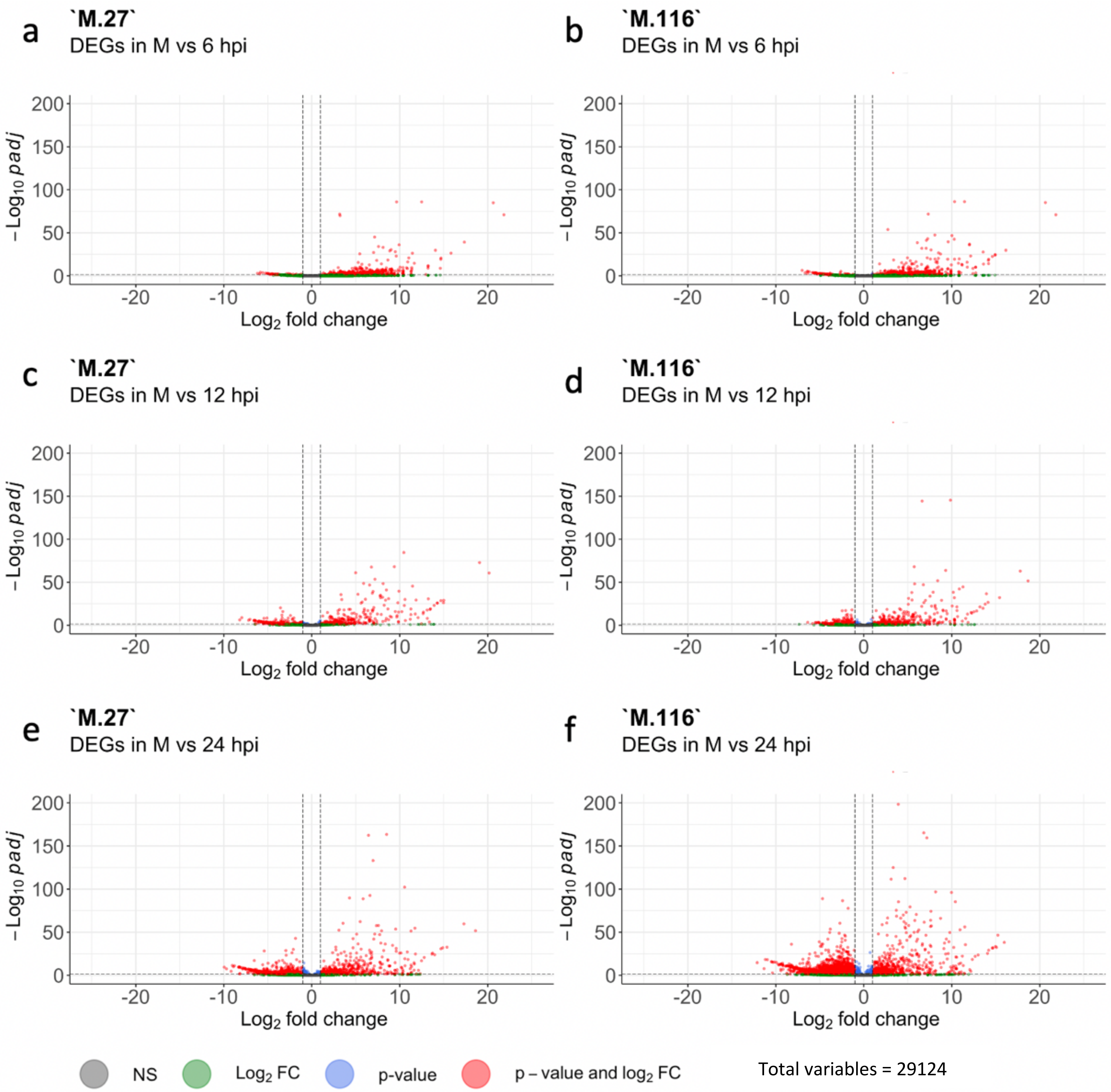


Figure 6.1. Volcano plots highlight a sharp increase in the number of differentially expressed genes (DEGs) by *Phytophthora cactorum* at 24 hours post inoculation (hpi) in *Malus* x *domestica*.

Genes are plotted according to their adjusted *p*-value (y-axis) on a -Log_{10} scale and their change in expression (x-axis) presented on a Log_2 scale. Genes are coloured according to whether the differential expression is significant by *p*-value (blue), fold change (Log_2(FC); green), both (*p*-value and log_2 (FC); red) or is not significant (NS; grey). Gene expression in the mycelia (M) is compared against expression at each timepoint in each cultivar.

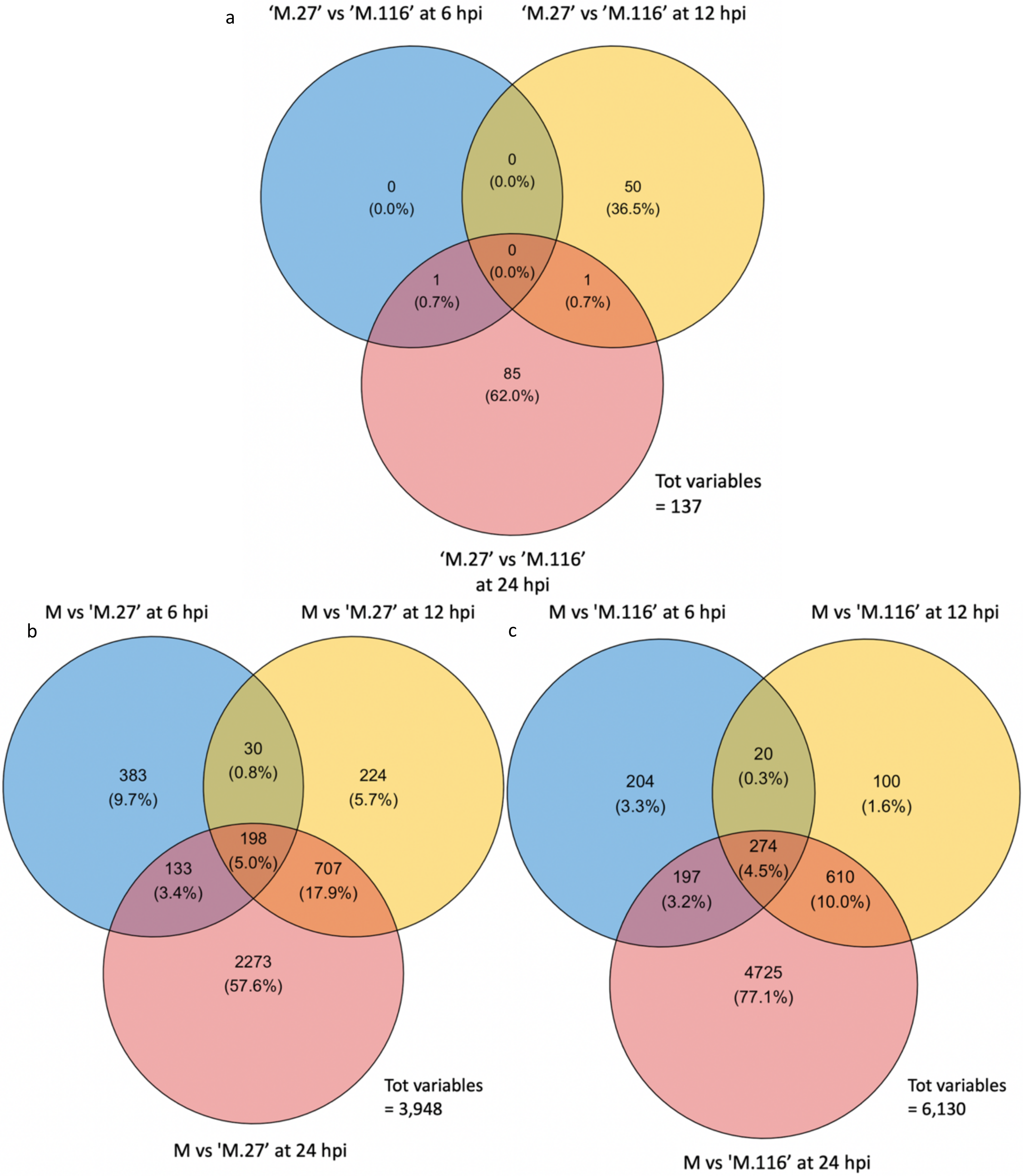


Figure 6.2. Venn diagram of the differentially expressed genes (DEGs) in *Phytophthora cactorum* isolate R36/14 between the cultivars 'M.116' and 'M.27' and timepoints in the mycelia and *in planta* at 6, 12 and 24 hours post inoculation (hpi).

a) Differentially expressed (DE) genes between the two cultivars at each timepoint. b) Genes DE in 'M.27', c) Genes DE in 'M.116'. Comparisons between mycelia and 6, 12, or 24 hpi are encircled in blue, yellow, and red, respectively. The percentage of genes is shown below the number of genes in each group.

6.4.3 Functional annotation and gene set enrichment analysis

The annotation software eggNOG was used to annotate the 29,124 predicted gene models in the apple infecting *Pc* genome. A total of 22,797 were given a putative annotation, 15,431 of which were found to be expressed in this study and 5,114 were DE between one or more of the comparisons analysed. No GO terms were found to be enriched in any of the gene sets analysed in this study. Only two KEGG pathways were significantly enriched. The "Fatty acid degradation" pathway (ko00071) was enriched in the DE gene-set between the 0 and 24 hpi R36/14 samples from 'M.27'. Similarly, the "Pyruvate metabolism" pathway (ko00620) was enriched in the DE gene-set up-regulated between the 0 and 24 hpi R36/14 samples from 'M.27'.

6.4.3.1 Secreted proteins and putative effectors are highly represented in DEGs sets

The annotation software SignalP (v6.0) identified a total of 1,857 genes encoding putative secreted proteins containing a Sec/SPI signal peptide (SP), 687 of which were found to be DE during this study (Supplementary Table 25). A total of 495 of the DE genes encoding proteins containing a putative SP were annotated as putative effectors, including 128 apoplastic effectors and 193 cytoplasmic effectors (Figure 6.3). Of the 280 putative effectors containing an RxLR motif, 119 (42.5%) were identified amongst the DEGs with 32 of them containing an EER motif, as well as 33 of 75 CRNs (44%; Figure 6.4), and 117 of 291 CAZymes genes (40.21%). Amongst the other prominent classes of MAMP-containing genes found to be DE throughout this study there were 32 elicitins (74%), nine necrosis inducing proteins (81.1%), seven cysteine rich secretory proteins (7.2%), five protease inhibitors (23.8%), and two cutinases (100%; Table 6.4.1).

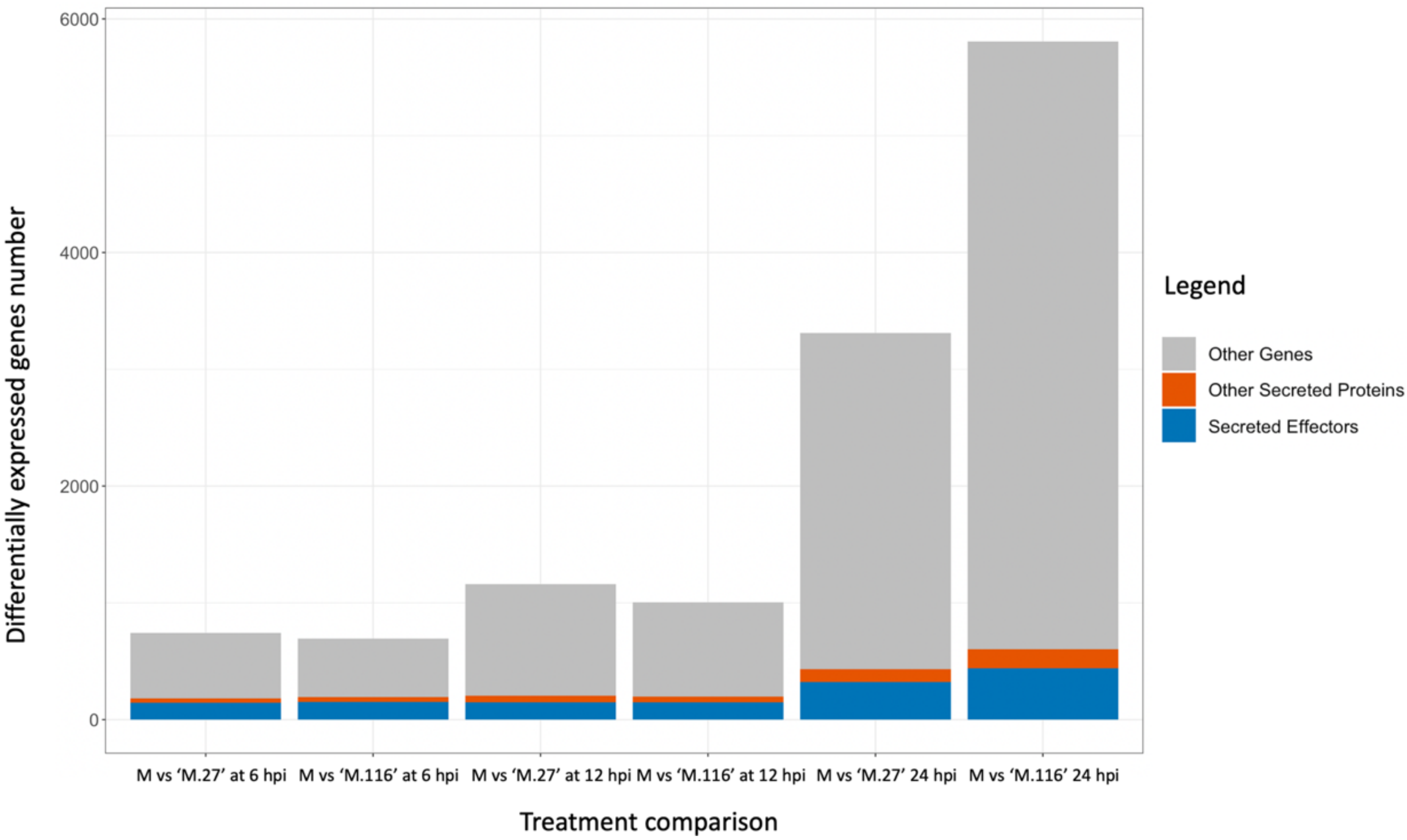


Figure 6.3. Differential expression of genes encoding putaitve secreted proteins increases as infection progresses.

The portion of genes differentially expressed (DEGs) by *Phytophthora cactorum* in each cultivar at each timepoint compared to mycelia (M) which was annotated as a putative secreted effector is reported in blue. Genes putatively encoding other types of secreted proteins are shown in orange, while the remainder of the DEGs are shown in grey.

Table 6.1. *Phytophthora cactorum (Pc)* deploys a wide array of virulence genes during *Malus* x *domestica* tissue colonisation. The number of genes annotated to each category is reported at the timepoint when differential expression (DE) is initiated by *Pc,* in each of the two apple cultivars.

Category	**Family**	**Timepoint of DE (hpi)**			**Host**
		6	12	24	
MAMP[a]	Elicitin	12	3	19	'M.116'
		13	5	5	'M.27'
	Transglutaminase elicitor	5	3	4	'M.116'
		5	2	1	'M.27'
Apoplastic effector	Protease (kazal)	4	-	-	Both
	Protease (cathepsin)	-	-	1	Both
	CAZy[b]	31	22	52	'M.116'
		31	31	34	'M.27'
	NIP[c]	7	1	1	'M.116'
		7	-	-	'M.27'
Cytoplasmic effector	RxLR	45	17	53	'M.116'
		44	14	40	'M.27'
	CRN[d]	1	5	27	'M.116'
		-	7	11	'M.27'

[a]Microbe-associated molecular pattern
[b]Carbohydrate-active enzyme
[c]Necrosis inducing protein

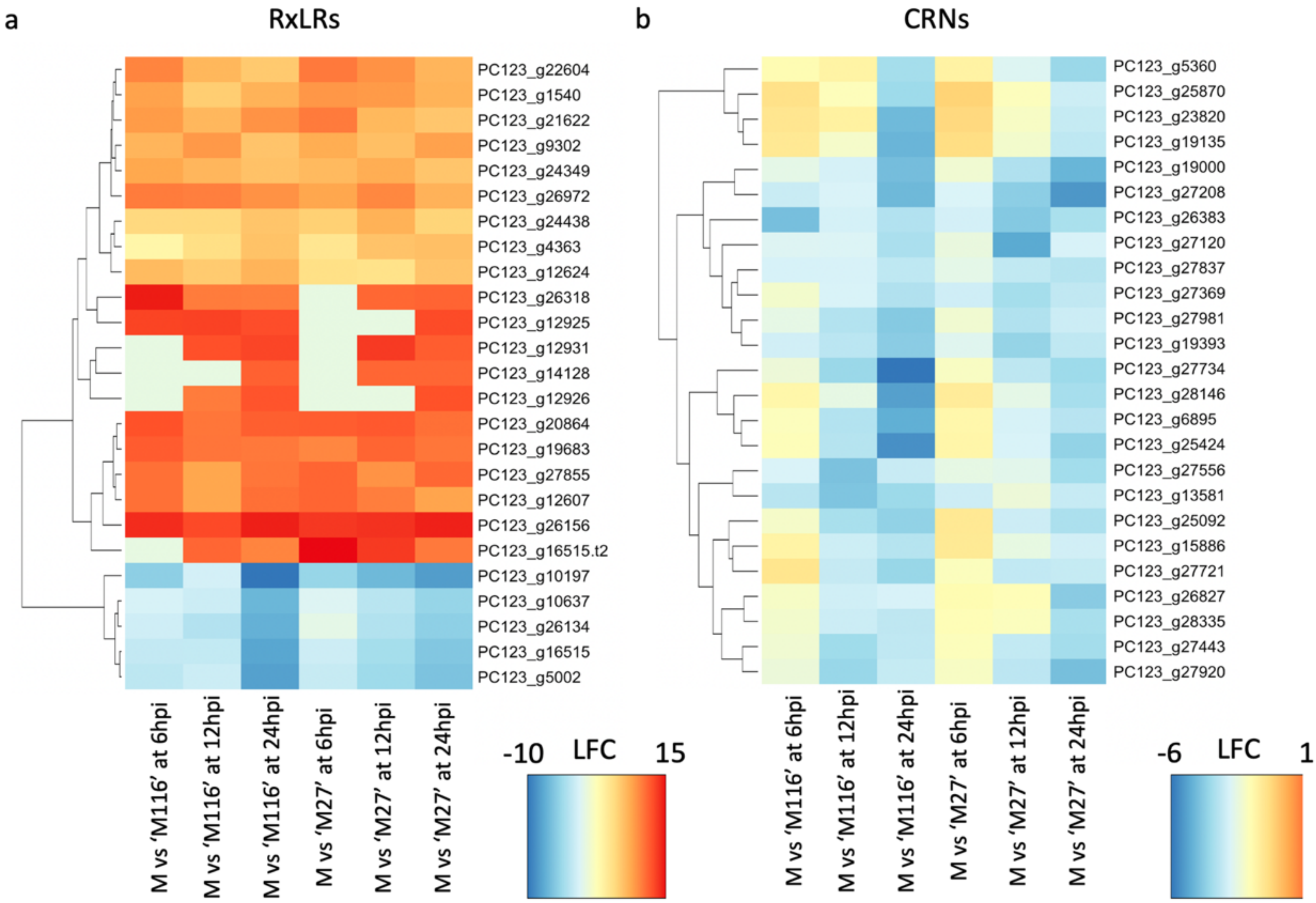


Figure 6.4. The two major classes of cytoplasmic effector genes show opposing expression patterns upon infection of apple (*Malus* x *domestica*).

The expression profiles of the 25 most highly differentially expressed genes (DEGs) ranked by log_2Fold change (LFC) between mycelia (M) and 24 hpi in 'M.27' putatively annotated as RxLR (a) or crinkler (CRN; b) effector genes are plotted in a heatmap. Genes are grouped together based on expression patterns.

6.4.3.2 The most highly up-regulated genes in planta are enriched for putative effectors

The top 100 genes DE ranked by LFC between the mycelial samples and the *in planta* samples in the susceptible cultivar 'M.27' were further investigated in an effort to identify genes involved in the initial establishment and progression of infection (Supplementary Table 26). Of the 100 most highly DEGs between mycelium and *in planta* samples at 6 hpi (LFC > 8), 45 were found to code for putatively secreted proteins, with 15 being RxLR candidates. In addition, 14 CAZymes (GH6, GH7, GH12, GH17, GH28, GH88, pectinesterase and carbohydrate-binding families), two kazal-type proteases, two jacalin-like lectin domain containing genes, a necrosis-inducing protein-coding gene, and a putative cysteine-rich secretory protein-coding gene were all highly up-regulated. Fifteen of the 100 most highly DEGs at 6 hpi showed no differential expression at any other timepoint, including six putative effectors. Only seven of the 100 most highly DEGs between mycelium and *in planta* samples at 12 hpi were not DE at 6 hpi. Five of them encoded candidate effectors, including three CAZymes (GH12, GH28 and pectinesterase families) and two RxLRs both possessing an EER motif. Similarly, 15 of the 100 most highly DEGs between mycelium and *in planta* samples at 24 hpi were not DE in any of the previous timepoints. Eleven were putatively annotated as effectors, including four candidate RxLRs (all containing an EER motif), and three CAZymes (GH12 and GH43 families).

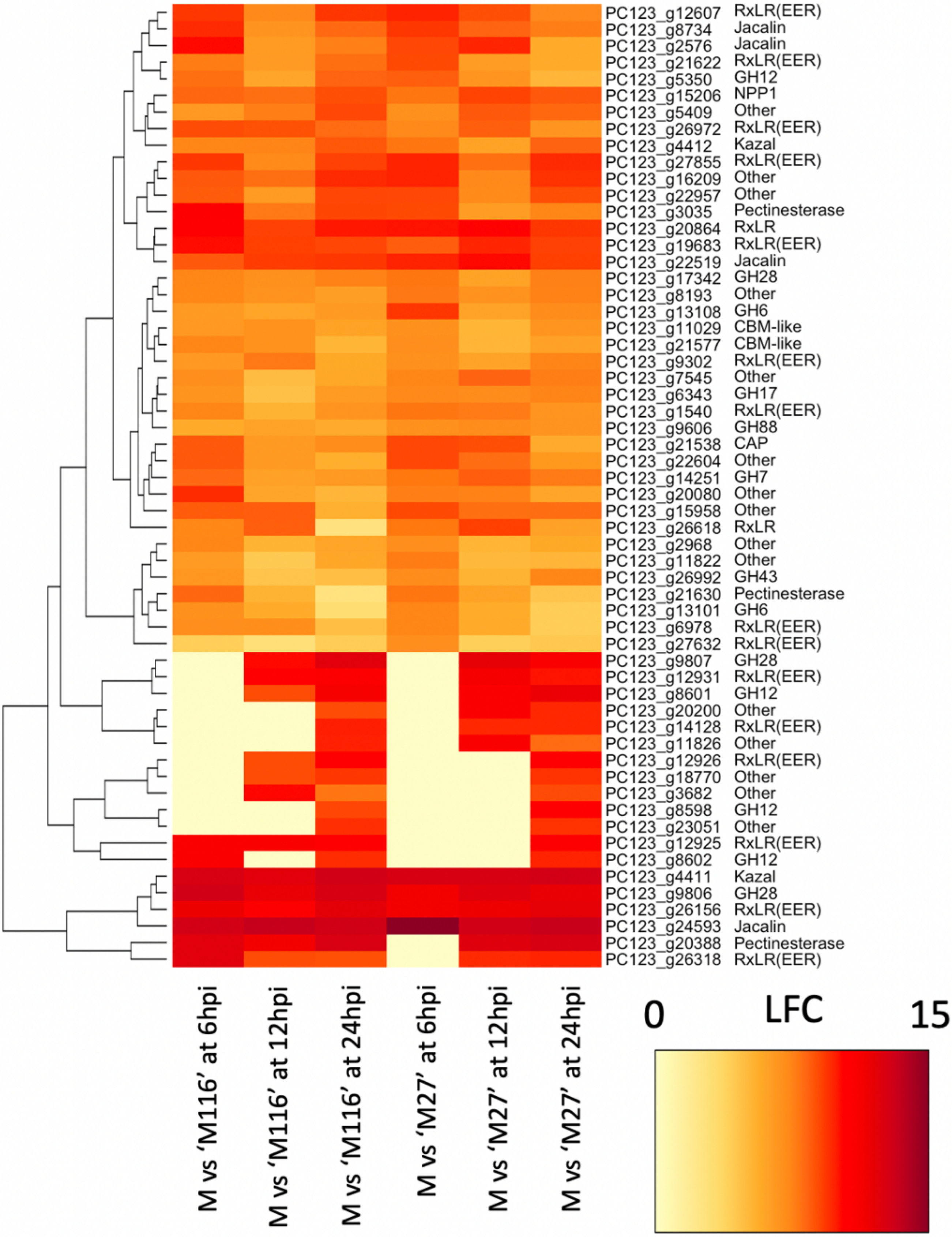


Figure 6.5. Putative effector genes are highly represented in the top 50 most highly differentially expressed genes (DEGs) ranked by log_2Fold change (LFC) between mycelia (M) and each of the sampled timepoints in 'M.27'.

The expression profiles of the top 50 most differentially regulated genes between varieties at each timepoint sampled are plotted in a heatmap of the LFCs for each comparison. Descriptions of the putative function of the protein encoded by the gene are given on the righthand side of each.

6.4.3.3 Genes differentially expressed by Pc in the two host cultivars

The top 50 DEGs between cultivars at each timepoint ranked by LFC were selected for further analysis to explore differences between *Pc*'s transcriptional response in the two host cultivars (Supplementary Table 27). At 6 hpi, there was only one significantly DEG (*PC123_g12959),* found to be more highly expressed by *Pc* in 'M.27' (|LFC| = 1.43, *p*-adj = 5.06 x 10^{-5}). *PC123_g12959* was putatively annotated as coding for a ribosomal protein S27a. At 12 hpi, the five most highly DEGs more highly expressed by *Pc* in 'M.116' were *PC123_g1043, PC123_g20916, PC123_g14545, PC123_g10197, PC123_g5279*; putatively annotated as a pyruvate phosphate dikinase, a phosphoglycerate kinase, a sinapyl alcohol dehydrogenase, a cytoplasmic effector containing an RxLR motif, and a flavodoxin-like fold-containing gene. While the five most highly DEGs more highly expressed by *Pc* in 'M.27' were *PC123_g4393, PC123_g377, PC123_g2779, PC123_g17561, PC123_g7452*; putatively annotated as a PrsW-protease, a broad-complex tram track and bric-a-brac coding gene, a structural constituent of cytoskeleton, a non-SMC mitotic condensation complex subunit 1 containing gene, and a lung seven transmembrane receptor. A total of nine genes DE by *Pc* at 12 hpi were putatively annotated to contain a SP, five of which were annotated as putative effectors, as well as one putative elicitin that was not annotated as an effector. Of the putative effectors, two contained an RxLR motif, one was annotated as a kazal-type serine protease inhibitor, one as putatively containing a fungal-type cellulose-binding domain, and one as an elicitin. All of them were more highly expressed in 'M.116'. At 24 hpi, the five most highly DEGs more highly expressed by *Pc* in 'M.116' were *PC123_g6161, PC123_g12883, PC123_g16317, PC123_g16022, PC123_g2952*; putatively annotated as a 2-oxoacid dehydrogenases acyltransferase, a gene containing the middle domain of eukaryotic initiation factor 4G (*eIF4G*), an acetyl-CoA carboxylase, a gene in the dehydratase family, and a transglutaminase elicitor. While the five most highly DEGs more highly expressed by *Pc* in 'M.27' were *PC123_g9680, PC123_g13356, PC123_g10652, PC123_g3832, PC123_g1719*; putatively annotated as a sugar (and other) transporter, a gene coding for a guanylate-binding protein, a gene coding for a protein with helicase activity, a gene belonging to the short-chain dehydrogenases reductases (SDR) family, and an apoplastic effector. A total of six genes DE by *Pc* at 24 hpi were putatively annotated to encode SPs, all of which were annotated as putative effectors. Two putative apoplastic effector genes were found to be more highly expressed in ‘M.116’, while the rest were more expressed in ‘M.27’. A putative CRN gene was also more highly expressed in ‘M.27’ (Figure 6.4).

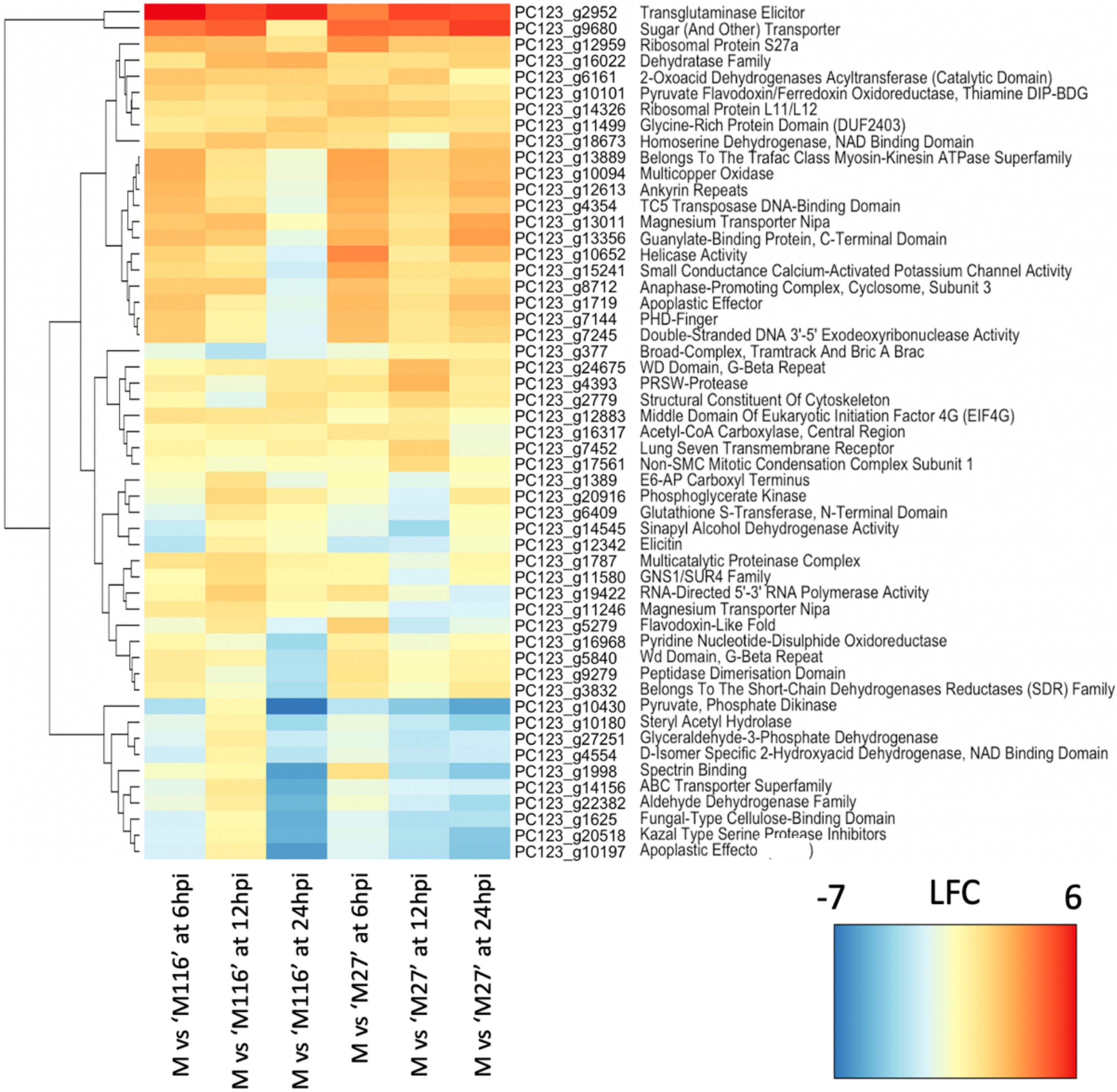


Figure 6.6. Heatmap of notable genes in the top 50 most highly differentially expressed genes (DEGs) ranked by $\log_2$(Fold change) (LFC) at each timepoint between cultivars.

The expression profiles of the top 50 most differentially regulated genes by *Phytophthora cactorum* between apple cultivars at each timepoint (in hours post inoculation; hpi) are plotted in a heatmap of the LFCs between mycelia (M) and each timepoint. Descriptions of the putative function of the protein encoded by the gene are given on the righthand side of each.

6.4.4 Host specificity factors and known virulence genes

6.4.4.1 Expression of genes in expanded and contracted orthogroups between *Pc* isolates

In an effort to identify factors involved in *Pc*'s host specificity, a selection of genes specific to *Pc* apple isolates were further investigated in this data set. A previous study conducted by Nellist *et al.* (2021) identified 119 expanded orthogroups in the *Pc* apple lineage compared to the strawberry lineage, for a total of 241 genes uniquely present in the genomes of *Pc* apple isolates. Moreover, they reported a contraction of 21 orthogroups in the strawberry crown rot (CR) isolates, for a total of 33 genes. Seventy-eight genes in the expanded apple orthogroups were expressed during this study, with 30 of them being expressed *in planta* and 16 showing significant differential expression in one or more comparisons. Of the genes in the contracted CR orthogroups, 21 were expressed in this study, with 13 expressed *in planta* and nine showing significant differential expression in one or more comparisons. A putative effector gene (*PC123_g14333*) belonging to one of the expanded orthogroups was found to be significantly down-regulated by *Pc* compared to the mycelium (LFC of mycelium vs 'M.27' at 24 hpi = -4.17, *p*-adj = 4.57×10^{-4}), while another putative effector gene containing an RxLR motif and belonging to one of the contracted orthogroups was found to be significantly up-regulated (*PC123_g27632;* LFC of mycelium vs 'M.27' at 24 hpi = 5.07, *p*-adj = 1.34×10^{-17}; Table 6.4.2).

Table 6.2. Genes in expanded or contracted orthogroups of *Phytophthora cactorum* isolates from *Malus* x *domestica* (*Md*) and *Fragaria* x *ananassa* crown rot (CR) differentially expressed in this study are presented below. Annotations from all software employed in this study are given for each gene.

Gene ID	Description	SignalP 6.0[a]	EffectorP 3.0	Motif	CR Contraction	*Md* Expansion
g2541	POT family	-	-	-	✓	
g4300	POT family	-	-	-	✓	
g7098	Protein tyrosine kinase	-	-	-		✓
g7310	Protein tyrosine kinase	-	-	-		✓
g9675	Sugar (and other) transporter	-	-	-		✓
g9680	Sugar (and other) transporter	-	-	-		✓
g9681	Sugar (and other) transporter	-	-	-		✓
g11140	-	-	-	-		✓
g11958	-	-	-	-		✓
g12573	POT family	-	-	RxLR	✓	
g12740	Domain of unknown function (DUF3342)	-	-	RxLR		✓
g14333	-	SP	Cytoplasmic effector	-		✓
g14933	-	-	-	-	✓	
g15995	Sugar (and other) transporter	-	-	-		✓
g19493	POT family	-	-	-	✓	
g19541	-	-	-	-		✓
g19740	Calcium-activated chloride channel	-	-	RxLR		✓
g19805	-	-	-	-	✓	
g19876	-	-	-	-		✓
g20416	Calcium-activated chloride channel	-	-	RxLR		✓
g22557	-	-	-	-	✓	
g25057	-	-	-	-		✓
g25247	POT family	-	-	-	✓	
g27599	Prokaryotic RING finger family 4	-	-	-		✓
g27632	-	SP	Cytoplasmic effector	RxLR (EER)	✓	

[a]*Signal peptide*

6.4.4.2 Known effectors and avirulence genes from other *Phytophthora* species

A selection of genes previously reported as avirulence genes in other *Phytophthora* species, including ones whose expression profiles had previously investigated in three *Pc* isolates during infection of *Fxa* (R36/14, P414, 17-21; Nellist *et al.,* 2021)*,* were investigated. The homologue of the *P. infestans* RxLR effector *PiAvramr1* (*PC123_g12607*; Lin *et al.,* 2020) had previously been found to be expressed during infection of *Fxa* in all three *Pc* isolate investigated, a similar pattern was observed upon infection of *Md* (LFC of mycelium vs 'M.27' at 24 hpi = 8.48, *p*-adj = 1.72×10^{-2})*. PC123_g3490* and *PC123_g17704*, homologues of *P. infestans* effectors *PiAvr4* and *PiAvr8* that had not been found to be expressed during infection of *Fxa* by any of the *Pc* isolates, were instead found to be up-regulated at 6 hpi by R36/14 in *Md*, before going back to non-significantly different levels of expression. *PC123_g26318*, the homologue of the *P. infestans* avirulence gene *PiAvrblb1* was also found to be up-regulated by *Pc* R36/14 in both apple cultivars, with DE becoming significant at 6 hpi in 'M.116' and 12 hpi in 'M.27'. Both *P. infestans* avirulence genes *PiAvr1* and *PiAvrSmira1* homologues *PC123_g15192* and *PC123_g18579,* which had been found to be expressed in *Pc* P414 isolate in *Fxa* were not expressed in *Md* (Nellist *et al.,* 2021). Neither was *PC123_g27333,* the homologue of *PiAvrblb2,* which had been expressed by both the P414 and 17-21 *Pc* isolates during *Fxa* infection (Nellist *et al.,* 2021). Interestingly, *PC123_g18579,* a homologue of *PiAvrSmira1* that was found to be expressed by R36/14 during infection of *Fxa* (Nellist *et al.,* 2021)*,* showed no expression at any of the timepoints analysed in this study*.*

Known effectors genes from *Pc* and other *Phytophthora* species were also assessed to identify genes likely to encode virulence factors. The *P. capsici PcAvh1* homologue *PC123_g12038* was found to be down-regulated by *Pc* in 'M.116' at 24 hpi. Similarly, the *P. capsici PcCBP3* homologue *PC123_g23529* was down-regulated by *Pc* in both cultivars compared to expression in the mycelia. In 'M.116' down-regulation started at 6 hpi, while in 'M.27' it started at 12 hpi. *PC123_g19084* and *PC123_g20321*, the homologues of two *Pc* small cysteine repeat-containing genes (*SCR96* and *SCR121*), were both found to be expressed during this study. *PC123_g19084* was up-regulated by *Pc* in both cultivars; in 'M.116' it was transiently up-regulated at 12 hpi, while in 'M.27' up-regulation became significant at 24 hpi. *PC123_g20321* was instead found to be down-regulated by *Pc* in 'M.116' at 24 hpi, while not being significantly regulated at any of the sampled timepoints in ‘M.27’. Interestingly, the known *Pc* effector gene *PcF* was not found to be expressed in either of the apple cultivars at any of the sampled timepoints (Table 6.4.3).

Table 6.3. Known virulence genes from other *Phytophthora* species in *Phytophthora cactorum* isolates.

Homologues of a selection of known effector genes from other *Phytophthora* species are presented below. Annotations for each gene are given, along with indication of whether they were found to be expressed by *Phytophthora cactorum* isolates during infection of *Malus* x *domestica (Md)* and/or *Fragaria* x *ananassa (Fxa)* root tissue.

[a]Signal peptide.
[b]Avirulence.

Gene ID (PC123_)	Genbank ID	Known Avh/Avr[a] gene	SignalP 6.0[b]	EffectorP 3.0	RxLR Motif	Expressed in	
						R36/14	P414
g3490	EF672355.1	*PiAvr4*	SP	Cytoplasmic effector	RxLR (ERR)	*Md*	-
g11949	KT215392.1	*PcF*	SP	Cytoplasmic/ apoplastic effector	-	-	-
g12038	MF975713.1	*P. capsici PcAvh1*	SP	Apoplastic effector	-	*Md*	-
g12607	XM_002904507.1	*PiAvramr1*	SP	Cytoplasmic effector	RxLR (ERR)	*Md, Fxa*	*Fxa*
g15192	DS028168.1	*PiAvr1*	SP	Cytoplasmic effector	RxLR (ERR)	-	*Fxa*
g17704	XM_002904498.1	*PiAvr8 (PiAvrSmira2)*	SP	Cytoplasmic effector	RxLR	*Md*	-
g18579	KX887490.1	*PiAvrSmira1*	-	-	-	-	Fxa
g19084	KT215393.1	*SCR96*	SP	Apoplastic/ cytoplasmic effector	-	*Md*	-
g19522	AEH27535.1	*PiAvr3a*	SP	Cytoplasmic effector	RxLR (ERR)	*Fxa*	-
g20321	KT215395	*SCR121*	SP	Cytoplasmic/ apoplastic effector	-	*Md*	-
g23529	MT774126.1	*P. capsici PcCBP3*	SP	Cytoplasmic/ apoplastic effector	-	*Md*	-
g26318	EEY61733.1	*PiAvrblb1*	SP	Cytoplasmic effector	RxLR (ERR)	*Md*	*Fxa*
g27333	XM_002895872.1	*PiAvrblb2*	SP	Cytoplasmic effector	RxLR (ERR)	-	*Fxa*

6.5 Discussion

6.5.1 *Pc* R36/14 regulates a vast array of putative effectors during infection

Oomycetes, including *Phytophthora* species and fungal pathogens, are known to have large effector repertoires, a factor believed to enable their wide host range (Schulze-Lefert and Panstruga, 2011; Stam *et al.,* 2014; Ökmen *et al.,* 2018; Panstruga and Moscou, 2020). Differences in effector repertoires between *Phytophthora* species and isolates are thought to be important determinants of host specificity, with specific effectors enabling colonisation of host tissues as well as being underlying factors to non-host resistance (Rojas-Estevez *et al.,* 2020; Panstruga and Moscu, 2020; Nellist *et al.,* 2021). *P. infestans* possess the largest of the *Phytophthora* genomes sequenced to date, with a size of 240 Mb (Haas *et al.,* 2009). Among other notable *Phytophthora* species, *P. sojae* has an estimated genome size of 95 Mb, followed by *P. ramorum* with 65 Mb (Tyler *et al.,* 2006), and *P. capsici* with 63.8 Mb (Lamour *et al.,* 2012). A Chinese ginseng isolate of *Pc* possesses the second largest genome of the *Phytophthora* genus at 121.5 Mb (Yang *et al.,* 2018), though strawberry and apple isolates' genomes range between 59 Mb and 66 Mb. Estimates of the number of effector genes present in each of them have varied depending on the methodology employed, ranging from 563 RxLR and 196 CRN effectors for *P. infestans* to as low as 108 RxLRs and 26 CRNs for *P. capsici* (Haas *et al*. 2009; Yang *et al.,* 2018). Different estimates for effector prediction in *Phytophthora* species have been summarised by recent studies and reviews (Yang *et al.,* 2018; Wang and Jiao, 2019). *Pc* isolates from different host species reportedly carry comparable numbers of effector genes (Nellist *et al.,* 2021), ranging from 158 RxLRs and 127 CRNs in CR isolate P414 to 132 RxLRs and 70 CRNs in leather rot isolate 11-40. Following a similar pattern to what was observed in the CR isolate P414 during *Fxa* infection, R36/14 was found to differentially regulate between 40-50% of the putative cytoplasmic effectors that were predicted to be present in the genome, with different classes of effectors showing different expression trends during *Md* infection.

6.5.1.1 Cytoplasmic effectors regulated upon infection

RxLR motif-containing secreted proteins are a well-known family of oomycete virulence factors (Kamoun and Morgan, 2007; Anderson *et al.,* 2015). This class of cytoplasmic effector proteins are translocated inside the host cell to suppress the host's immune response and promote infection (Deb *et al.,* 2018; Liang *et al.,* 2021). Though the details of the translocation mechanism remain controversial, the RxLR motif has been extensively shown to be necessary for host cell entry, potentially by interacting with cell-membrane phospholipid proteins (Kale *et al.,* 2010; Kale and Tyler, 2011; Wawra *et al.,* 2012). They are mainly associated with the initial biotrophic phase of infection and are often found to be up-regulated in these early stages of plant-*Phytophthora* interaction (Want *et al.,* 2011). In this study, all 40 of the RxLR motif-containing genes DE between the mycelia and 6 hpi in the susceptible apple cultivar 'M.27' except for one were up-regulated (LFC = 1.34-14.61, p-adj < 0.05). It is also noticeable that 17 of the most highly up-regulated genes *in planta* were putatively annotated as RxLR effectors (LFC in 'M.27' > 4.5, p-adj < 0.05), reinforcing the idea that this class of effectors plays a major role during *Pc* infection. The number of down-regulated RxLR genes then increases with time, with 39 down-regulated putative RxLR genes at 24 hpi against the 38 up-regulated ones. This is consistent with what was observed by Murphy *et al.* (2018), Wang *et al.* (2019), Joubert *et al.* (2021) and Nellist *et al.* (2021) and indicates a potential shift from biotrophy to necrotrophy is being initiated at 24 hpi (Murphy *et al.,* 2018; Wang *et al.,* 2011). In particular Pi*Avrblb1* has previously been shown to be expressed predominantly during the biotrophic phase of infection of *P. infestans,* suggesting it may likewise be a biotrophy marker in other *Phytophthora* species (Zuluaga *et al.,* 2016). A large number of RxLR avirulence genes have been described in *Phytophthora* species, with many of the corresponding resistance genes also having been identified (Champouret *et al.,* 2009; Chen *et al.,* 2019b; Stefańczyk *et al.,* 2017; Lin *et al.,* 2020; Waheed *et al.,* 2021). The homologue of the avirulence gene *PiAvramr1* (*PC123_g12607)* and of *PiAvrblb1* (*PC123_ g26318*) were up-regulated by *Pc* isolates in both *Fxa* and *Md* suggesting their role may be conserved across different plant hosts and *Phytophthora* species. In contrast, the homologues of *P. infestans* effectors *PiAvr4* and *PiAvr8* (*PC123_g3490* and *PC123_g17704*), which had not been found to be expressed during *Fxa* infection, showed early (6 hpi) regulation in *Md*. The homologue of *PiAvrSmira1* (*PC123_g18579),* which had been found to be expressed by R36/14 during infection of *Fxa,* was not regulated during *Md* infection. Moreover, *Pc123_g27632,* a putative RxLR cytoplasmic effector gene that is absent from the genomes of strawberry CR *Pc* isolates, was found to be up-regulated during infection of apple tissue by R36/14. These expression patterns indicate the possibility that these genes may serve a host-specific function, making them promising candidates for analysis in future efforts to elucidate determinants of host-specificity in *Pc*.

CRNs are another important family of cytoplasmic effectors (Stam *et al.,* 2013), with several examples of them being virulence as well as avirulence factors (Rajput *et al.,* 2015; Ai *et al.,* 2021; Stam *et al.,* 2021). In this study, all the putative CRN genes identified as significantly DE were found to be down-regulated *in planta* compared to mycelia*,* which is congruent to what was observed during *Pc* isolate's P414 infection of *Fxa* (Nellist *et al.,* 2021)*.* Interestingly, the down-regulation was more accentuated in 'M.116', with 33 CRN genes being significantly down-regulated against the 15 down-regulated in 'M.27'.

6.5.1.2 Apoplastic effectors regulated upon infection

The CAZy gene family include some encoding carbohydrate-active proteins used by pathogenic oomycetes to degrade hosts cell walls during tissue colonisation (Blackman *et al.,* 2014; Brouwer *at al.,* 2014; Rafiei *et al.,* 2021; Bradley *et al.,* 2022). In this study, a vast array of CAZy genes were found to be differentially regulated upon *Md* infection. In susceptible *Md* cultivar 'M.27', an initial (6 hpi) up-regulation of 31 CAZy-family genes was observed upon infection. It was followed by differential regulation of individual genes in later timepoints, with genes being either up- or down-regulated compared to mycelia. Indicating a potentially temporally-specific function served by these genes. There were several genes putatively belonging to notable glycosyl hydrolase (GH) families associated with virulence in other pathogenic microbes, including GH families 5, 6, 7, 10, 12, and 18 (Wang *et al.,* 1995; Nguyen *et al.,* 2011; Mentlak *et al.,* 2012; Van Vu *et al.,* 2012; Ma *et al.,* 2015; Tan *et al.,* 2020). Of particular interest are families 7 and 12, to which two and five genes up-regulated in 'M.27' from 6 hpi (LFC = 3.33-13.65, *p*-adj < 0.05) were putatively annotated, respectively. Genes belonging to both GH7 and GH12 families have been reported as virulence factors in *P. sojae* (Ma *et al.,* 2015; Tan *et al.,* 2020), suggesting they may serve a similar function in *Pc.*

Noticeably, four of the putative GH12-family genes were among the most highly up-regulated genes in 'M.27'. Of those, *PC123_g5350* shows early (6 hpi) up-regulation, *PC1223_g8601* is up-regulated at 12 hpi, while *PC123_g8598* and *PC123_g8602* are up-regulated between mycelia and 24 hpi. Additionally, a GH7-family gene (*PC123_g14251*) was also among the most highly up-regulated genes in 'M.27', showing consistent up-regulation from 6 hpi. These data suggests that GHs play a major role during *Pc* infection of *Md,* and that different genes belonging to these families are expressed at different infection stages thus potentially serving different functions.

Additionally, a number of other putative apoplastic effector genes were found to be highly up-regulated during infection. Two putative kazal-type serine protease inhibitor genes (*PC123_g4411* and *PC123_g4412*), were found to be highly up-regulated early (6 hpi) upon infection (LFC in 'M.27' > 4, *p*-adj < 0.05). This type of protease inhibitors has been extensively associated with pathogen virulence, including in *Phytophthora* species (Tian *et al.,* 2004; Tian *et al.,* 2005; Chinnapun *et al.,* 2009; Gumtow *et al.,* 2018; Guo *et al.,* 2019). Moreover, three putative effectors containing a jacalin-like lectin domain (*PC123_g15206, PC123_g24593, PC123_g22519*), the first of which was also putatively annotated as coding for a necrosis inducing protein, were all found to be highly up-regulated (LFC = 9-17.34, *p*-adj < 0.05) soon after infection. A jacalin-like lectin domain-containing protein (*SG06536*) has recently been identified as an apoplastic effector of oomycete pathogen *Sclerospora graminicola* (Kobayashi *et al.,* 2022). Heterologous expression of this protein in *Nicotiana benthamiana* leaves was also found to enhance *P. palmivora* virulence, suggesting it may have a conserved function in oomycetes (Kobayashi *et al.,* 2022).

6.6 Conclusions

This study details the first report of *Pc* whole-genome transcriptional changes during *Md* infection. The highly virulent *Pc* isolate R36/14 was employed to inoculate the resistant ('M.116') and the moderately susceptible ('M.27') apple rootstock cultivars, in an effort to determine the virulence factors involved in the early stages of infection, as well as help elucidate the factors underlying *Pc* host specificity. We uncovered a large array of effector genes differentially regulated by *Pc* upon infection, including ones that display a clear temporal regulation. Moreover, we identified several homologues to known virulence factors from other *Phytophthora* species that were differentially expressed during this experiment. In particular, the difference in expression of the homologues of *PiAvr4, PiAvramr1, PiAvr1, PiAvr8, PiAvrSmira1, PiAvr3a*, and *PiAvrblb2* between the strawberry and apple *Pc* isolates provide valuable insights into effector-determined host specificity, as well as providing a set of candidates for future studies.

Chapter 7: General Discussion

7.1 Key Findings

Despite the ubiquity of *Phytophthora cactorum* (*Pc*) across the temperate growing regions and forests of the world, much is still unknown about the genetic basis of resistance to this pathogen. Production of horticulturally important species can be, and has been, severely impacted by this pathogenic oomycete. Thus, it was the aim of this study to identify sources of resistance to *Pc* and elucidate the mechanisms underlying it in two of the most economically important horticultural plant species in the world: strawberry (*Fragaria* x *ananassa; Fxa*) and apple (*Malus* x *domestica; Md*).

While some recent studies have investigated *Pc* resistance in strawberry, there is virtually no recently published data regarding the genetics of apple resistance to *Pc* (Tojliamo *et al* 2016; Mangandi *et al;* 2017; Nellist *et al.,* 2019; Nellist *et al.,* 2021). One of the main objectives of this project was therefore to assess the current levels of resistance/susceptibility to *Pc* in the UK apple rootstock germplasm in an effort to identify potential sources of resistance. The results presented in this thesis revealed a great range of variation in the severity of responses to *Pc* infection exhibited by UK apple varieties, which largely reflected reports of previous observations in the field. However, there are exceptions. Notably the four varieties 'Northern Spy', 'Red Melba', 'M.9 (EM)' and 'M.27' which had previously reported as resistant (Sewell and Wilson, 1959; Alston, 1969; Utkhede, 1986) were all found to be susceptible to various degrees in the assessment described in Chapter 1. In the case of 'M.9', this is congruent with what was previously reported by Bessho and Soejima (1992) and supports the idea that the isolate utilised in this study may have broken the resistance carried by these older rootstock varieties. It is worth noting that a discrepancy in the response of two different 'M.9' clones was observed in the first assessment of rootstock varieties performed during this project, which could be due to clonal variation or to imperfect inoculation. The case of 'M.9' is particularly interesting as Gómez Cortecero and collaborators working at the same site this study was carried out at also observed clonal variation in resistance levels to *Neonectria dititssima* in this variety (Gómez Cortecero, personal communication). Despite the unresolved issues posed by clonal variation, subsequent assessments have yielded consistent results for all varieties tested. In light of these and past results, the detached shoot assay utilised in this study can be considered a useful tool to assess resistance to *Pc* in apple varieties. It presents some notable advantages in scalability compared to whole plant inoculation, such as cost effectiveness and labour reduction. Moreover, using detached shoots allows for better replication in each round of phenotyping; although some caution must be taken when considering the applicability of these results to field conditions. Future efforts into the optimisation of this phenotyping technique, as well as comparative studies with other inoculation techniques will allow to better assess its applicability.

The assessment of the UK apple rootstock germplasm allowed for the identification of a bi-parental population (M432), originated from a ‘M.27’ X ‘M.116’ cross, segregating for resistance to *Pc.* Using M432 as a mapping population, a large effect quantitative trait locus (QTL; *MdRPc1*) located on chromosome 6 of the apple genome was identified, spanning ~3Mbp. The *MdRPc1* locus remained significant in two consecutive years of phenotyping (2019 and 2020) and accounted for 54.6% and 58.2% of the variation observed in each year, respectively. Following the discovery of this QTL, a total of 99 accessions including both rootstock and scion varieties were included in a preliminary genome-wide association study (GWAS) to assess the presence of sources of resistance in the wider apple germplasm. Again, the varieties included in the experiment showed a varied range of responses to *Pc* infection, going from highly susceptible to completely resistant. No statistically significant difference between rootstock and scion varieties was observed in this instance. This is interesting as it may have been postulated that the breeding selection pressure on rootstock varieties would have produced generally higher levels of resistance. On the other hand, scion varieties may benefit from the lack of specific adaptations of the soilborne *Pc* isolate used in this study. The preliminary GWAS led to the discovery of two additional loci significantly associated with resistance (*MdRPc2* and *MdRPc3*) on chromosomes 5 and 15, respectively, as well as the previously reported *MdRPc1* which remained significant in this experiment. All of them contained several genes associated with pathogen detection and resistance. Notably, two of the single nucleotide polymorphism (SNP) markers located on chromosomes 5 and 6 that were significantly associated with resistance in this study also resulted in non-synonymous mutations located within genes’ coding sequences (F_0990003 and RBbinsnp0265, respectively). Both genes, an API5-like gene and a putative transcription factor containing an AT-hook motif, represent strong candidates for further characterisation.

The independent identification of the *MdRPc1* locus through the QTL mapping and GWAS experiments, as well as with the use of a KASP marker panel in a related apple population (MCM007), strongly supports the idea that this locus is associated with resistance to *Pc*. Despite this, the limited number of genotyped individuals and accessions available for this study has negatively impacted the statistical power of this analysis. The markers thus identified will therefore need to be validated using larger and independent apple populations in order to better estimate the effect of the *MdRPc1* locus. In addition to the aforementioned factors the use of a single *Pc* isolate is to be taken into consideration when assessing these results. It was selected due to the higher virulence levels it demonstrated, compared to the other isolates in our collection, during preliminary screens (Nellist *et al.,* 2021). Despite this, testing the efficacy of the *MdRPc1* locus against a wider range of *Pc* isolates will be essential before it is integrated in rootstock-breeding programs.

As well as understanding the genetic sources or resistance, it is also essential to unravel the mechanisms that underlie it. For this reason, the transcriptional response to *Pc* inoculation of the two parents of the M432 population were investigated in a time course experiment. The results of the differential gene expression and pathway enrichment analyses has highlighted substantial differences in the two varieties. The resistant 'M.116' variety was found to show a transcriptional response to *Pc* infection within hours of inoculation, and significantly regulated the salicylic acid (SA)-mediated defence response pathway leading to the up-regulation of several systemic acquired resistance (SAR)-related genes. In contrast the susceptible 'M.27' variety showed a markedly lower transcriptional response. This is likely to indicate that *Pc* is able to avoid detection in 'M.27', which is then unable to mount an effective resistance response to the pathogen. In addition, activation of the SAR pathway has been strongly associated with resistance to biotrophic pathogens (Yang *et al.,* 2015; Ullah *et al.,* 2019; Kou *et al.,* 2021; Islam *et al.,* 2021). As *Pc* is known to have a hemi-biotrophic lifestyle, it is likely to indicate that the timepoints sampled in this study captured the initial biotrophic phase of colonisation and the corresponding plant immune response. This is congruent with what was observed in *Phytophthora cinnamomi* during infection of *Castanea sativa* and in

Phytophthora infestans during infection of *Solanum tuberosum* (Saiz-Fernández *et al.,* 2020; Soliman *et al.,* 2021).

Similarly to what was observed in apple, the two parental varieties utilised to map resistance to *Pc* in strawberry were employed in a time course experiment aimed at exploring their transcriptional response to *Pc* inoculation show distinct pathway regulation patterns. In particular, the results of the transcriptome data analysis highlight the role of cell death regulation as a possible determinant for resistance. The resistant variety 'Fenella' was in fact shown to up-regulate genes responsible for the negative regulation of cell death. As necrotrophic pathogens are known to hijack the plant immune system to induce uncontrolled necrosis in order to facilitate tissue colonisation, it appears plausible that negatively regulating cell-death may result in resistance to the pathogen (Pitsili *et al.,* 2020). These results also point towards the possibility that the later timepoint sampled in this study captured the necrotrophic stage of *Pc* infection. Conversely, the earlier timepoint analysed in this study did not appear to have captured statistically significant enrichment of pathways related to the biotrophic infection stage. They also appear to indicate that while the susceptible apple variety 'M.27' is unable to recognise *Pc* infection in the initial phases of tissue colonisation and mount an effective immune response, the susceptible strawberry variety 'Emily' is able to activate immune response pathways which are then hijacked by the pathogen to aid infection in the necrotrophic phase. It is hard to determine whether the differences in response observed between apple and strawberry are caused by an intrinsically different immune response, different pathogen infection strategies or sampling. It would therefore be interesting to repeat the apple time course experiment extending the sampling range to later timepoints, potentially allowing to capture the transition from the initial biotrophic stage to the postulated later necrotrophic stage of tissue colonisation.

The data from the apple time course experiment offered a chance to also explore the transcriptional changes occurring in the pathogen during host infection. The results obtained have highlighted the vast effector repertoire regulated by *Pc* during apple infection. Different classes of effectors were found to have specific patterns of regulation. In particular apoplastic RxLR effectors showed a dramatic initial up-regulation followed by a gradual down-regulation at 24 hours post inoculation (hpi). This subsequent down-regulation could be an indicator of a shift to necrotrophy being initiated by the pathogen (Murphy *et al.,* 2018; Wang *et al.,* 2011) and is consistent with what has been previously reported by Murphy *et al.* (2018), Wang *et al.* (2019), Joubert *et al.* (2021), as well as what was observed by Nellist *et al.* (2021) during *Pc* infection of strawberry plants. Thus, exploring later timepoints of infection could help elucidate the timing of the switch to necrotrophy in apple as well as the factors involved in it.

7.2 Conclusions

The work presented in this thesis explored the basis of resistance to the plant pathogen *Pc* in two economically important plant species, strawberry and apple. The apple germplasm used in modern breeding programs in the UK and the world was found to have ranging levels of susceptibility to this pathogen, highlighting the need for robust resistance markers. Genetic mapping performed in apple identified the first reported genomic loci putatively associated with resistance to *Pc.* Moreover, efforts towards marker validation for future deployment in rootstock breeding programs were initiated during the course of this study. It was also possible to study resistance mechanisms in both apple and strawberry through whole-transcriptome sequencing of a time course experiment performed in both species which identified several candidate genes for future study. Moreover, the analysis of transcriptional changes in *Pc* during infection of apple has led to the identification of elements underpinning virulence as well as potential candidates for host-specificity. In summary, these results provide a valuable platform for further investigation of *Pc* resistance in apple and strawberry which will help future resistance breeding efforts in both species.

7.3 Future directions

7.3.1 Further validation of the putative resistance markers

This analysis has identified a panel of markers associated with the putative resistance to *Pc* locus *MdRPc1* through a QTL mapping and a GWAS. These were found to be significantly associated with resistance in an additional apple population, genetically close to the one originally used for mapping. Thus, it will be important to test these markers in more genetically distant populations in order to better assess the effect on resistance before they are deployed in breeding programs.

7.3.2 Functional characterisation of candidate resistance genes

The results obtained from the analysis of both RNAseq data sets have highlighted several strong resistance gene candidates for functional characterisation. A platform for CRISPR-based genome editing in octoploid strawberry has recently been developed at NIAB (Wilson *et al.,* 2019) , and a similar resource is currently being developed in apple with promising results (data not published). This could be used to assess the effect of knock-outs (KOs) on resistance, as well as knock-ins (KIs) on susceptibility. Potentially allowing to introgress the resistance trait observed in this study in elite commercial cultivars through genome editing if future UK legislation on the matter was to allow the commercialisation of genome edited food crops.

7.3.3 Functional characterisation of candidate *Pc* virulence genes

Similarly, several effectors with putative virulence roles were identified analysing *Pc* transcriptome data during apple infection. Though CRISPR-based genome editing techniques have been developed for *Phytophthora* species (Fang *et al.,* 2017; Ah-Fong *et al.,* 2021), no reliable methods for *Pc* transformation have been reported. *Pc* transformation was attempted during the course of this study, but no appreciable results were produced. The development of such a resource would greatly facilitate the study of gene function in *Pc* and thus warrants future efforts. KOs of candidate virulence genes highlighted as host specificity determinant would be of particular interest to observe the effect on pathogenicity. While KIs could be employed to introgress those virulence genes into non-pathogenic isolates to confirm their role in virulence.

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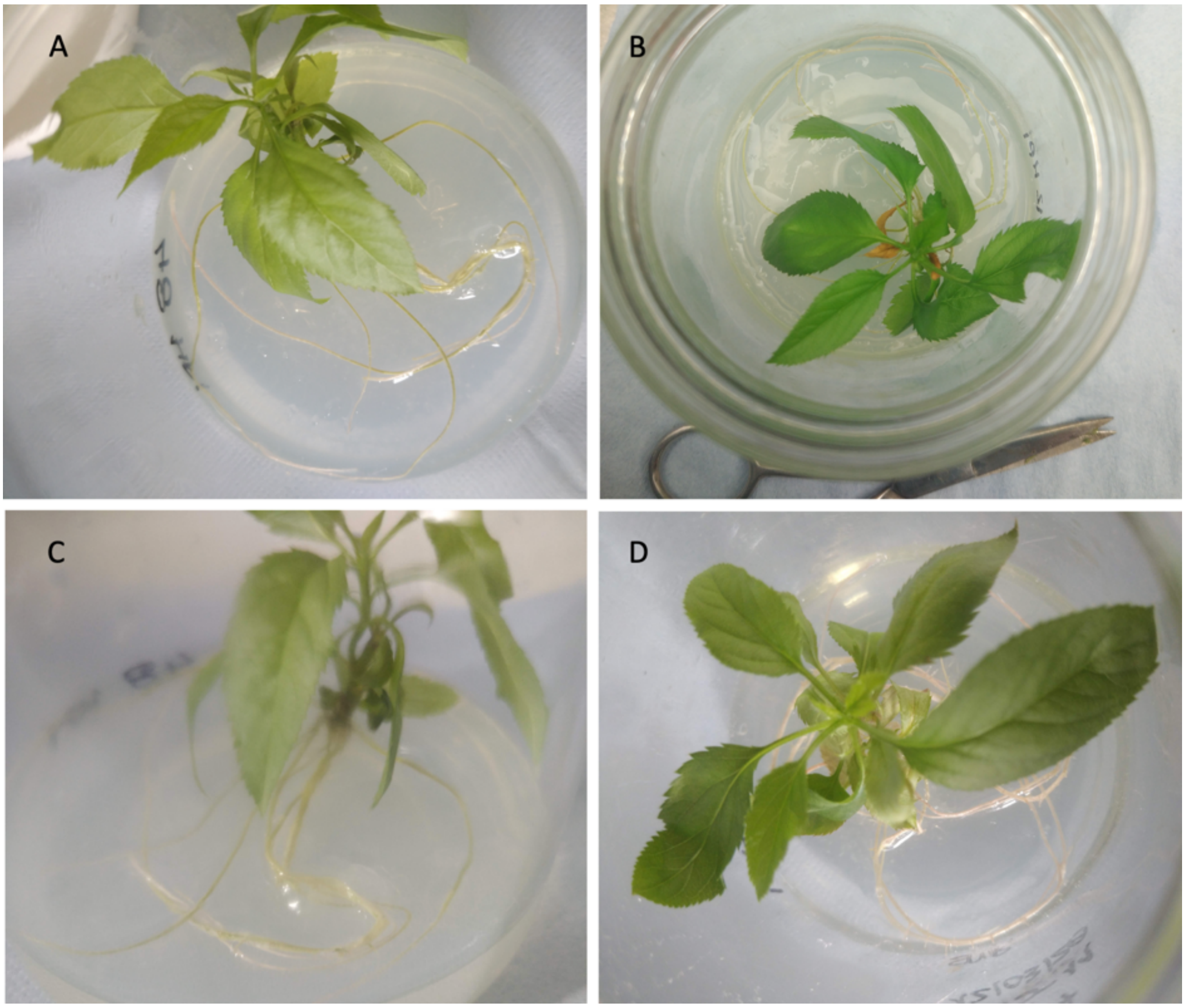
Supplementary Figures



Supplementary Figure 0.1. Example of experimental set-up.

Plants were rooted in sterile jars (cylindrical, clear glass honey jars, ø = 9 cm; medium thickness = ~4 cm) on a modified Driver and Kuniyuki Walnut (DKW)/Juglans medium (0.44% DKW, 0.75% agar, 5 nM IBA, 0.35 nM GA3, 3% sucrose, pH 5.6), in a controlled environment chamber at 21 ˚C ±2 ˚C with 16/8 h, day/night light cycle (Driver and Kuniyuki, 1984). On the day of the inoculation, they were transferred to fresh medium, the root system was laid flat and homogeneously covered with a with 1 mL of $2{\times}10^4$ R36/14 zoospore suspension. They were then incubated in a controlled environment chamber at 21 ˚C ±2 ˚C with 16/8 h, day/night light cycle for up to 48 h. 'M.27' plants are represented in panels A and C, 'M.116' plants are represented in panels B and D.

Supplementary Tables

See http://dx.doi.org/10.17864/1947.000460 for details.

Supplementary table 1. Details of mapped individuals.
Supplementary table 2. Linkage map used in QTL mapping of M432 population.
Supplementary table 3. KASP marker panel used for MCM007 population.

Supplementary table 4. Genes found within putative QTL loci.
Supplementary table 5. RNA data quality control for apple samples.
Supplementary table 6. Alignment results for apple samples against the *Malus* x *domestica* genome.
Supplementary table 7. Differentially expressed genes with resistance associated motifs (apple).
Supplementary table 8. Differentially expressed genes annotated as transcription factors (apple).
Supplementary table 9. Gene Ontology terms enriched between varieties at each timepoint (apple).
Supplementary table 10. Gene Ontology terms enriched between control samples and each timepoint for ach variety (apple).
Supplementary table 11. Most highly differentially expressed genes (apple)
Supplementary table 12. Differentially expressed genes found within the *MdRPc1* locus.
Supplementary table 13. RNA data quality control for strawberry samples.
Supplementary table 14. Alignment results for strawberry samples.
Supplementary table 15. Gene Ontology terms enriched between control samples and 48 hours post inoculation in each variety (strawberry).
Supplementary table 16. KEGG terms enriched between control samples and 48 hours post inoculation in each variety (strawberry).
Supplementary table 17. REVIGO clustering of Gene Ontology terms.
Supplementary table 18. Gene Ontology terms enriched in up- and down-regulated differentially expressed gene sets between control samples and 48 hours post inoculation in each variety (strawberry).
Supplementary table 19. Gene Ontology terms enriched in each co-expression module (strawberry).
Supplementary table 20. KEGG terms enriched in each co-expression module (strawberry).
Supplementary table 21. Differentially expressed genes annotated as transcription factors (strawberry).
Supplementary table 22. Differentially expressed genes with putative resistance associated motifs (strawberry).
Supplementary table 23. Differentially expressed genes found within previously described QTL loci (strawberry).
Supplementary table 24. Alignment results for apple samples against the *Phytophthora cactorum* genome.
Supplementary table 25. Differentially expressed genes coding for putative secreted proteins (*Phytophthora cactorum*).
Supplementary table 26. Most highly differentially expressed genes compared to mycelia in each variety (*Phytophthora cactorum*).
Supplementary table 27. Most highly differentially expressed genes compared between varieties at each timepoint (*Phytophthora cactorum*).

Appendix

The published article which was filed at the end of the thesis has been redacted for copyright reasons. The article can be found at the following reference:

Luberti, M, Litthauer, S. Dunwell, J.M., Fernández Fernández, F., Nellist, C.F. (2021) *Response of apple (Malus domestica) accessions to UK Phytophthora cactorum isolates in cut-shoot tests.* In: Sedlak, J., Kellerhals, M. (eds.) Proceedings of the XV EUCARPIA Symposium on Fruit Breeding and Genetics. Acta Horticulturae 1307. IHS, Gent, pp. 369-373. ISBN 9789462613065.

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